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IN VITRO AND *IN SILICO* CHARACTERIZATION OF THE ANTICHOLINESTERASE ACTIVITY OF SELECT TERPENOIDS AGAINST *ANOPHELES* VECTORS

Thankhoe Abram Rants'o



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

A thesis submitted to the Faculty of Health Sciences,

University of the Witwatersrand,

in fulfilment of the requirements for the degree

of

Doctor of Philosophy

Johannesburg, 2023

DECLARATION

I Thankhoe Abram Rants'o declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

_15th day of March 2023_at__Parktown_____

DEDICATION

Dedicated to everyone who loved education, devoted to their studies, and hoped for a better future but never made it due to financial constraints.

I KNOW YOUR PAIN.

~ Thankhoe A Rants'o

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Published articles

1. Rants'o, T.A., Panayides, J-L., Koekemoer, L.L., van Zyl, R.L. Potential of essential oil-based anticholinesterase insecticides against *Anopheles* vectors: A review. Molecules, 2022; 27(20): 7026; doi: 10.3390/molecules27207026.

   Student's contribution to the paper

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- Thankhoe A. Rants'o, Lizette L Koekemoer, Jenny-Lee Panayides, Robyn L. van Zyl. The differential acetylcholinesterase binding of larvicidal donepezil analogues. Wits Research Institute for Malaria Day 2022. Faculty of Health Sciences, University of the Witwatersrand, 20 April 2022. [Oral].
- Thankhoe A. Rants'o, Jenny-Lee Panayides, Lizette L Koekemoer, Robyn L. van Zyl. Multi-stage activity of essential oils on *Anopheles arabiensis*. 6th Malaria Research Conference 2021. SAMRC. 3-4 August 2021 [Oral].
- Thankhoe A. Rants'o, Jenny-Lee Panayides, Lizette L Koekemoer, Robyn L. van Zyl. The potential of essential oils to overcome insecticide resistance in malaria control. IPUF 2021. University of Johannesburg. 5-7 July 2021. [Oral].
- Thankhoe A. Rants'o, Jenny-Lee Panayides, Lizette L Koekemoer, Robyn L. van Zyl. Structure-activity relationships for *Anopheles* acetylcholinesterase inhibition. Faculty of Health Sciences Research Day & Postgraduate Expo 2020. 15 October 2020. [Oral].

- Thankhoe A. Rants'o, Jenny-Lee Panayides, Lizette L Koekemoer, Robyn L. van Zyl. The anticholinesterase effects of camphor on *Anopheles*. 53rd Annual Conference of the South African Society of Basic and Clinical Pharmacology 2019. Kievits Kroon Faircity Hotel Pretoria, South Africa. 5 - 7 October 2019. [Oral].
- Thankhoe A. Rants'o, Jenny-Lee Panayides, Lizette L Koekemoer, Robyn L. van Zyl. Identification of *Anopheles*-specific donepezil analogues through molecular modelling. School of Therapeutic Sciences Research Day 2019. Faculty of Health Sciences, University of the Witwatersrand. 10 September 2019. [Poster].
- Thankhoe A. Rants'o, Jenny-Lee Panayides, Lizette L Koekemoer, Robyn L. van Zyl. The isomeric effects of camphor on *Anopheles*. 5th Malaria Research Conference 2019. University of Pretoria, South Africa. 30 July - 1 August 2019. [Oral]

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ABSTRACT

Malaria is a life-threatening plasmodial disease that is transmitted by female *Anopheles* mosquitoes. Major African malaria vectors include *Anopheles arabiensis*, *An. funestus*, *An. gambiae* and *An. coluzzii*. Malaria vector control programs have shown effectiveness in reducing the *Anopheles* populations. The main insecticide classes used in these interventions include pyrethroids, organochlorines, organophosphates, carbamates, and neonicotinoids. Nevertheless, the development of *Anopheles* resistance to these insecticide classes has greatly reduced the effectiveness of these interventions. A common resistance mechanism is through rapid detoxification of insecticides by overexpressed P450 monooxygenases. Although acetylcholinesterase (AChE) is a valid target in *Anopheles* vector, current anticholinesterase insecticides suffer from resistance and low selectivity between insect and mammal AChE targets. This indicates the urgent need to discover novel AChE inhibitors with higher affinity to *Anopheles* AChE compared to the mammal target, and less prone to resistance caused by the overexpressed monooxygenases. Identification of novel AChE inhibitors from natural sources and their potential to kill *Anopheles* during all its different life stages, presents a cost-effective approach. This PhD study aimed to identify such novel AChE inhibitors from essential oil sources and assess them for consistent activity against *Anopheles* species with hyperactive P450 monooxygenases. In this study, molecular differences between *Anopheles* and human AChEs were identified showing the opportunity to develop selective *Anopheles* AChE inhibitors. A novel approach was used to integrate the *in silico* and *in vitro* assays in assessing the *Anopheles* AChE inhibitory potential of select terpenoids and coupled these to the *in vivo* assays against different life stages of *Anopheles*. The terpenoids, farnesol, (-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, and methyleugenol were identified as potent *Anopheles* AChE inhibitors and larvicidal agents with moderate adulticidal effects. Farnesol and (-)-α-bisabolol also displayed pupicidal activity, while methyleugenol inhibited the hatching of *Anopheles* eggs. Generally, farnesol and (-)-α-bisabolol were highly active across the *Anopheles* species, except in the strain with P450-based metabolic resistance. In contrast, the efficacy of *cis*-nerolidol, *trans*-nerolidol, and methyleugenol was not affected by this resistance mechanism. This research suggests that *cis*-nerolidol, *trans*-nerolidol, and methyleugenol are potential candidates for further development as anticholinesterase bioinsecticides.

Keywords: Malaria, *Anopheles*, life cycle, terpenoids, monooxygenases

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Table of Contents

DECLARATION i

DEDICATION ii

PUBLICATIONS AND PRESENTATIONS iii

PUBLICATIONS iii

PRESENTATIONS iv

ABSTRACT vi

ACKNOWLEDGEMENTS vii

LIST OF FIGURES xi

LIST OF TABLES xv

LIST OF ACRONYMS AND ABBREVIATIONS xvii

CHAPTER 1 1

Introduction 1

1. Background 1

1.1. *Anopheles* life cycle 1

1.2. Malaria control 2

1.3. *Anopheles* vectors 4

1.4. Insecticide resistance 5

1.5. Acetylcholinesterase and inhibitors 6

1.6. Monooxygenases 6

1.7. Novel AChE inhibitors 7

1.8. Rationale of the study 8

1.9. Aim and objectives 10

2. Structure of the thesis 10

References 14

CHAPTER 2 27

Literature review 27

1. Introduction 29

2. Malaria Vector Control 30

3. Conclusions and Future Perspective 46

References 48

CHAPTER 3 70

Optimization of the *in silico* approach to assess the acetylcholinesterase inhibitory potential of chemical entities using molecular modelling and molecular mechanics simulations 70

1. Introduction 72

2. Material and methods 74

3. Results and discussion **79**

4. Conclusions **90**

References **92**

CHAPTER 4 ..... **100**

The *in vitro* and *in silico* assessment of donepezil derivatives for the *Anopheles* acetylcholinesterase inhibition **100**

1. Introduction **103**

2. Materials and Methods **104**

3. Results and Discussion **109**

4. *In silico* studies **111**

5. Conclusions **122**

References **124**

CHAPTER 5 ..... **132**

The *in vitro* and *in silico* assessment of select terpenoids for the *Anopheles* acetylcholinesterase inhibition **132**

1. Introduction **135**

2. Methods and materials **137**

3. Statistical analyses **140**

4. Results and discussion **141**

5. Conclusions **149**

References **152**

CHAPTER 6 ..... **163**

The activity of terpenoids against multiple stages of *Anopheles* life cycle **163**

CHAPTER 7 ..... **192**

The insecticidal activity of essential oil constituents against insecticide resistant *Anopheles* with overexpressed P450 monooxygenases **192**

1. Introduction **194**

2. Materials and methods **196**

3. Results **198**

4. Discussion **206**

5. Conclusion **208**

References **209**

CHAPTER 8 ..... **216**

Integrative discussion **216**

8.1 Establishment of *Anopheles* acetylcholinesterase as a critical target **216**

8.2 Hypothesis of essential oils as sources of novel *Anopheles* acetylcholinesterase **217**

8.3 Optimization of the *in silico* approach for assessment of novel AChE inhibitors **218**

8.4 *In silico*, *in vitro* and *in vivo* analysis of anticholinesterase activity of terpenoids .. 219

8.5 Terpenoids bioactivity against the *Anopheles* life cycle 222

8.6 Structure-activity relationships 223

8.7 Terpenoids activity against resistant *Anopheles* strains 226

References 228

CHAPTER 9 236

Conclusions and recommendations for future research 236

Concluding remarks 236

Limitations of the current study 236

Recommendations for future research 236

CHAPTER 10 238

Appendices 238

Appendix A.1: Potential of essential oil-based anticholinesterase insecticides against *Anopheles* vectors: A review (Chapter 2; published paper) 238

Appendix B.1: Optimization of covalent docking for organophosphates interaction with *Anopheles* acetylcholinesterase (Chapter 3; published paper) 261

Appendix B.2: Supplementary information for Chapter 3 (published paper)……272

Appendix C.1: The *in silico* and *in vitro* analysis of donepezil derivatives for *Anopheles* acetylcholinesterase inhibition (Chapter 4; published paper)……...279

Appendix C.2: Supplementary information for Chapter 4 (published paper) 300

Appendix D.1: *In vitro* and *in silico* analysis of the *Anopheles* anticholinesterase activity of terpenoids (Chapter 5; published paper)…….305

Appendix E.1: The insecticidal activity of essential oil constituents against pyrethroid-resistant *Anopheles funestus* (Diptera: Culicidae) (Chapter 7; published paper)……...310

Appendix F.1: Author declarations 318

Appendix G.1: Approval of change of title 324

Appendix H.1: Human Research Ethics waiver 325

Appendix I.1: Biosafety Ethics certificate 326

Appendix J.1: Animal Research Ethics waiver 327

LIST OF FIGURES

CHAPTER 1

Figure 1: Four *Anopheles* stages of growth…….……..2

Figure 2: The parasite developmental stages within the *Anopheles* host (Gitta and Kilian, 2020)……..…….4

CHAPTER 2

Figure 1: The nervous system ganglia of *Anopheles*……33

Figure 2: Acetylcholine release, postsynaptic receptor binding, and hydrolysis by acetylcholinesterase ……...……..34

Figure 3: Molecular comparison of *An. gambiae* wild-type (**A**) and resistant (**B**) AChE catalytic sites (PDB IDs: 5YDI and 6ARY, respectively) to the human AChE (PDB ID: 7E3H) (**C**), generated by Schrodinger's Maestro 2018-2 software. The G280S mutation is shown (red arrow) in the resistant *Anopheles* AChE phenotype (B) .....…….……..36

CHAPTER 3

Figure 1: General OP hydrolysis by catalytic serine in the AChE catalytic site……..…..….72

Figure 2: Chemical structures of ligand OPs……...76

Figure 3: Generated binding site on the *Ag*AChE……79

Figure 4: Malathion (A) and malaoxon (B) docked at *Ag*AChE with a noncovalent function……...……..80

Figure 5: Acephate (A) and methamidophos (B) docked at *Ag*AChE with a noncovalent function……...……..80

Figure 6: Malathion (A) and malaoxon (B) re-docked at *Ag*AChE with MM-GBSA method……..82

Figure 7: Acephate (A) and methamidophos (B) re-docked at *Ag*AChE with MM-GBSA method. ……..……..82

Figure 8: Malathion (A) and malaoxon (B) docked at *Ag*AChE with new covalent function.……..……..83

Figure 9: Acephate (A) and methamidophos (B) docked at *Ag*AChE with new covalent function.……..……..83

Figure 10: Superimposed dimethoate in noncovalent (green) and covalent (violet) binding states...……..86

Figure 11: Superimposed malathion (A) and malaoxon (B) in noncovalent (green) and covalent (violet) binding states…….86
Figure 12: Superimposed *Ag*AChE active site amino acid residues non-aged (green) and aged by malathion or malaoxon (red) versus acephate or methamidophos (violet). **(A)** Shows the catalytic serine reorientation (arrow) in malathion or malaoxon phosphorylation (red) and **(B)** shows the reorientation distance from the non-aged catalytic serine (2.55 Å) and increased distance from histidine (3.42 Å). Ligands have been undisplayed to enhance visibility……..……..87

CHAPTER 4

Figure 1: Chemical structure of donepezil showing two common sides of derivatization: A) indanone moiety and B) methyl linker…… .103
Figure 2: Chemical structures of the nine donepezil derivatives screened. 1-benzyl-*N*-(2-morpholinoethyl) piperidine-4-carboxamide **1**, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2**, 1-benzyl-*N*-(3,4,5-trimethoxyphenethyl) piperidine-4-carboxamide **3**, 1-benzyl-*N*-(pyridine-3-ylmethyl) piperidine-4-carboxamide **4**, 1-benzyl-*N*-(furan-2-ylmethyl) piperidine-4-carboxamide **5**, *N*-[2-(1H-imidazol-4-yl)ethyl]-1-benzylpiperidine-4-carboxamide **6**, 1-benzyl-*N*-(1,2,3,4-tetrahydro-naphthalen-1-yl) piperidine-4-carboxamide **7**, 1-benzyl-*N*-(2-(piperidin-1-yl)ethyl)piperidine-4-carboxamide **8**, and 1-benzyl-*N*-(pyridine-4-ylmethyl) piperidine-4-carboxamide **9**……..……..……...105
Figure 3: Quality of the *An. arabiensis* homology model. A) Shows the quality estimate of the generated model based on its similarity to the template and B) shows its comparison to non-redundant 3D structures…….……...……..113
Figure 4: Validity of the model displayed by Verify3D tool (A) and scored by MolProbity (B) and the corresponding Ramachandran plot (C)…….……115
Figure 5: Alignment of the catalytic sites of the generated *An. arabiensis* AChE (sky blue) and reference *An. gambiae* (plum)……...……..116
Figure 6: Molecular interactions of a derivative **2** with electric eel AChE (A; Score: -11.8) and *An. arabiensis* (B; Score: -7.5)……..……117
Figure 7: Superimposed amino acid residues showing the catalytic site entrance and the catalytic triad of electric eel (red), human (green) and *Anopheles* (blue) AChE. The distinct Cys^{447} at the *Anopheles* AChE catalytic entrance and Asp^{602} at the catalytic site base are shown by circles. ……..……118

Figure 8: Comparison of the molecular interactions of donepezil with the AChE catalytic sites of electric eel (A; PDB: 1EVE; score: -15.0), human (B; PDB: 4EY7; score: -16.2) and An. gambiae (C; PDB: 5YDI; score: -10.2)……..……....119

Figure 9: Comparison of the molecular interactions of derivative **2** with the AChE catalytic sites of *An. arabiensis* model (A; score: -7.5), wild-type *An. gambiae* (B; score: -9.2), target site mutated *An. gambiae* (C; score: -7.8), *An. coluzzii* model (D; score: -9.0) and *An. funestus* model (E; score: -9.1)……..122

CHAPTER 5

Figure 1. Chemical structures of the terpenoids……..……...141

Figure 2. Differences in farnesol poses against *Anopheles* AChE (A) and electric eel AChE (B)…….……..146

Figure 3. Comparison of propoxur binding pose against *Anopheles* (A) and electric eel (B)…….……..146

Figure 4. Crystal poses of (-)-α-bisabolol (A), *cis*-nerolidol (B), *trans*-nerolidol (C) and methyleugenol (D) in the *Anopheles* AChE binding site…….…….……..147

Figure 5: Superimposition of terpenoid poses on the active site of *Anopheles* AChE. (*R*)-(+)-Limonene lodged at the active site entrance is shown in green…….…….……148

CHAPTER 6

Figure 1: Chemical structures of the EOCs and a positive control, propoxur, investigated for insecticidal activity……...……..168

Figure 2: The IC_{50}-based comparison of the egg hatching inhibitory potential of EOCs…….…….……171

Figure 3: The structural comparison of methyleugenol and propoxur, a standard insecticide. These molecules share a 1,2-dimethoxybenze moiety (circled).…….…….……..172

Figure 4: The close structural similarities between farnesol and (-)-α-bisabolol is indicated by the former cyclising to the latter….…….175

CHAPTER 7

Figure 1: Cytochrome C oxidase standard curve (inset graph) used to determine the linearity of TMB substrate peroxidation (main graph) (cytochrome C concentration: 1.036 to 66.600 μM)…….……199

Figure 2: Monooxygenase activity in *An. funestus*. **(A)** Comparison of the equivalent units of cytochrome P450/mg protein in *An. funestus* FUMOZ-R and *An. funestus* FANG**; (B)** Comparison of the P450 activity in *An. funestus* FUMOZ-R and *An. funestus* FANG…..200

Figure 3: The susceptibility status of FANG and FUMOZ-R against three most active EOCs assessed at their double LC_{99} concentrations (a). The comparison of FANG and FUMOZ-R susceptibility to three less active EOCs and one EO (b). The terpenoid-induced mortalities were compared to deltamethrin. *Only deltamethrin in (a) showed significantly different mortality rates between FANG and FUMOZ-R strains (p<0.05) while all test compounds in (b) showed significant difference in the mortality against FANG versus FUMOZ-R (p<0.05)…….....202

CHAPTER 8

Figure 1: Chemical similarity of propoxur and methyleugenol. The common 1,2-dimethoxybenze group is enclosed in a rectangular box…….223

Figure 2: Propoxur (A) and methyleugenol (B) in the *Anopheles* AChE binding site……...……...224

Figure 3: The close similarity of farnesol and (-)-α-bisabolol chemical structures. Farnesol is aliphatic while (-)-α-bisabolol is cyclic……..224

Figure 4: Comparison of the molecular interactions of farnesol (A) and (-)-α-bisabolol (B) with the *Anopheles* AChE binding site……...225

Figure 5: Comparison of the *Anopheles* AChE binding profiles of farnesol *cis*-nerolidol (A) and *trans*-nerolidol (B)……...……226

LIST OF TABLES

CHAPTER 2

Table 1: Anticholinesterase and insecticidal activities of essential oils……..……..39

Table 2: Major constituents of EOs with anticholinesterase insecticidal activity……41

Table 3: Anticholinesterase and insecticidal activities of essential oil constituents……..….44

CHAPTER 3

Table 1: Glide docking scores for OPs bonded to 5YDI target *Ag*AChE protein……...……84

Table 2: Comparison of amino acid residues involved in the binding of OPs to 5YDI target *Ag*AChE protein in different docking methods……...…..85

CHAPTER 4

Table 1: Larvicidal activities of the assessed compounds……..110

Table 2: Comparison of the *Anopheles* AChE inhibitory potential of derivatives **1, 2, 5** and **8**……...…...111

Table 3: Comparison of the electric eel AChE inhibitory potential of derivatives **1, 2, 5** and **8**……..……112

CHAPTER 5

Table 1: Comparison of the larvicidal and artemicidal activities of the assessed terpenoids.…….....143

Table 2: Comparison of the *Anopheles* and electric eel anticholinesterase activity of the identified terpenoids…….…..145

Table 3: The binding scores and amino acid interactions of terpenoids and *Anopheles* AChE

Table 4: The comparison of the docking scores and amino acid interactions of farnesol and propoxur against electric eel AChE……..…..149

CHAPTER 6

Table 1: Concentration-based comparison of the *Anopheles* egg hatching inhibitory activities of the select EOCs compared to propoxur…….……170

Table 2: Concentration-dependent larval lethality of the assessed EOCs compared to propoxur…….173

Table 3: The EOC-induced pupal sensitivity to standardized concentrations compared to propoxur……...……..174
Table 4: Concentration-dependent adulticidal activity of EOCs against *An. arabiensis*……...……176
Table 5: Concentration-dependent adulticidal activity of propoxur against *An. arabiensis*……...……176

CHAPTER 7

Table 1: WHO susceptibility analysis of *An. funestus* colonies, FANG and FUMOZ-R, to % deltamethrin……..……..198
Table 2: The lethality concentrations of EOCs against pyrethroid-susceptible *An. funestus*…..……..……..…..201
Table 3: Activities of select EOCs against *An. funestus* FUMOZ-R……..203
Table 4: Comparison of terpenoid potencies between pyrethroid-susceptible and resistant *An. funestus* strains……204
Table 5: Evaluation of *An. funestus* FUMOZ-R susceptibility to deltamethrin and EOCs with and without a synergist……...…205
Table 6: Co-toxicity effects of four select EOCs in combination with PBO against *An. funestus* FUMOZ-R……..205

LIST OF ACRONYMS AND ABBREVIATIONS

3D:	3-dimensional
Å:	Angstrom
ACh:	Acetylcholine
AChE:	Acetylcholinesterase
*Ag*AChE:	*Anopheles gambiae* Acetylcholinesterase
ANOVA:	Analysis of variance
BChE:	Butyrylcholinesterase
CDC:	Centers for Disease Control and Prevention
CNS:	Central Nervous System
CYP450:	Cytochrome P450
DDT:	Dichloro-diphenyl-trichloroethane
DEET:	*N*,*N*-diethyl-meta-toluamide
DF:	Degree of Freedom
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic acid
DTNB:	Dithiobis(2-nitrobenzoic acid)
EO:	Essential Oil
EOC:	Essential Oil Constituent
et al:	et alia (and others)
FDA:	Food and Drug Administration
GABA:	γ-Aminobutyric Acid
GMQE:	Global Model Quality Estimate
IC_{50}:	50% Inhibitory Concentration
IRS:	Indoor Residual Spraying
ITNs:	Insecticide-Treated Mosquito Nets
kdr:	knockdown resistance
L:	Litre
LC_{50}:	50% Lethal Concentration
LD_{50}:	50% Lethal Dose
LC_{90}:	90% Lethal Concentration
LC_{99}:	99% Lethal Concentration
LLINs:	Long-Lasting Insecticidal Nets

µg: microgram

µg/ml: microgram per millilitre

mg: milligram

mg/ml: milligram per millilitre

µL: microlitre

mL: millilitre

µM: micromolar

mM: millimolar

MM-GBSA: Molecular Mechanics - Generalised Born Surface Area

nAChR: Nicotinic Acetylcholine Receptor

NPs: Natural Products

OP: Organophosphate

PBO: Piperonyl Butoxide

PDB: Protein Data Bank

QSAR: Quantitative Structure-Activity Relationship

QSQE: Quaternary Structure Quality Estimation

TMB: 3,3′,5,5′-Tetramethylbenzidine

U/mL: Units per millilitre

v/v: volume per volume

w/v: weight per volume

WHO: World Health Organization

CHAPTER 1
Introduction

1. Background

Malaria is a life-threatening disease caused mainly by *Plasmodium falciparum* parasites that are further transmitted to humans by infected female mosquitoes of the *Anopheles* genus (WHO, 2017). The recent World Health Organization (WHO) Malaria Global Report indicated a fourteen million increase in 2020 malaria cases (241 million) compared to 2019 (227 million), accompanied by a 12% increase in deaths (627 000 deaths (2020) versus 558 000 deaths (2019)). The WHO African Region reported exceedingly high malaria burdens in the African region encompassing 95% and 96% of global cases and deaths, respectively (WHO, 2021a). The main challenge in malaria treatment is the escalating and recurring resistance to antimalarial drugs and insecticides (WHO, 2016; CDC, 2020). Resistance of *P. falciparum* to previously potent antimalarial drugs including sulfadoxine-pyrimethamine and chloroquine has become widespread in Africa (Roux et al., 2021). Likewise, the *Anopheles* resistance to various classes of insecticides has been reported across the African continent (Pinda et al., 2020; WHO, 2021b).

1.1. *Anopheles* life cycle

As for all mosquitoes, the *Anopheles* mosquitoes' life cycle is composed of four stages namely, egg, larva, pupa, and adult, with the first three stages being aquatic (Figure 1). At adult stage, certain species of the female *Anopheles* mosquitoes are a potential malaria vector (Service, 1980). The adult females lay eggs in water and these hatch in a period of two to three days. The larvae stages progress through four instars where moulting occurs as each instar is completed to allow for the next stage of growth. Fourth instar larvae develop into a comma-shaped pupa before maturing into an adult mosquito within a few days. A fully developed adult mosquito has three body sections: head, thorax and abdomen (Figure 1) (Service, 1980; Williams and Pinto, 2012).

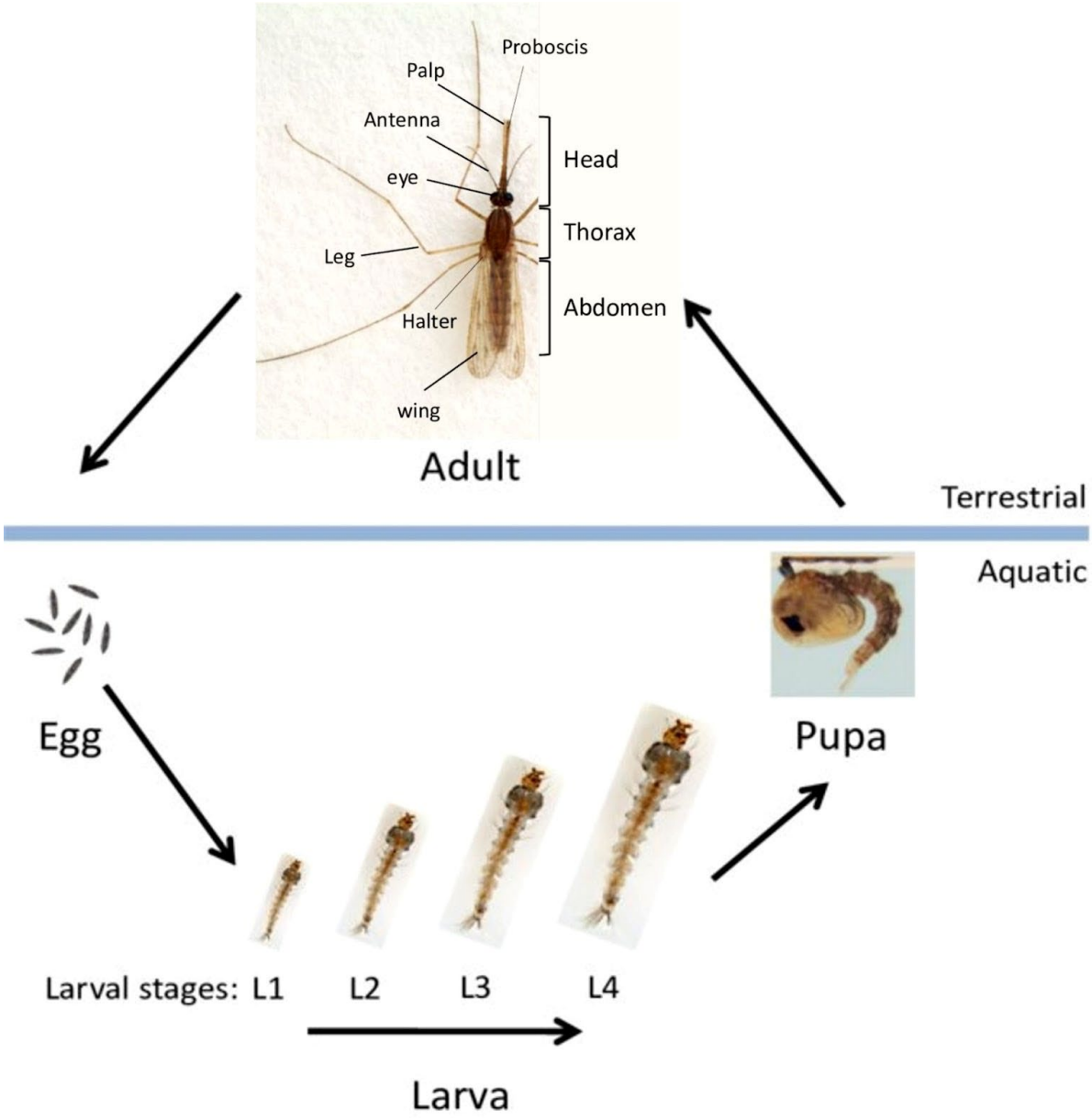


Figure 1: Four *Anopheles* stages of growth (Adapted from Williams and Pinto, 2012).

1.2. Malaria control

The control of malaria includes three integrated interventions: vector control, early diagnosis and treatment. Vector control is an essential strategy for reduction of the mosquito population resulting in a reduction in malaria transmission. Equally so, early diagnosis and malaria treatment with standard antimalarial drugs are important in preventing and reducing malaria transmission (WHO, 2018). Therefore, vector control interventions are used together with antimalarial drugs in the standard malaria control strategies (WHO, 2016). This thesis focused on vector control as an essential element in preventing malaria transmissions.

1.2.1. Malaria vector control

Vector control involves two main strategies, which are the use of insecticidal agents and insect repellents. The repellents can be impregnated in clothing, nets or be applied as creams, lotions, soaps, candles, or sprays for personal (topical repellent) or area (spatial/airborne repellent) protection (Nguyen et al., 2018; Maia et al., 2018). The common constituent in the insect repellent products is *N*,*N*-diethyl-3-methylbenzamide (DEET). This synthetic repellent is generally safe and has been used as a repellent since the 1950s. The alternative repellents include natural products such as citronella oil, eucalyptus oil, and catnip oil (Nguyen et al., 2018; Swale and Bloomquist, 2019). However, the potential effectiveness of insect repellents in reducing malaria transmissions has limited evidence (Chen-Hussey et al., 2013; Maia et al., 2018).

The vector control intervention with public health impact relies on the use of insecticides. With insecticides, there are mainly two interventions: indoor residual spraying (IRS) and insecticide-treated mosquito nets (ITNs) or long-lasting insecticidal nets (LLINs) (WHO, 2016). Insecticide classes include pyrethroids, organochlorines, carbamates, organophosphates, neonicotinoids, bacterial larvicides, insect growth regulators, pyrroles and spinosyns (Sparks and Nauen, 2015; WHO, 2021c). The major insecticide classes used in malaria vector control are pyrethroids, organochlorines, carbamates, and organophosphates. Recently, the combination of a pyrethroid with a P450 monooxygenase synergist, piperonyl butoxide, has been added to the vector control intervention list (WHO, 2021a). The ITNs use pyrethroids and the IRS relies mostly on organophosphates and more recently, neonicotinoids (WHO, 2021a). Pyrethroids and organochlorines target voltage-gated sodium channels in which they cause sustained depolarization state of the neurons, which in turn leads to paralysis and death (Field et al., 2017). The carbamates and organophosphates inhibit the key enzyme in the cholinergic system, namely, acetylcholinesterase (AChE), leading to unregulated cholinergic activity with consistent activation of the post-synaptic membrane which eventually results in paralysis and death (Fukuto, 1990). Similarly, the neonicotinoids modify the insect's cholinergic activity by antagonizing the binding of a neurotransmitter acetylcholine to the post-synaptic membrane receptors, namely nicotinic acetylcholine receptors (Simon-Delso et al., 2015). The focus of the current study was on the cholinergic activity of *Anopheles* nervous system, particularly the AChE activity. More detailed discussion on *Anopheles* AChE activity, challenges with current

AChE inhibitors and potential for natural product-based novel AChE inhibitors is found in Chapter 2.

1.3. *Anopheles* vectors

1.3.1. *Anopheles*-human host interaction

The female *Anopheles* mosquitoes transmit malaria through taking a blood meal from an infected human host in which they ingest *Plasmodium* gametocytes (sexually matured (male and female) erythrocytic stage of the *Plasmodium* parasite) (Figure 2) (CDC, 2020). The male (micro-gamete) and female (macro-gamete) gametocytes mate in the mosquito's midgut to form zygotes. The zygotes develop into ookinetes that then penetrate the *Anopheles* midgut wall and form oocysts. These oocysts rupture and form sporozoites that are ready to infect the next human host. To enhance the disease transmission, sporozoites get concentrated in mosquito's salivary glands in which they get released upon the bite of the human host (CDC, 2020; Gitta and Kilian, 2020). This parasitic development process within the mosquito host is known as a sporogonic cycle (CDC, 2020).

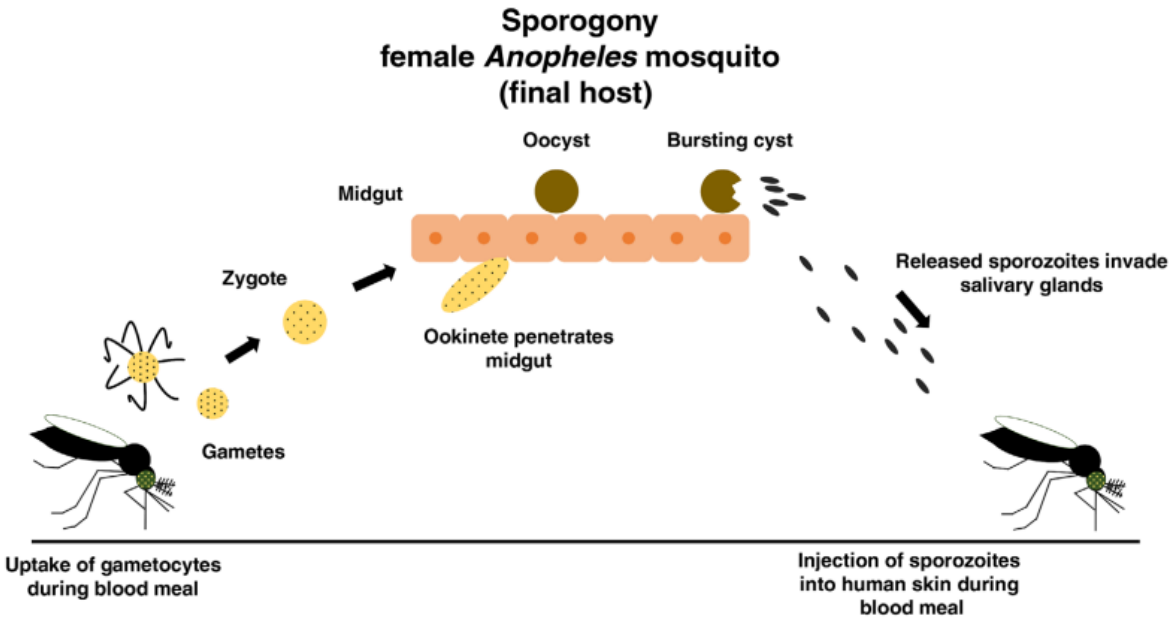


Figure 2: The parasite developmental stages within the *Anopheles* host (Adapted from Gitta and Kilian, 2020).

1.3.2. *Anopheles* species in Africa

Species from the *Anopheles gambiae* complex; *An. gambiae*, *An. arabiensis*, and *An. coluzzii* as well as *An. funestus* from the *An. funestus* group are the main malaria vectors in Africa (WHO, 2021a; Dahan-Moss et al., 2020; Sinka et al., 2012). In the recent literature, other

Anopheles species such as *An. stephensi* and *An. merus* are becoming more common in malaria transmissions (Bartilol et al., 2021; WHO, 2021a). *Anopheles stephensi* was localized in Asia before spreading to Africa where it was first reported from Djibouti in 2012 (Faulde et al., 2014; Kweka, 2022; Mnzava et al., 2022). *Anopheles merus* is a member of the *An. gambiae* complex that is fast becoming an important malaria vector in East and Southern Africa including South Africa (Mbokazi et al., 2018; Bartilol et al., 2021). Other species identified in the African region include *An. melas*, *An. nili s.l.*, *An. moucheti*, as well as *An. pharoensis* to mention but a few (Sinka et al., 2012; Antonio-Nkondjio and Simard 2013; Moyo et al., 2021; WHO, 2021a).

1.4. Insecticide resistance

Significant resistance to the commonly used insecticides for vector control; organophosphates, organochlorides, carbamates and pyrethroids, has been reported (Engdahl et al., 2015; Brooke et al., 2013). This resistance can be due to various mechanisms such as target site mutation, metabolic enzymes detoxification, thickened cuticle, and behavioural avoidance (Weill et al., 2003; Witzig et al., 2013; Namias et al., 2021). Target site mutations mutations have been detected in the insecticide targets such as AChE and sodium channels which prevents effective binding of the concerned insecticides to their target site, thus affecting their effectiveness. The overexpressed metabolic enzymes such as P450 monooxygenases, glutathione *S*-transferases and nonspecific esterases have been shown to detoxify the insecticides, and this affects the effectiveness of these insecticide (Witzig et al., 2013; Prasad et al., 2017; Asadi et al., 2019; Tchouakui et al., 2019; Namias et al., 2021). On the other hand, the cuticular mechanism reduces the penetration capacity of insecticides through the *Anopheles* epicuticle. The behavioural mechanism is observed whereby mosquitoes develop a sense of identification and avoidance of insecticide-treated areas such as pyrethroid ITNs (Ariaratnam et al., 1975; Lewis, 1980; Wood et al., 2010; Kreppel et al., 2020; Namias et al., 2021). The latter mechanism is not fully understood (He et al., 2019; Namias et al., 2021).

Among these resistance routes, this thesis focused on P450 monooxygenases; major metabolic enzymes that detoxify pyrethroids, the only class of insecticides approved for use on bed nets (Brooke et al., 2001; Hunt et al., 2005; WHO, 2021a). Therefore, while aiming to identify novel *Anopheles* AChE inhibitors, this study also monitored a change in their insecticidal activity against *Anopheles* species that had upregulated P450 monooxygenases.

1.5. Acetylcholinesterase and inhibitors

1.5.1. Acetylcholinesterase in *Anopheles*

Two types of cholinesterase, AChE and butyrylcholinesterase (BChE), are physiologically present in both humans and mosquitoes. To avoid co-activity in humans, insecticide specificity for the mosquito's AChE is essential (Dou et al., 2013). Localization of AChE is mainly the neuronal synapses while BChE is mainly found in blood plasma (Taylor et al., 2009). While AChE can be found in the central nervous system and neuromuscular junctions in humans, it is localized only in the central nervous system on insects; whereby it serves to maintain normal neuronal impulse transmission (Casida and Durkin, 2013).

Inhibition of AChE has been the target site for two of the four major classes of insecticides: organophosphates and carbamates. Exposure to these insecticides leads to insect death from neuronal hyperexcitation (English and Webster, 2012). However, mutations in the AChE genes have greatly decreased the effectiveness of these insecticides (Casida et al. 2004). Unlike humans where a single *ace* gene codes for AChE, mosquitoes have two *ace* genes (*ace*-1 and *ace*-2) coding for AChE1 and AChE2 genotypes, respectively (Weill et al., 2002; Hoffmann et al., 1992). The DNA sequence showed that there is about 53% similarity between *ace*-1 and *ace*-2 genes in *Anopheles* mosquitoes (Weill et al., 2002; Elamathi et al., 2016). The AChE1 is responsible for the enzymatic activity in the cholinergic system while AChE2 is involved in the reproductive system development of female mosquitoes (Lu et al., 2012). The mutation responsible for insecticide resistance has been reported in AChE1 (*ace-1*^{R}) whereby a glycine was converted to serine at position 119 (G119S) in *An. gambiae*, and at lower intensities on *An. coluzzii* and *An. arabiensis* (Essandoh et al., 2013; Weill et al., 2003; Keita et al., 2020).

1.6. Monooxygenases

Multiple P450 monooxygenases have been associated with insecticide resistance (Cassone et al., 2014; Witzig et al., 2013). These vary between species and even geographical variations within individual species. The amplified P450 monooxygenases mainly reduce the activity of pyrethroids though other insecticides including organochlorines, carbamates and organophosphates are affected at comparatively lower intensities (Nardini et al., 2013; Matowo et al., 2022). The main P450 genes implicated in pyrethroid resistance include *CYP6P4*,

CYP6M2, *CYP6P3* (Müller et al., 2008; Wagah et al., 2021; Matowo et al., 2022), *CYP6P9*, *CYP12F2*, *CYP6AG1*, *CYP6Z1* and *CYP6Z3* (Nardini et al., 2013; Chiu et al. 2008, Ibrahim et al., 2016; Norden et al., 2022) just to name a few. The overexpressed P450 monooxygenases have been detected in *An. funestus* and *An. arabiensis*, *An. gambiae* and *An. coluzzii* (Christian et al. 2011; Koekemoer et al., 2011, Munhenga and Koekemoer, 2011; Venter et al., 2017; Ingham et al., 2017; Wagah et al., 2021; Nolden et al., 2022).

1.7. Novel AChE inhibitors

Due to the increasing resistance to insecticides, there is an urgent need for the discovery of novel insecticides with capacity to overcome resistance (Pinda et al., 2020; WHO, 2021b). AChE serves as a critical target for the neuroactive agents (discussed extensively in Chapter 2) (Ĉolović et al., 2013). A molecular mechanism of current AChE inhibitors shows that they form an irreversible complex with the AChE catalytic serine residues that is responsible for acetylcholine hydrolysis (Hörnberg et al., 2007; Rants'o et al., 2022). However, this catalytic serine residue is also present in mammals and is often targeted by the AChE inhibitors used in the treatment of Alzheimer's disease (Dou et al., 2013). Using the *in silico* studies, this thesis showed key differences between the AChE binding sites of mammals and *Anopheles* mosquitoes, indicating the possibility of identifying *Anopheles* selective anticholinesterases.

The AChE inhibitors that have been originally designed for the treatment of Alzheimer's disease but showed no activity against the human target have been suggested as a promising group of novel compounds that may act against the *Anopheles* AChE (Pang et al., 2009; Hartsel et al., 2012; Dou et al., 2013; Souto et al., 2021; Montgomery et al., 2022). Structural analogues of the most potent AChE inhibitor, donepezil, a piperidine derivative used for Alzheimer's disease, that lacked significant activity against mammal AChE (van Greunen et al., 2019) have been explored for potential activity against *Anopheles* AChE in Chapter 4 of this thesis.

Among the plant-derived natural products (NPs), essential oils (EOs) have been widely studied for their insecticidal activity (Gnankiné and Bassolé, 2017; Luo et al., 2022; Wangrawa et al., 2022). Though these NPs have been implicated in AChE inhibition of other mosquito species (Seo et al., 2015; Liu et al., 2021; Santos et al., 2021), AChE inhibition as the mechanism of insecticidal activity in *Anopheles* species by EOs and essential oil constituents (EOCs) has not been well studied and characterized.

In this study, the anticholinesterase activity of select EOCs was studied *in vitro* and their insecticidal activities evaluated using *in vivo* bioassays. With molecular modelling being a substantial tool for studying the mechanism of action of compounds at a molecular level (Kanitkar et al., 2021; Rants'o et al., 2022), various techniques of molecular modelling have been used to elucidate the *Anopheles* AChE inhibition by terpenoids in this thesis for the first time. Moreover, the effectiveness of *Anopheles* anticholinesterases against *Anopheles* colonies with overexpressed monooxygenases is reported in this study.

In general, AChE is a critical enzyme in the *Anopheles* central nervous system. Biochemical and molecular studies have suggested critical differences between mammal and *Anopheles* that create opportunity for identification of *Anopheles* selective AChE inhibitors. In order to identify selective *Anopheles* anticholinesterases, molecular modelling technology can be used to study the binding differences of potential AChE inhibitors between the *Anopheles* and mammal targets. On the other hand, the upregulated P450 monooxygenases is the most concerning *Anopheles* resistance mechanism that compromises vector control interventions. Hence, the goal of this project was to identify novel *Anopheles* AChE selective inhibitors with sustained insecticidal activity against *Anopheles* species with overexpressed P450 monooxygenases.

1.8. Rationale of the study

The resistance against insecticides by all main African malaria vectors has increased in both intensity and geographical distribution (WHO, 2021b). The target site for almost all approved insecticides including pyrethroids, organochlorines, organophosphates, carbamates, and neonicotinoids is the insect nervous system. Due to small sizes, the mosquitoes have a short pathway to the nervous system making it easy for the penetration and subsequent systemic distribution of an insecticide from contact exposure (Davies et al., 2007). The pyrethroids and organochlorines target the voltage-gated sodium channels in the peripheral and central nervous system (Silver et al., 2014). The organophosphates, carbamates, and neonicotinoids modify the cholinergic activity of the central nervous system through alteration of the AChE activity (organophosphates and carbamates) or agonism at nicotinic acetylcholine receptors (neonicotinoids). Acetylcholine is a key excitatory neurotransmitter in the insect nervous system making AChE the main nervous system enzyme (English and Webster, 2012; Simon-Delso et al., 2015). AChE is therefore a critical target for the discovery of novel insecticides

hence current studies are aimed at identifying novel AChE inhibitors with high selectivity towards *Anopheles* over the corresponding mammalian targets (Engdahl et al., 2016; Carlier et al., 2017).

A potent human AChE inhibitor, donepezil, has been shown to be active against insect AChE, but is about 40 times more selective to human than the corresponding *Anopheles* target (Williamson et al., 2013; Engdahl et al., 2015). The current study assessed the donepezil derivatives prepared by van Greunen et al. (2019) for potential selective *Anopheles* AChE inhibition and rationalised their binding profiles through molecular docking. On the side of NPs, previous studies have identified essential oils as potential sources of cost-effective and eco-friendly bioinsecticides (Pavela, 2015; Isman, 2020). Various terpenoid classes including sesquiterpenes, monoterpenes, and diterpenes form the main chemical constituent repository of essential oils (Sharifi-Rad et al., 2017). Jankowska et al. (2017) stated that some essential oils display the insect neurotoxic effects characterized by rapid paralysis and death. This is consistent with neurotoxic effects caused by AChE inhibition (Colović et al., 2013). Indeed, some essential oils and EOCs have shown the anticholinesterase potential (Miyazawa and Yamafuji, 2005; Alout et al., 2012). This study assessed several EOCs including sesquiterpene alcohols, monoterpenes and phenylpropanoids for *Anopheles* AChE. All *in vitro* AChE screening assays were carried out on both *Anopheles* and electric eel AChE targets for selectivity analysis.

In this study, a novel *in silico* approach of the integrated homology modelling, molecular modelling, and molecular dynamic simulations was used to characterize the AChE binding profiles of donepezil derivatives and terpenoids. Molecular structures of human, electric eel and *Anopheles* AChE binding sites have been extensively studied to identify key differences that may explain the differences observed in the *in vitro* activities between these targets and permit the rational design of selective *Anopheles* AChE inhibitors for future studies.
For relevance to field application, the identified AChE inhibitors from *in vitro* and *in silico* studies were assessed for *in vivo* activity against all four stages of *Anopheles* life cycle including egg, larva, pupa and adult. The laboratory strains of four main African malaria vectors *An. gambiae* (COGS), *An. arabiensis* (KWAG), *An. funestus* (FANG, FUMOZ and FUMOZ-R) and *An. coluzzii* (G3) were used in this study. The cytotoxicity potential of the identified *Anopheles* AChE inhibitors was assessed through artemicidal assay using *Artemia franciscana* (Hübsch et al., 2014).

1.9. Aim and objectives

1.9.1. Aim

The overall aim of this thesis was to identify novel *Anopheles* AChE selective inhibitors with unique molecular binding profiles coupled to the insecticidal activity that is sustained in *Anopheles* species with upregulated P450 monooxygenases.

1.9.2. Specific objectives

To achieve the aim of this project, the following objectives were set:

A. To determine and characterize the AChE inhibition of select terpenoids by evaluating them against AChEs from electric eel and *Anopheles* using *in vitro* enzyme assays and *in silico* modelling technology.
B. To assess the aquatic toxicological profile of the identified AChE inhibitors against *Artemia franciscana.*
C. To evaluate the identified *Anopheles* anticholinesterases for insecticidal activity against *Anopheles* vector life stages including egg, larva, pupa and adult.
D. To assess the change in insecticidal activity of novel *Anopheles* AChE inhibitors when screened against the *Anopheles* species with upregulated P450 monooxygenases.
E. To assess the combination effect of novel *Anopheles* AChE inhibitors with synergists on *Anopheles* species with overexpressed P450 monooxygenases

2. Structure of the thesis

The structure of this thesis follows the integrated format outlined in the University of the Witwatersrand's Faculty of Health Sciences guide for PhD thesis formats. Other chapters of this thesis are presented in the form of published work, manuscript submitted for publication and final manuscript ready for submission to the identified journal. For this reason, the general formatting and referencing styles of such chapters are aligned with the requirements of their specific journals.

Chapter 1: The introduction chapter discusses the background information of the study, the study rationale, significance of the study, outline the study aim and objectives, and conceptual framework.

Chapter 2: The literature review chapter. This chapter is presented as a published paper entitled "Potential of essential oil-based anticholinesterase insecticides against *Anopheles* vectors: A review". The paper discusses acetylcholinesterase as a critical target in the *Anopheles* nervous system and essential oils as potential sources of novel inhibitors of this target. Therefore, this paper establishes the base for this thesis. It has been published in the special edition (Essential Oils: Characterization, Biological Activity and Application) in Molecules (Molecules 2022;27(20): 7026; https://doi.org/10.3390/molecules27207026).

Chapter 3: This chapter outlines the study methodology. The chapter consists of a published paper. As indicated in objective A, this study aimed to evaluate the acetylcholinesterase inhibition potential through both the *in vitro* and *in silico* methods. The paper presents the optimization of *in silico* methods including molecular modelling and molecular mechanics simulations. These optimized methods were further used in the following chapters of this thesis to assess the binding modes of the identified novel *Anopheles* AChE inhibitors. This paper was published in the Journal of Molecular Graphics and Modelling (J Mol Graph Model. 2022;110: 108054; https://doi.org/10.1016/j.jmgm.2021.108054).

Chapter 4: This chapter is presented as a published paper. As done in Chapter 3, molecular modelling is performed using a 3-dimensional (3D) structure of the target protein. If available, these proteins can be obtained in Protein Data Bank (PDB). In the case where the required protein is unavailable, the method named homology modelling is used to generate new 3D protein structures. These new models should be validated and meet the set quality standards before use in the molecular modelling work. Among the *Anopheles* species used in this thesis (*An. gambiae*, *An. arabiensis*, *An. coluzzii* and *An. funestus*), only *An. gambiae* had its AChE crystal structure available in the PDB repository at the time of writing this thesis. Therefore, Chapter 4 focuses mainly on the application of homology modelling to generate AChE crystal structures for *An. arabiensis*, *An. coluzzii* and *An. funestus*.

Moreover, the *in vitro* assessment of *Anopheles* AChE inhibition, *in vivo* larvicidal activity against the assessed *Anopheles* species along with aquatic toxicity assessment through artemicidal assay were conducted in this published paper. This paper used a set of compounds derived from a known potent human AChE inhibitor, donepezil. These compounds were used to optimize the methodology before assessing the EOCs. Moreover, this was also based on the hypothesis that donepezil derivatives that lack activity against mammal AChE target may

selectively possess activity against insect target. This paper was published in PLoS ONE (PLoS ONE, 2022;9;17(11): e0277363; https://doi.org/10.1371/journal.pone.0277363). Both the objectives A and B have been fulfilled in Chapter 4.

Chapter 5: This chapter consists of a published paper. The paper outlines the *in vitro* and *in silico* assessment of select terpenoids for *Anopheles* AChE inhibition. This chapter utilized the same methods used in Chapter 3 but with a different set of compounds being the terpenoids. This chapter therefore addresses both objectives A and B by identifying selective *Anopheles* AChE inhibitors and assessing their artemicidal toxicity potentials. The paper has been published in Parasitology International (Parasitol Int. 2023;93: 102713; https://doi.org/10.1016/j.parint.2022.102713).

Chapter 6: The chapter presents a manuscript titled "Bioactivity of select essential oil constituents against life stages of *Anopheles arabiensis* (Diptera: Culicidae)". The manuscript has been submitted to Experimental Parasitology and is currently undergoing the review process (Manuscript ID: EXPARA-D-22-00337). This chapter addresses the objective C by identifying the *Anopheles* anticholinesterase EOCs with egg hatching inhibitory activity, larvicidal, pupicidal and adulticidal effects.

Chapter 7: This chapter presents evidence on the potential of essential oil constituents to reverse insecticide resistance. It is presented in the form of a published paper entitled "The insecticidal activity of essential oil constituents against pyrethroid-resistant *Anopheles funestus* (Diptera: Culicidae)". The paper has been published in Parasitology International (Parasitol Int. 2023;95: 102749; https://doi.org/10.1016/j.parint.2023.102749). Chapter 7 addresses objective D by identifying the *Anopheles* anticholinesterase EOCs that were not affected by the upregulated *Anopheles* CYP450 monooxygenases. It also addresses objective E by identification of the *Anopheles* anticholinesterase EOCs whose considerable activities against resistant *Anopheles* were only obtained through combination with standard CYP450 synergist, piperonyl butoxide.

Chapter 8: In this chapter, the broader study findings are discussed.

Chapter 9: In this chapter the study implications are outlined, and the conclusions are drawn. Furthermore, the study limitations as well as future recommendations are outined.

Chapter 10: Appendices. This section presents the published papers for Chapters 2 and 3, supplementary information for Chapters 3 and 4, author declarations, title approval letter and the ethical clearance certificates.

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CHAPTER 2

Literature review

This chapter presents a detailed literature review that was carried out in line with the aim of this study to assess the potential of identifying novel AChE inhibitors from the natural sources, mainly the essential oils. The chapter is presented in the form of a completed review manuscript.

This manuscript paved a way for the assessment of essential oil constituents in this study, for the *Anopheles* acetylcholinesterase inhibitory activity.

Chapter type: Published ISI paper (Appendix A.1)

Title of manuscript: Potential of essential oil-based anticholinesterase insecticides against *Anopheles* vectors: A review

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Conceptualization	✓			✓
Literature search	✓			
Data analysis	✓			
Writing – original draft preparation	✓			
Writing - review and editing	✓	✓	✓	✓
Supervision		✓	✓	✓
Funding acquisition		✓	✓	✓

Abstract: The insect nervous system is critical for its functional integrity. The cholinergic system, of which acetylcholinesterase (AChE) is a key enzyme, is essential to the *Anopheles* (consisting of major malaria vector species) nervous system. Furthermore, the nervous system is also the primary target site for insecticides used in malaria vector control programs. Insecticides, incorporated in insecticide-treated nets and used for indoor residual spraying, are a core intervention employed in malaria vector control. However, *Anopheles* resistance against these insecticides has grown rapidly. Due to this major setback, novel agents with potential activity against resistant *Anopheles* and/or capacity to overcome resistance against current WHO-approved insecticides are urgently needed. The essential oils have the potential to be natural sources of novel insecticides with potential to inhibit the *Anopheles* AChE target. In the current review, the scientific evidence highlights the ability of essential oils and specific essential oil constituents to serve as anticholinesterase insecticides. For this reason, the published data from scientific databases on the essential oils and essential oil constituents on anticholinesterase, ovicidal, larvicidal, pupicidal and adulticidal activities were analyzed. The identification of major constituents in active essential oils and their possible influence on the biological activity have also been critically evaluated. Furthermore, the toxicity to mammals as well as potential activity against the mammalian AChE target has also been reviewed. The importance of identifying novel potent insecticides from essential oils has been discussed, in relation to human safety and cost-effectiveness. Finally, the critical insights from this review can be used to inform future researchers towards potent and safe anticholinesterase insecticides for the management of *Anopheles* malaria vectors.

Keywords: malaria; insecticides; terpenoids; acetylcholinesterase

1. Introduction

Malaria is a devastating disease caused by a protozoan parasite, namely *Plasmodium falciparum* which is the major causative agent in the pathogenesis of this infectious disease [1–3]. *Anopheles* vectors are infected with malaria after they ingest blood from an infected human host. The female *Anopheles* vectors effectively bite the human hosts between dusk and dawn [3] and it is during this time that she ingests gametocytes. The *Plasmodium* gametocytes develop into an oocyst in the mosquito midgut, which then matures into sporozoites. The sporozoites are released into the hemolymph and migrate to the salivary glands [1,4]. This parasite developmental process within the vector takes approximately 11–16 days before the female mosquito is able to transmit the parasite to the next human host during a blood feeding. This means that a long lifespan of the *Anopheles* vector is required for the successful completion of the parasite development and reinfection of the human host. Vertebrate blood is needed every 2–3 days by the female mosquito for nutrition, as well as egg development. The eggs are oviposited into water and fertile eggs hatch into larvae a few days later. Larvae will develop into pupae and finally adults will emerge after a few days [3]. There are more than 400 *Anopheles* species of which about 30 are major malaria vectors. The African *Anopheles* vectors have both long lifespans and a higher preference for human feeding and, collectively, these account for the high malaria cases and mortality that is recorded in Africa [3,5]. Other factors, such as climate conditions and political and economic stability, also affect the intensity of transmission and enhance the problem [3].

The malaria vectors have long been controlled by using insecticides. Insecticide classes include organophosphates, carbamates, pyrethroids, organochlorines and neonicotinoids [6]. Larvicides including insect growth inhibitors as well as bacterial larvicides, such as *Bacillus thuringiensis* subspecies *israelensis, Bacillus sphaericus* and spinosyns from *Saccharopolyspora* species, have also gained popularity in mosquito control activities [6–8]. The implementation of large-scale larviciding is however challenging in Sub-Saharan Africa and these may be used as a complementary intervention [6,9–11]. The *Anopheles* vectors have developed substantial resistance against almost all current insecticides [12–15]. To compound the issue, the commercial development of insecticides through various and often complicated synthetic mechanisms is expensive and time-consuming [16,17]. We propose that the identification of potential insecticides from natural product resources, such as essential oils (EOs), is a relatively cost-effective and faster alternative. Target identification and the corresponding mechanism of action are critical components of the drug discovery process [18].

Acetylcholinesterase (AChE) is a validated target in the insect nervous system and inhibitors of this critical enzyme have been useful in the control of malaria vectors for over eighty years [6,19,20].

To facilitate future research in EOs and potential insecticidal activity through AChE inhibition, this review will first discuss malaria vector control systems and current challenges, and we will explore the insect nervous system with relevance to a specific neurotransmitter, acetylcholine (ACh), and provide an in-depth discussion on AChE function and inhibition. Finally, we identify the potential of various EOs and essential oil constituents (EOCs) as anticholinesterase agents against *Anopheles* vectors. This was achieved by collecting scientific data using keywords such as anticholinesterase/acetylcholinesterase inhibition, EOs, terpenoids/terpenes, *Anopheles*, larvicidal, pupicidal, adulticidal and insecticidal in the search engines of PubMed, Google Scholar, ScienceDirect, SciFinder, SCOPUS and Web of Science.

2. Malaria Vector Control

The early vector control strategies adopted vast activities to reduce larval populations, which included, amongst others, the drainage of breeding sites such as swamps or the application of copper (II) acetoarsenite (Paris green), a highly toxic inorganic compound to the breeding sites [21,22]. In addition, the screening of windows and doors to prevent vectors entering houses and the use of mosquito nets have been at the forefront in protecting people against mosquito bites [6]. The plant-based insecticides were the first preparations used historically. Pyrethrins extracted from the flowers of *Chrysanthemum cinerariifolium* and *Chrysanthemum roseum* were used against indoor *Anopheles* mosquitoes in the 19th century [23,24]. However, the structural modifications of the natural pyrethrins and the generation of first synthetic pyrethroids were first reported in the period 1924 to 1970 [25]. The discovery of an organochloride, namely, dichlorodiphenyltrichloroethane (DDT), was reported in 1939 [25,26]. DDT has been highly effective against malaria vectors. However, in recent times increasing safety concerns have seen it being replaced in many countries by newer insecticides with reduced toxicity profiles [27–29].

Currently, malaria vector control adopts an integrated vector management program through the use of insecticides targeting both the larval and adult stages [6,27]. This is achieved through two main interventions, namely, insecticide-treated mosquito nets and indoor residual spraying, with additional interventions including larviciding [6]. The insecticide-treated nets provide both a physical barrier and insecticidal activity against *Anopheles* vectors. Indoor residual spraying (IRS) on the other hand, provides host protection through the *Anopheles*

insecticidal effect [3]. Pyrethroids, pyrethroid-PBO combinations and pyrroles are the only insecticide classes used for the insecticide-treated nets, as the latter insecticides pose a low toxicity risk to humans [30,31]. The pyrethroids used in IRS include deltamethrin, alpha-cypermethrin, etofenprox, lambda-cyhalothrin, bifenthrin and cyfluthrin, while the organochlorines include DDT. On the other hand, the organophosphates approved by WHO for IRS include malathion, fenitrothion, pirimiphos-methyl and the carbamates such as propoxur and bendiocarb [6,32–34].

2.1. Insecticide Resistance in Main African Malaria Vectors

Insecticide resistance has been reported in all of the main African malaria vectors and this resistance against WHO approved insecticidal agents is rapidly increasing in intensity and geographical distribution [5,35,36]. An overview of mosquito resistance has been highlighted below. To keep this brief, only a few examples will be provided to explain the extent of the problem mainly on the African continent. Insecticide resistance in the main vector species has been reported for pyrethroids [35,37–41], organochlorides [38,41–43], organophosphates [12,44] and carbamates [20,38,43,45].

Common insecticide resistance markers associated with pyrethroid and organochloride resistance include the L1014F and L1014S mutation of the voltage-gated sodium channel gene, known as knockdown resistance (*kdr*). These mutations shift activation voltage dependence of sodium channels stabilizing them in the closed state. This antagonizes the action of pyrethroids and organochlorines since these compounds bind to open sodium channels [25,41,46,47]. Apart from the *kdr* mutations, elevated metabolic enzymes, including P450 monooxygenases, glutathione-S-transferases and non-specific esterases, also convey high resistance to pyrethroids and organochlorides [13,39,40,44,48,49]. On the other hand, the resistance mechanism commonly conferring organophosphate and carbamate resistance is a single point polymorphism resulting from glycine conversion to a serine residue at position 119 (G119S; *Torpedo californica* AChE numbering) or more precisely, position 280 (G280S; *Anopheles gambiae* AChE numbering) in the AChE target [41,47,50,51]. Resistance mechanisms often prevent the intended biological activity of a specific insecticide; therefore, it is important to discuss modes of action of the major insecticide classes.

2.2. Modes of Action of Main Insecticides Used in Malaria Vector Control

Regardless of their small size, insects have a high surface area for the penetration and subsequent systemic distribution of an insecticide from contact exposure. Furthermore, the

small size generates short pathways to the insect's nervous system and as a result, most insecticides act on the insect's nervous system [52]. Organochlorines act specifically on the peripheral nervous system, where they bind and stabilize the open voltage-gated sodium channels [25]. The stabilized open state of the sodium channels allows for continuous sodium influx and prolonged action potentials leading to spontaneous neuronal firings succeeded by muscle twitches and sustained body tremors [46]. In contrast to the organochlorines, pyrethroids act on both the peripheral and central nervous systems; however, they act in a similar manner to prevent the closing of the voltage-gated sodium channels, resulting in continuous neuronal discharges followed by paralysis [46]. Similarly, the organophosphates and carbamates also exert their effects on the central nervous system. However, these insecticide classes inhibit acetylcholinesterase, a principal enzyme in the insect nervous system, which leads to an increase in the neurotransmitter ACh levels in the synapse. This leads to enhanced ACh effects on the cholinergic receptors resulting in constant neurotransmission and neuronal hyperexcitation [53]. On the other hand, the neonicotinoids enhance cholinergic activity by acting as agonists on nicotinic acetylcholine receptors (nAChRs). Similarly, this disrupts neuronal transmission in the insect nervous system, causing paralysis and subsequent insect death [54]. Since the insect nervous system is the target site for all of the major insecticide classes, it is worth discussing it in more detail.

2.3. Insect Nervous System

The insect nervous system is composed of central, visceral and peripheral nervous systems [55]. The insect central nervous system (CNS) is composed of the ventral nerve cord and brain connected to various ganglia including supra- and sub-esophageal ganglia, thoracic ganglia and abdominal ganglia (Figure 1). The sub-esophageal ganglion transmits impulses to the mouthparts and salivary glands. The insect brain is composed of three cephalic neuromeres, including the protocerebrum, deutocerebrum and tritocerebrum. The deutocerebrum carries out olfactory and sensory functions through the antennae; where the olfactory signal transduction is important in host identification and interaction by the insect. The tritocerebrum nerves innervate the ventral nerve cord and internal organs including the anterior digestive canal [55–57]. The insect's peripheral nervous system, commonly referred to as a stomatogastric nervous system is composed of the peripheral ganglia complex and nerves that innervate visceral organs. This system mainly controls food intake and digestion. Generally, the insect CNS ganglia receive sensory impulses from the appendages and body cuticle after which the efferent signals are sent to the body muscles, internal organs and genitalia [58,59]. The protocerebrum

controls the insect's vision through compound eyes and ocelli. Most importantly, neurosecretory cells are located in the protocerebrum [59,60]. Most insecticides including the organophosphates and carbamates affect neurotransmitter secretion and action [61,62]. As a result, the insect neurotransmitters, more specifically ACh and its transmission cascade, will be discussed in more detail.

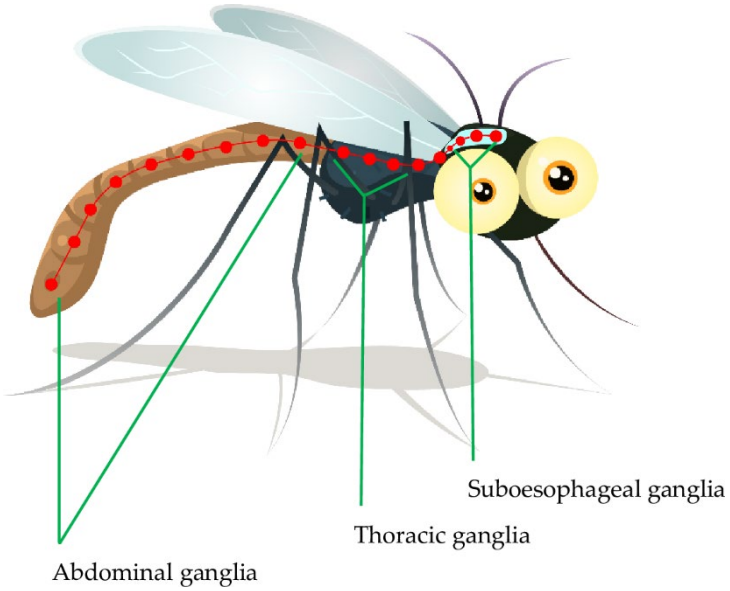


Figure 1. The nervous system ganglia of *Anopheles* [55].

2.3.1. Insect Neurotransmitters

The protocerebrum of the insect brain has nerves that innervate the corpora cardiaca, an organ located posterior to the brain that is composed of the neurohemal and endocrine sections. This enables the corpora cardiaca to perform the important role of neurotransmitter storage and release [55]. Biogenic amines play an important role in the insect nervous system as neuromodulators, neurohormones and neurotransmitters. Moreover, biogenic amines are also a key role player in associative learning and memory for insects. Common neurotransmitters found in the insect CNS include biogenic amines such as dopamine, 5-hydroxy-tryptamine, octopamine, noradrenaline and ACh [58,63,64]. In addition, other neurotransmitters important in the insect nervous system are histamine, glutamate and gamma-aminobutyric acid (GABA). GABA is a main CNS inhibitory neurotransmitter, while glutamate plays an excitatory role both centrally and peripherally [55,65]. For example, the insect heart rate and contraction are regulated by glutamate and other neurotransmitters including ACh, norepinephrine, dopamine, serotonin and octopamine [66]. ACh is a key excitatory neurotransmitter in the insect nervous system [67].

2.3.2. Acetylcholine and Its Function in Insects

ACh is synthesized from acetyl-coenzyme A and choline in insect cholinergic neurons and stored in nerve terminals inside presynaptic vesicles from which they are released upon detecting the nerve impulse. This impulse activates voltage-gated calcium channels leading to the influx of calcium ions that then induce ACh release from vesicles via exocytosis [68]. After being released in the synapse, ACh exerts its actions by binding to the postsynaptic nAChRs [69]. ACh is the main excitatory neurotransmitter in insects and its binding to nAChRs and generation of excitatory action potential controls the rapid synaptic neurotransmission process [70]. The nAChRs are ligand-gated channels controlled by binding of ACh and its subsequent removal from the binding site. As long as ACh is bound on the postsynaptic nAChRs, the nerve impulses are transmitted. However, this is short-lived as ACh is rapidly hydrolyzed from the binding site by a specialized enzyme, namely AChE. This ensures that the action potentials are initiated at precise and accurate intervals for efficient neurotransmission [71,72].

2.3.3. Acetylcholinesterase and Its Function in Insects

AChE belongs to the general family of cholinesterases; these are the specialized hydrolase enzymes that hydrolyze the choline ester bonds [73]. Therefore, AChE rapidly hydrolyzes the neurotransmitter ACh, to the resultant products, choline and acetate (Figure 2). Through this, it prevents constant nerve firings and maintains normal neuronal impulse transmission at cholinergic synapses and neuromuscular junctions [74,75].

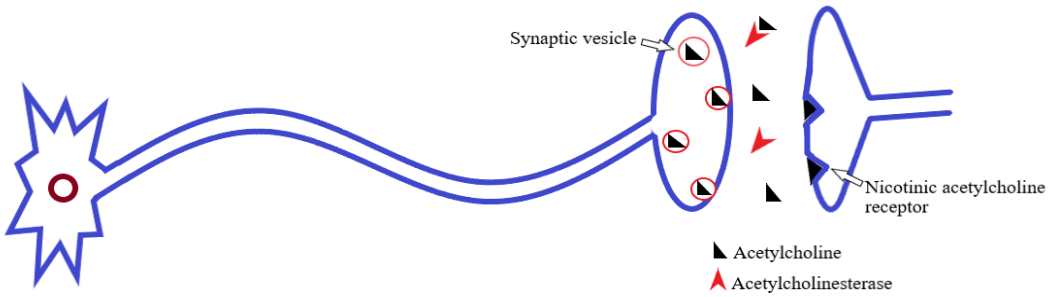


Figure 2. Acetylcholine release, postsynaptic receptor binding, and hydrolysis by acetylcholinesterase [74].

Exclusively in insects, the cholinergic system is localized centrally and is absent at the neuromuscular junctions [76]. The cholinergic system is essential for the functioning of the insect nervous system and AChE is a key enzyme in this system [77]. The functional integrity of insects is maintained by its nervous system, and it is for this reason that most insecticides act on the insects' nervous system [72,78].

2.4. Molecular Characterization of Acetylcholinesterase

There are apparent AChE structural differences between insects and mammals. These span from their distinct genomics, amino acid sequences to their active and peripheral anionic site conformations [76]. Recent biochemical studies have revealed critical differences between the *Anopheles* AChE and human AChE that could serve as potential drug targets for directed insecticide design.

The amino acid sequence of *Anopheles* AChE is reported to be 48–49% identical to that of the human AChE [79,80]. Unlike humans where there is a single *ace* gene coding for AChE, mosquitoes have two *ace* genes, *ace*-1 and *ace*-2, coding for AChE1 and AChE2 enzymes, respectively [81,82]. These genes are crucial in all life stages of the mosquito, ranging from egg through to adult stages [83]. AChE1 is the main catalytic enzyme, while AChE2 is involved in non-catalytic activities such as reproduction. As a result, target site insensitivity on insect AChEs, such as G280S genotype, is linked to mutations in *ace*-1 but not *ace*-2 [51,62,81]. AChE is characterized by a deep and narrow active-site gorge (Figure 3). There are differences in these gorge structures between *Anopheles* and human AChEs and this may affect ligand binding and specificity [51,79,84]. Notably, a free cysteine residue (Cys^{447}) is available at the entrance to the active site gorge of *Anopheles* AChE (Figure 3A,B), but not in human AChE. Instead, a human AChE has a bulky phenylalanine (Phe^{295}) at the active site entrance (Figure 3C). Additionally, in *Anopheles* AChE, a smaller aspartic acid residue (Asp^{602}; Figure 3A,B) replaces a larger tyrosine residue (Tyr^{449}) at the base of the active site gorge [51]. Moreover, a conserved arginine residue (Arg^{339}; not shown in order to maintain the catalytic side resolution) has also been identified in *Anopheles* AChE [85]. In addition, the displayed *An. gambiae* AChE catalytic site in Figure 3B shows a G280S mutated site (pointed).

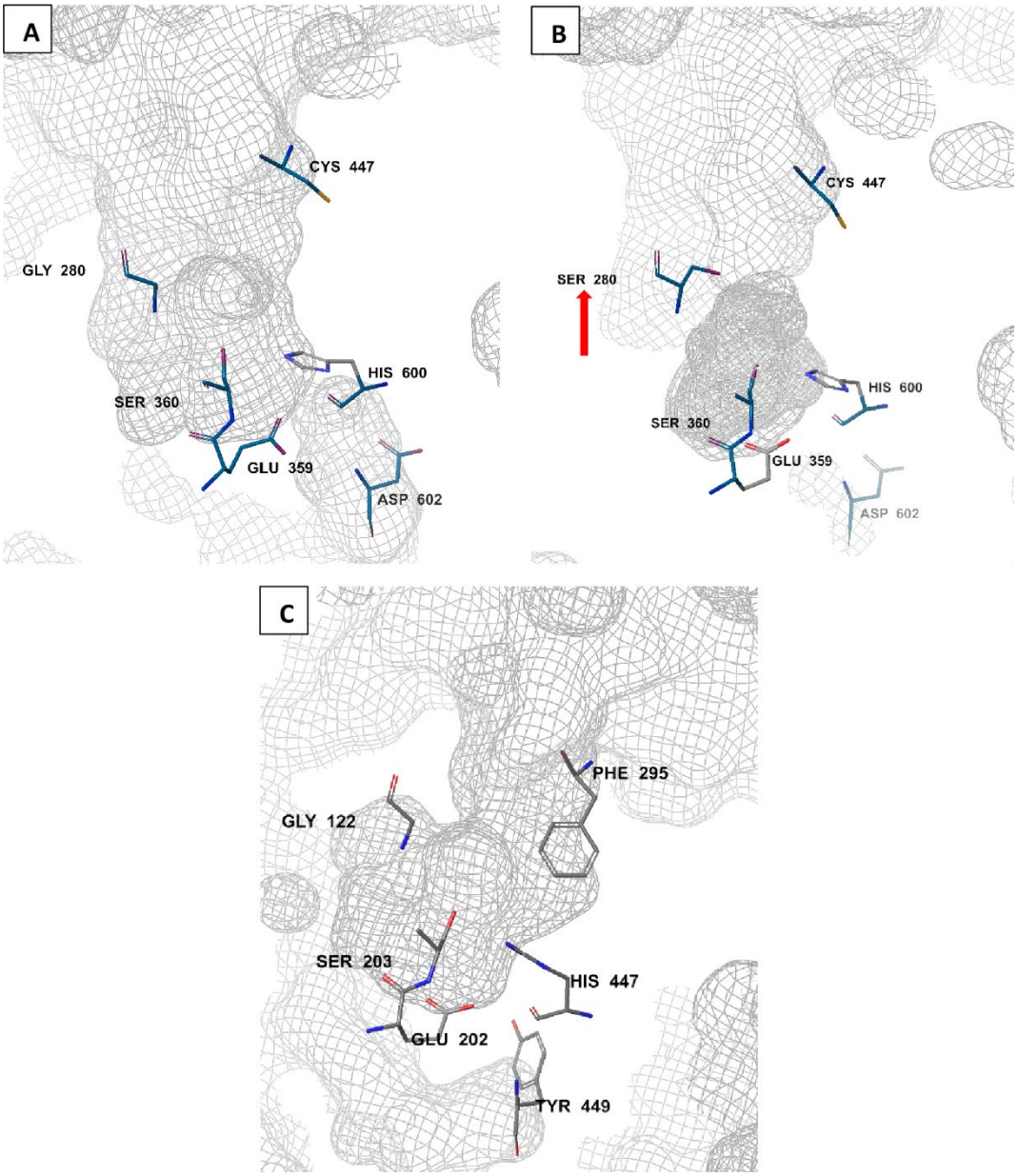


Figure 3. Molecular comparison of *An. gambiae* wild-type (**A**) and resistant (**B**) AChE catalytic sites (PDB IDs: 5YDI and 6ARY, respectively) to the human AChE (PDB ID: 7E3H) (**C**), generated by Schrodinger's Maestro 2018-2 software. The G280S mutation is shown (red arrow) in the resistant *Anopheles* AChE phenotype (B) [51,75].

2.4.1. Acetylcholinesterase Inhibition in *Anopheles*

The catalytic site in *Anopheles* is characterized with a catalytic triad made of His-Ser-Glu (His^{600}-Ser^{360}-Glu^{359}; Figure 3A) amino acid combination. The catalytic serine (Ser^{360}; Figure 3A) is the target for covalent insecticides, including organophosphates and carbamates [61,62]. These insecticides establish a covalent bond with AChE through phosphorylation and carbamoylation, respectively [67]. The inhibition of AChE leads to ACh accumulation that eventually results in over-stimulation of postsynaptic cholinergic receptors [76]. This

neuroexcitation causes rapid insect paralysis and death [72,76]. The *Anopheles* resistance to the anticholinesterase insecticide classes is usually caused by *ace*-1 G280S mutation (Figure 3B) and metabolic resistance resulting from the elevated levels of monooxygenases, glutathione-S-transferases and general esterases [62,86–88].

Given the widespread resistance that has largely rendered organophosphates and carbamates non-effective, there is an urgent need to identify novel anticholinesterase insecticides. AChE has proven to be a valid target in *Anopheles* vectors [79] and EOs have also shown to be the promising sources of novel insecticides [89–91]. Interestingly, the EOs have shown activity against resistant *Anopheles* species and a capability to synergize conventional insecticides including the pyrethroids [92,93]. Additionally, the EOs and their constituents inhibit the P450 monooxygenase and glutathione-S-transferase detoxification enzymes involved in multiple insecticide resistance including the pyrethroids, organophosphates and carbamates. The insect toxicity of pyrethroids, organophosphates and carbamates has been greatly enhanced by synergy with EOs including cedarwood oil, geranium oil, clove oil, patchouli oil, cinnamon oil, basil oil, oregano oil, purple nutsedge oil, thyme, coriander and galangal oil [94–96]. The identified individual EOCs capable of synergizing conventional insecticides, especially pyrethroids, include thymol, eugenol, carvacrol, geraniol and linalool [96,97].

2.5. Essential Oils and Constituents as Potential *Anopheles* AChE Inhibitors

EOs are hydrophobic secondary metabolites extracted from different aromatic plant parts by steam distillation, hydro-distillation, head-space analysis, solvent extraction or liquid carbon dioxide extraction [93,98]. Various terpenoids including sesquiterpenes, monoterpenes, diterpenes and phenylpropanoids are major phytoconstituents in EOs [99,100]. Many EOs and certain EOCs have exhibited significant anticholinesterase activity in in vitro studies. In insects, many EOs have been reported to exhibit neurotoxic effects characterized by rapid paralysis and death [98], which links them to AChE inhibition. Interestingly, most EOCs can only inhibit mammalian AChE very weakly. In addition, most EOs and EOCs are relatively non-toxic to mammals with very low potency for acute oral toxicity, whereby lethal doses for pure compounds range from 800 to 3000 mg/kg and more than 5000 mg/kg for EOs or EOCs incorporated in pharmaceutical formulations [93,101,102]. The EOs are natural resources and therefore, the identification of novel insecticides from such sources is relatively cost-effective. Production of insecticides from EOs can be relatively less expensive translating into low costs for the malaria endemic countries [103,104]. In addition, the discovery of insecticides is

relatively rapid since the lead compounds are screened on the actual insect as opposed to pharmaceuticals intended for human use that undergo a lengthy translational science process from the in vitro to the in vivo studies including animal models and human clinical trials [105].

In vitro anticholinesterase activity is usually assessed with the Ellman assay, a globally accepted method to assess the AChE activity and potential inhibition thereof [106]. For such studies, the insect homogenate may be used as a crude enzyme source [107–109]. However, some studies use AChE from *Electrophorus electricus* (electric eel) to estimate insect AChE activity [110,111]. The electric eel and *Anopheles* AChEs are reported to have nearly the same backbone conformation [85]. For the purpose of this review, EOs or EOCs inhibiting AChE in the in vitro studies are only considered to have potential *Anopheles* anticholinesterase activity if they have shown *Anopheles* insecticidal activity in addition to the observed AChE inhibition. Furthermore, where available, the selectivity between human and *Anopheles* AChE targets, as well as human toxicity potential for the EOs and EOCs, are extensively discussed.

2.5.1. The EOs as Potential Anticholinesterase Insecticides

Several EOs have shown insecticidal activity against *Anopheles* species [93]. Given their high volatility, the EOs can reach their target through inhalation by the insect species, ingestion or contact [112]. The absorption of EOs is facilitated by the lipophilic character and low molecular weights of their active constituents [100,112]. This behavior is also important for the partitioning of EOs into the insect midgut plasma membrane [112,113].

Some of the EOs have not only shown the insecticidal effects, but also exhibited anticholinesterase activity indicating a potential to be anticholinesterase insecticides [102]. With increasing resistance against current insecticides, the potential use of EOs as alternative insecticides has been suggested [93]. Various EOs have shown potential to be active against resistant colonies including pyrethroid resistant *Anopheles* species, such as *An. gambiae* and *An. stephensi* [93,114,115]. Furthermore, the EOs and EOCs have shown potential to synergize with conventional insecticides and to inhibit those enzymes responsible for insecticide detoxification, which shows promise in overcoming resistance [88,92,94]. This study identified essential oils from 16 plant species with anticholinesterase potential and corresponding insecticidal capabilities (Table 1). Seven of these exhibited both anticholinesterase and insecticidal activities at IC_{50} and LC_{50} values less than 100 µg/mL, indicating high potencies [116]. These highly potent EOs with potential of being anticholinesterase insecticides include *Hyptis suaveolens*, *Hyptis spicigera*, *Ocimum canum*, *Lantana camara*, *Ferulago carduchorum, Ferulago trifida*, *Salvia officinalis* and *Curcuma longa* (Table 1); the latter EOs

belong to the general plant families of Lamiaceae, Verbenaceae, Apiaceae and Zingiberaceae [93,117,118].

Table 1. Anticholinesterase and insecticidal activities of EOs.

Essential Oil	Anticholinesterase IC_{50}	Insecticidal Activity	Insecticidal LD_{50}	*Anopheles* Species (Laboratory Strain)	Reference(s)
Carum carvi	0.82 ± 0.05 mg/mL	Larvicidal	72.00 µg/mL	*An. dirus*	[93,119]
Citrus limon	0.85 ± 0.01 mg/mL	Larvicidal	13.75 µg/mL	*An. gambiae (Kisumu)*	[120,121]
			32.28 µg/mL	*An. gambiae Logbessou*	
Curcuma longa	34.70 ± 3.10 µg/mL	Larvicidal	33.61 µg/mL	*An. cracens*	[122,123,124]
		Larvicidal	1.8–3.7 µg/mL	*An. quadrimaculatus*	
Eucalyptus globulus Labill.	0.13 ± 0.01 mg/mL	Larvicidal	0.09 mg/mL	*An. arabiensis*	[121,125]
Ferulago carduchorum	23.6 µg/mL	Larvicidal	12.00–12.78 µg/mL	*An. stephensi*	[93,1265]
Ferulago trifida	21.5 ± 2.2 mg/mL	Larvicidal	10.46 µg/mL	*An. stephensi*	[127]
Foeniculum vulgare Mill.	1.19 ± 0.01 mg/mL	Larvicidal	35.30 µg/mL	*An. dirus*	[93,121,128]
		Larvicidal	20.10 µg/mL	*An. stephensi*	
Hyptis spicigera Lam.	6.3 ± 0.43 µg/mL	Ovicidal Larvicidal Adulticidal	69.61 µg/mL 45.18 µg/mL 1.45% *w/v*	* *An. gambiae*	[110,129]
		Adulticidal	1.04% *w/v*	*An. gambiae* (Kisumu)	
Hyptis suaveolens Poit.	0.55 ± 0.12 µg/mL	Ovicidal Larvicidal Adulticidal	61.71 µg/mL 159.5 µg/mL 1.86% *w/v*	* *An. gambiae*	[110,129]
		Adulticidal	0.85% *w/v*	*An. gambiae* (Kisumu)	
Lantana camara L.	1.75 ± 0.12 µg/mL	Ovicidal Larvicidal Adulticidal	53.59 µg/mL 61.69 µg/mL 0.84% *w/v*	* *An. gambiae*	[110,129]
		Adulticidal	0.24% *w/v*	*An. gambiae* (Kisumu)	
Mentha pulegium L.	108.75 µg/mL	Larvicidal	40.13–113.6 µg/mL	*An. stephensi*	[130–132]
		Larvicidal	118.0 µg/mL	*An. gambiae*	
		Larvicidal	58.9 µg/mL	*An. atroparvus*	
Ocimum canum Sims.	0.21–26.16 µg/mL	Ovicidal Larvicidal Adulticidal	71.56 µg/mL 138.7 µg/mL 1.84% *w/v*	* *An. gambiae*	[93,110,129,133]
		Adulticidal	1.22% *w/v*	*An. gambiae* (Kisumu)	
		Larvicidal	74.12 µg/mL	*An. funestus*	
Salvia leucantha	>250 µg/mL	Larvicidal	10.9 µg/mL	*An. quadrimaculatus*	[93,134]
Salvia officinalis	47.68–77.51 µg/mL	Larvicidal	14.1 µg/mL	*An. quadrimaculatus*	[93,135]
Schinus molle L.	6.54 ± 0.15 mg/mL	Larvicidal	21.0 µg/mL	*An. arabiensis*	[136,137]
Thymus vulgaris	0.22–0.54 mg/mL	Larvicidal	351.63 µg/mL	*An. labranchiae*	[121,138,139]

* Wild population.

Notably, one EO could exhibit considerable differences in anticholinesterase and/or insecticidal activity (Table 1). This is probably due to variations in the identity or quantity of its specific phytoconstituents. Three *Salvia officinalis* EOs from different locations in Italy exhibited anticholinesterase activity at IC_{50} values of 47.68, 58.35 and 77.51 µg/mL [135]. While these EOs had a similar content of camphor (16.84%, 16.16% and 18.92%, respectively) as the main constituent, it was notable that the borneol content in the third EO (IC50: 77.51 µg/mL) was approximately half (2.34%) of that yielded from the former EOs (4.48% and

4.68%, respectively) [135]. Borneol has been previously shown to inhibit AChE [121]. Using similar experimental conditions and AChE enzyme concentrations, the *Ocimum canum* Sims. EOs from Burkina Faso inhibited AChE at the IC_{50} value of 0.21 µg/mL [110] and a relatively higher value of 36.16 µg/mL [133]. The latter EO had 59.9% content of 1,8-cineole as the main constituent, while the composition of the former and more active EO was not mentioned [110,133]. *Mentha pulegium L.* EO from Iran exhibited the larvicidal activity against *An. stephens*i with the LC_{50} value of 40.13 µg/mL [130], while that from Portugal attained the LC_{50} of 113.6 µg/mL against the same *Anopheles* species [132]. *Mentha pulegium* L. EO from Portugal had 61.4% pulegone and 20% menthone as the main constituents, while the phytochemical analysis was not reported for the *Mentha* species from Iran [130,132].

Major constituents in EOs are often responsible for the observed biological activity associated with such a sample [100,140,141]. For this reason, this review collected phytochemical data on the major components in the identified bioactive EOs (Table 2). Some of these major constituents possess anticholinesterase and insecticidal activity as displayed in Table 3. Common constituents in most EOs include α-pinene, β-pinene, p-cymene, γ-terpinene and β-caryophyllene. Certain EOCs such as terpinen-4-ol, 1,8-cineole, menthone, menthol, fenchone, γ-terpinene, (-)-bornyl acetate, linalool, citral and pulegone have been reported as competitive AChE inhibitors [98,142–144]. Additionally, common constituents in seven of the most active EOs, especially *Hyptis suaveolens*, *Hyptis spicigera*, *Ocimum canum*, *Lantana camara*, *Ferulago carduchorum, Ferulago trifida* and *Salvia officinalis* are α-pinene, β-pinene, β-caryophyllene and γ-terpinene (Table 2). The *Curcuma longa* EO has a unique composition profile of ar-turmerone, tumerone, curlone, α-curcumene and β-sesquiphellandrene [123]. The main constituent ar-turmerone has only attained anticholinesterase activity against human AChE with an IC_{50} value of >100 µg/mL (IC_{50}: 191.1 ± 0.3 µg/mL), indicating a low potency [116]. *Curcuma longa* EO has been reported as possessing an anticholinesterase with an IC_{50} value of 34.70 ± 3.10 µg/mL. The *Curcuma longa* EO has also been reported to possess strong larvicidal activity with the LC_{50} range of 1.5 to 34 µg/mL [122,123], indicating that it may be selectively targeting the insect.

Table 2. Major constituents of EOs with anticholinesterase insecticidal activity.

Essential Oil	Major Constituents	References
Carum carvi	γ-Terpinene, β-pinene, bornyl acetate, carvone, *p*-cymene	[119]
Citrus limon	Limonene	[121]
Curcuma longa	ar-Turmerone, tumerone, curlone, α-curcumene, β-sesquiphellandrene	[123]
Eucalyptus globulus Labill.	Eucalyptol, α- pinene, *p*-cymene, β-cymene, 1,8-cineole, limonene	[121,145,146]
Ferulago carduchorum	(*Z*)-β-ocimene, α-pinene, bornyl acetate	[126]
Ferulago trifida	Isobornyl acetate, trans-verbenol, (*E*)- β-caryophyllene	[127]
Foeniculum vulgare Mill.	Thymol, estragole, α-phellandrene, limonene, (*E*)-anethole, fenchone,	[121,123,147]
Hyptis spicigera Lam.	β-caryophyllene, α-pinene, β-pinene, α-phellandrene, α-thujene, sabinene	[129,148,149]
Hyptis suaveolens Poit.	1,8-cineole, sabinene, terpinolene α-thujene, α- pinene, β-pinene	[129,150,151]
Lantana camara L.	β-caryophyllene, α-humulene, γ-curcumene, germacrene D	[152,153]
Mentha pulegium L.	Pulegone, neomenthol, menthone	[131,132]
Ocimum canum Sims.	Thymol, *p*-cymene, γ-terpinene, estragole, linalool	[154,155]
Salvia leucantha	Bornyl acetate, 6,9-guaiadiene, (*E*)-β-caryophyllene, bicyclogermacrene, camphene, α-pinene, β-pinene	[117,134]
Salvia officinalis	Camphor, camphene, α-thujone, 1,8-cineole, α- pinene, β-pinene	[135]
Schinus molle L.	α-Phellandrene, β-phellandrene, α- pinene, β-pinene, γ-terpinene, *p*-cymene	[136]
Thymus vulgaris	*p*-Cymene, borneol, thymol, carvacrol	[121]

2.5.2. The EOCs as Potential Anticholinesterase Insecticides

Some studies have isolated the active principles from EOs and demonstrated the potential of these to exert *Anopheles* anticholinesterase activity [98,156]. Moreover, some of these have also exerted *Anopheles* mortality in insecticide susceptibility assays [93,131,157]. Gnankiné and Bassolé (2017) reported that several constituents in EOs possess ovicidal, larvicidal and adulticidal effects against *Anopheles* species. In this study, 22 EOCs from various terpenoid classes such as sesquiterpene alcohols, sesquiterpene oxides, monoterpenoids and monoterpene alcohols, as well as phenylpropanoids, have been identified as possessing both anticholinesterase and insecticidal activity against *Anopheles* species (Table 3). Some of these EOCs, such as 1,8-cineole, α-pinene, camphor, linalool, borneol, (+)-3-δ-carene, γ-terpinene, caryophyllene oxide, *p*-cymene, (*E*)-anethole, terpinen-4-ol, pulegone and limonene, have shown low potency towards human AChE [98,102,158–160].

The α-pinene, estragole, carvacrol, (+)-δ-3-carene, eugenol and camphor were the most active in terms of both AChE inhibition and insecticidal activity (Table 3); thus, indicating that these EOCs show potential to serve as anticholinesterase insecticides. Interestingly, previous studies have shown that these EOCs have a lower activity against human AChE. For example, camphor and α-pinene could only inhibit AChE from human erythrocytes with IC_{50} values of >10 mM and 0.4–0.7 mM, respectively [159,161]. Similarly, an IC_{50} range of 0.2 to 0.3 mM of (+)-3-δ-carene was needed to inhibit bovine and human erythrocytes AChE [159,161]. These

EOCs may therefore produce insecticides with high selectivity towards *Anopheles* AChE inhibition over the human target.

Monoterpenoids: The monoterpenoid α-pinene is the main constituent in many essential oils [93]. Orhan et al. (2008) reported that α-pinene, but not β-pinene, has anticholinesterase properties [162]. α-Pinene has shown anticholinesterase activity at an IC_{50} value as low as 22 µg/mL [163]; however, this was higher than its parent EOs from *Hyptis suaveolens* and *Hyptis spicigera* that obtained IC_{50} values between 0.5 and 6.5 µg/mL [110]. Apart from the fact that different *Anopheles* species were used, this suggests the possibility of synergism among the EOCs in such EOs. Due to their smaller molecular weights, more than one monoterpenoid can bind to the AChE catalytic and/or peripheral site and, usually, binding of one monoterpenoid facilitates binding of the other [102]. On the other hand, α-pinene exhibited larvicidal activity (LC_{50} of 32.1 µg/mL) against *An. subpictus* that was comparable to its anticholinesterase activity [93,163]. In another study, Wojtunik-Kulesza et al. (2017) reported a higher anticholinesterase IC_{50} value of 102.0 mM for α-pinene along with declaration that insolubility of the EOCs affected the accuracy of spectrophotometric measurements [164]. Generally, the observed variations were caused by the use of different AChE types and protein contents. For example, using 0.25 U/mL AChE, Farag et al. (2016) determined an IC_{50} of 0.337 µM for estragole; meanwhile, Lopez et al. (2015) doubled the AChE concentration and obtained an IC_{50} value of 12.6 mM [102,165]. The purity and stability of reagents and EOs or EOCs used in AChE activity assays may also affect the outcome [166,167]. Additionally, AChE activity is a pH-dependent reaction [168] and variations in pH across studies may cause potential differences in inhibition kinetics.

A major constituent in the EO of *Echinophora lamondiana*, (+)-δ-3-carene, resulted in comparable anticholinesterase (IC_{50} value: 36 µg/mL) and larvicidal (LC_{50} value of 42.9 µg/mL) activity against *An. quadrimaculatus* [169,170]. The latter larvicidal LC_{50} value of the EOC was observed to be similar to that obtained for the EO of the flowers (LC_{50}: 46.9 µg/mL) with 61.9% (+)-δ-3-carene content, but higher than the EOC of the leaves (LC_{50}: 26.2 µg/mL) with comparable (+)-δ-3-carene content (75.0%) [169]. Though less predominant than (+)-δ-3-carene in the *Echinophora lamondiana* EO, both terpinolene (2.7 to 3.3%) and α-phellandrene (12.8 to 20.3%) were reported to be even more potent larvicidal agents than (+)-δ-3-carene, with LC_{50} values of 20.9 and 15.6 µg/mL, respectively [169].

Camphor, a monoterpenoid ketone and the major component in the EOs of *Ocimum africanum* and *Ocimum americanum*, was also shown to exhibit low anticholinesterase activity (IC_{50} value of 21.43 µM) [165]. Moreover, camphor is known to non-competitively inhibit

nAChRs [98,102,171]. Camphor is also active as a repellent against *An. culicifacies, An. gambiae* and *An. funestus* [172–174]. However, poor larvicidal activity by camphor was obtained with a LC_{50} value >100 µg/mL [175].

Aazza et al. (2011) reported that the EO of *Thymus vulgaris* consists of 16% carvacrol. Interestingly, carvacrol is more than three times as active (IC_{50} value of 63.0 µg/mL) as its parent EO (IC_{50} value of 216.9 µg/mL) in AChE inhibition studies [121,139]. This indicates the potential effect of antagonistic interactions within the complex mixture of the *Thymus vulgaris* EO resulting in decreased activity. However, the *Anopheles* anticholinesterase potential of carvacrol (IC_{50} value of 63.0 µg/mL) is non-specific as its activity is comparable to that against AChE from bovine serum (IC_{50} value of 70.3 µg/mL) [139,162]. Stereochemistry in AChE inhibition also plays a role, whereby carvone (a monoterpenoid ketone) attained weak AChE inhibition (IC_{50} value of 830 µg/mL), in comparison to the structurally related phenolic monoterpenoid, carvacrol (IC_{50} value of 63.0 µg/mL) [93,139,164]. Carvone has previously been reported to be a potent non-competitive AChE inhibitor of mammal AChE [102,162].

Another phenolic monoterpene, thymol, is ineffective in inhibiting AChE, but is a positive allosteric modulator of the insect's GABA-A receptors [118,164]. Thymol has also been reported to act at the octopamine receptors; this is where octopamine is an analogue of norepinephrine, functioning as a neurotransmitter, neuromodulator and neurohormone in insects [176]. Most studies have shown thymol to be as ineffective as its parent EO *Thymus vulgaris* against the AChE target [121,139].

Phenylpropanoids: Estragole is a phenylpropanoid isolated from the EOs of the *Ocimum* species. Along with strong anticholinesterase activity (IC_{50} value of 0.337 µM) [165], it has shown similarly potent larvicidal activity against *An. stephensi* (LC_{50} value of 11.01 µg/mL) and *An. atroparvus* (LC_{50} value of 15.7 µg/mL) [102,118]. Estragole was reported to be potentially selective for *Anopheles*, as it has shown no inhibition of mammal AChE [162].

Eugenol, an allyl chain-substituted guaiacol (phenol), is a major constituent (31.12%) in the *Plectranthus barbatus* EO, an EO with the LC_{50} value of 84.2 µg/mL, against *An. subpictus* larvae [177]. However, eugenol is three times more potent (LC_{50} value of 25.45 µg/mL) than this latter parent EO as a larvicide against *An. subpictus* [93]. Again, this suggests possible antagonism with other constituents. Eugenol and (*E*)-anethole (a phenylpropanoid) reportedly interacted in an antagonistic manner when tested for larvicidal activity [178]. Although eugenol had an IC_{50} value of 40.32 µg/mL for AChE inhibition, this inhibitory activity was however comparable to that attained against bovine erythrocytes AChE (42.44 ± 1.21 µg/mL) [165,179]. In contrast, Dohi et al. (2009) reported a much higher IC_{50} value for eugenol (480 µg/mL)

against electric eel AChE [163]. Based on these two findings, eugenol has a potential nonselective AChE inhibition, and to be an antagonist at insect octopamine receptors [93].

Table 3. Anticholinesterase and insecticidal activities of EOCs.

Essential Oil Constituent	Anticholinesterase IC_{50}	Insecticidal Activity	Insecticidal LD_{50}	*Anopheles* Species (Laboratory Strain)	Reference(s)
(E)-Anethole	1.324 ± 0.011 mg/mL	Larvicidal	25.11 µg/mL	* *An. sinensis*	[121,178]
Borneol	0.132 ± 0.012 mg/mL	Larvicidal	35.89 µg/mL	*An. anthropophagus*	[121,180]
Camphor	0.003–1.7 mg/mL	Larvicidal	129.17 µg/mL	*An. anthropophagus*	[102,165,175]
(+)-δ-3-Carene	0.036–0.64 mg/mL	Larvicidal	42.9 µg/mL	*An. quadrimaculatus*	[169,170]
Carvacrol	0.063–0.092 mg/mL	Larvicidal	21.1–24.06 µg/mL	*An. subpictus*	[121,139,181]
		Larvicidal	21.15 µg/mL	*An. stephensi*	
Carvone	0.437–0.83 mg/mL	Larvicidal	19.3 µg/mL	*An. stephensi*	[102,157,164]
Caryophyllene oxide	>200 µg/mL	Larvicidal	49.46 µg/mL	*An. anthropophagus*	[93,175,182]
Estragole	0.337 µM–12.6 mM	Larvicidal	15.7 µg/mL	*An. atroparvus*	[102,118,165]
			11.01 µg/mL	*An. stephensi*	
Eucalyptol	266.0 ± 11.87 mM	Larvicidal	>200 µg/mL	*An. anthropophagus*	[93,102,164]
Eugenol	40–480 mg/mL	Larvicidal	93.14 µg/mL	*An. stephensi*	[163,165,178,181,183]
			25.45 µg/mL	*An. subpictus*	
			31.09 µg/mL	* *An. sinensis*	
Isopulegol	233.0 ± 10.08 mM	Larvicidal	49.4 µg/mL	*An. gambiae s.s*	[93,102]
Limonene	220–586 µg/mL	Larvicidal	8.8 µg/mL	*An. stephensi*	[121,184–186]
		Larvicidal	18.91 µg/mL	*An. sinensis*	
Linalool	1.69–2.4 mg/mL	Larvicidal	35.87 µg/mL	*An. anthropophagus*	[102,164,180]
(Z)-*β*-Ocimene	4.7 ± 0.20 mM	Larvicidal	25.84 µg/mL	*An. stephensi*	[118,164]
			30.86 µg/mL	*An. subpictus*	
α-Phellandrene	0.12–3.68 mg/mL	Larvicidal	15.6 µg/mL	*An. quadrimaculatus*	[102,169]
α-Pinene	0.022–1.43 mg/mL	Larvicidal	32.1 µg/mL	*An. subpictus*	[163–165,187]
Pulegone	9.0 ± 0.41 mM	Larvicidal	48.9 µg/mL	*An. stephensi*	[141,164]
Sabinene	176.5 ± 2.8 µg/mL	Larvicidal	19.67 µg/mL	*An. stephensi*	[118]
Terpinen-4-ol	0.19–3.2 mg/mL	Larvicidal	47.73 µg/mL	*An. subpictus*	[143,156,163,186,188]
		Larvicidal	43.27 µg/mL	*An. stephensi*	
		Larvicidal	62.09 µg/mL	*An. sinensis*	
		Larvicidal	337.7 µg/mL	*An. gambiae s.s*	
γ-Terpinene	5.8 mM	Larvicidal	44.61 µg/mL	*An. anthropophagus*	[102,180,186]
		Larvicidal	36.42 µg/mL	*An. sinensis*	
α-Terpineol	1.3 ± 0.06 mg/mL	Larvicidal	39.98 µg/mL	*An. sinensis*	[163,186]
Terpinolene	156.4–550.0 µg/mL	Larvicidal	404.71 µg/mL	*An. gambiae s.s*	[93,118,156,169,170]
		Larvicidal	20.9–25.7 µg/mL	*An. quadrimaculatus*	
Thymol	0.05–0.74 mg/mL	Larvicidal	10.3–22.06 µg/mL	*An. subpictus*	[118,139,141,189,190]
		Larvicidal	48.88 µg/mL	*An. stephensi*	

* Wild population.

While showing potent insecticidal activity, some EOs and EOCs did not show any evidence that their mode of action involved targeting AChE. Pulegone has a potent insecticidal activity against *An. stephensi*, however, it could not efficiently inhibit AChE [141,164]. In contrast, the epoxide form of pulegone, the pulegone-1,2-epoxide isolated from the *Lippia steochadifolia* EO, has be reported to be an insect neurotoxin, acting as an irreversible inhibitor of AChE [191]. In addition, the *Citrus limon* EO and it major EOC, limonene (99%) both

possess weak AChE inhibition properties; whilst both are potent larvicides against *An. gambiae* and *An. stephensi* [120,121,184]. This suggests that a different mode of action may be responsible for their insecticidal activity. In *Cimex cimicidae* (bedbugs), limonene has been proposed as acting by destroying the wax layer of the insect respiratory system [192].

This review identified EOs and specific EOCs with anticholinesterase activity and indeed the capacity to cause *Anopheles* mortality. The EOs and EOCs spectrum of activity against *Anopheles* included ovicidal, larvicidal and adulticidal activities, where they were regarded as active if they obtained LC_{50} values less than 100 µg/mL [193]. The common *Anopheles* species involved in global malaria transmission that have been assessed include *An. stephensi*, *An. gambiae*, *An. arabiensis, An. subpictus*, *An. anthropophagus*, *An. quadrimaculatus*, *An. dirus, An. cracens*, *An.labranchiae, An. sinensis* and *An. atroparvus* [194,195]. Interestingly, almost all of these have already developed clinically significant resistance against current insecticides [194,196–198]. The commonly assessed *An. Stephensi*, that was susceptible to many of the EOs and EOCs, is predominant in Asia and recently invaded Africa [199,200]. This species is resistant to conventional AChE insecticides, including organophosphates and carbamates, and has also shown resistance to other insecticide classes such as pyrethroids and organochlorines [201,202].

The EO of *Ferulago carduchorum* has shown both larvicidal and anticholinesterase activity at LC_{50} and IC_{50} values around 12 µg/mL, which are extremely promising [126]. Interestingly, the EOs of *Hyptis suaveolens*, *Hyptis spicigera*, *Ocimum canum* and *Lantana camara* have shown activity against all three developmental stages of *An. gambiae,* a main African vector. Meanwhile, the EO of *Mentha pulegium* and *Ocimum canum* have been shown to target the larval stage of *An. gambiae* [93,131] and *An. funestus* (LC_{50} value of 91.2 µg/mL), respectively [93,195,203]. Of all the species, *An. arabiensis* is the main vector in Sub-Saharan Africa [48] against which the EOs of *Schinus mole* and *Eucalyptus globulus* have displayed larvicidal activity with LC_{50} values less than 100 µg/mL. However, the anticholinesterase activity of these latter EOs, as well as their major EOCs, α-phellandrene and eucalyptol, respectively, were in the millimolar range indicating that their larvicidal activity is not primarily through AChE inhibition. Both *Schinus mole* and *Eucalyptus globulus* EOs are non-toxic towards mammals and the *Eucalyptus globulus* EO is recommended for skin application as a repellent [93,204].

It is crucial to identify novel insecticides from EOs as most are relatively safe to mammals [93,118]. An EO of *Foeniculum vulgare* could only exhibit acute oral toxicity at the LD_{50} of 3120 mg/kg in the rat model [118]. This EO is active against *An. stephensi* and *An. dirus* with

LD_{50} values between 20 and 35 µg/mL, thus indicating a high safety index for human use [93]. The EOCs, (*E*)-anethole, eugenol, limonene, thymol and γ-terpinene, displayed acute oral toxicity at the LD_{50} values ranging from 980 to 4600 mg/kg [118] where all attained insecticidal LC_{50} values less than 100 µg/mL (Table 3); thus, indicating their potential as bioinsecticides.

3. Conclusions and Future Perspective

The *Anopheles* vectors are responsible for malaria transmission across the world; additionally, most of these vectors have acquired resistance against current insecticide classes. *Anopheles* AChE is a valid target by conventional AChE inhibitors in the market. Due to the current resistance status towards anticholinesterase insecticides, organophosphates and carbamates, the identification of novel insecticides is critical. The EOs and EOCs have shown potential to serve as anticholinesterase insecticides against *Anopheles* vectors. This is afforded by the capability of some EOs and EOCs to exhibit both anticholinesterase and insecticidal activity. In this review, seven EOs from *Hyptis suaveolens*, *Hyptis spicigera*, *Ocimum canum*, *Lantana camara*, *Ferulago carduchorum, Ferulago trifida*, *Salvia officinalis* and *Curcuma longa* plant species which showed potent anticholinesterase and insecticidal activities against various *Anopheles* species were summarized. Along with these, six EOCs, namely, α-pinene, estragole, carvacrol, (+)-3-δ-carene, eugenol and camphor, were identified as being the most active against the *Anopheles* vector and AChE target. All of these, except for eugenol and carvacrol, have the potential to be selective towards *Anopheles* AChE. This scientific data review is important for informing future research towards novel anticholinesterase insecticides for malaria vector control. Future studies in this area should focus on the EOs and EOCs identified in this review for possible development into insecticidal agents. Active constituents should be identified from the EO complex mixtures and possible synergistic interaction taken into consideration. The EOs and the identified EOCs in this review require further assessment against various stages of the *Anopheles* life cycles and potential activity against insecticide resistant *Anopheles* vectors. Moreover, these promising EOs and EOCs should be assessed for possible activity against human AChE, toxicity against other aquatic lives, as well as for mammal toxicity.

In general, this review supports the future development of EOs as a potential source for novel insecticides with AChE inhibitory potential; including the specific EOs with potential anticholinesterase and insecticidal activities outlined in this review, belonging to the families of Lamiaceae, Verbenaceae, Apiaceae and Zingiberaceae. Meanwhile, the promising EOCs for

development into novel anticholinesterase insecticides belong to broader classes of sesquiterpene alcohols, monoterpenoids, monoterpene alcohols and phenylpropanoids.

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CHAPTER 3

Optimization of the *in silico* approach to assess the acetylcholinesterase inhibitory potential of chemical entities using molecular modelling and molecular mechanics simulations

This chapter presents the investigation and optimization of the *in silico* methods required to reliably assess the binding profiles of acetylcholinesterase inhibitors. It is presented in the form of a published paper and the supplementary information related to the paper is available in Appendix B.2.

Chapter type: Published ISI paper (Appendix B.1)

Title of publication: Optimization of covalent docking for organophosphates interaction with *Anopheles* acetylcholinesterase

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Method	✓		
Data curation	✓		
Writing – original draft	✓		
Writing – review and editing	✓	✓	✓
Visualization	✓	✓	
Supervision			✓
Funding acquisition			✓

Abstract: Organophosphates (OPs) used as potent insecticides for malaria vector control, covalently phosphorylate the catalytic serine residue of *Anopheles gambiae* AChE (*Ag*AChE) in a reaction that liberates their leaving groups. In the recent 10-year insecticide use assessment, OPs were the most frequently used World Health Organization prequalified insecticides. Molecular modelling programs are best suited to display molecular interactions between ligands and the target proteins. The docking modes that generate ligand poses closer to the binding site show high accuracy in predicting the ligand binding mode. The implicit solvation approach such as molecular mechanics-generalized born surface area (MM-GBSA) is a more reliable method to predict ligand conformations and binding affinities. Apart from covalent docking studies being scarce, current molecular docking programs do not adequately possess the covalent docking reaction algorithm to display the molecular mechanism of OPs at the *Ag*AChE catalytic site. This results into OP docking studies commonly being conducted through noncovalent panels. The aim of this study was to establish the optimum covalent docking system for OPs through manual customization of Schrödinger's Glide covalent docking reaction algorithm. To achieve this, a newly customized covalent reaction algorithm was assessed on a set of ligands covering aromatic, non-aromatic and hydrophobic OPs and compared to the noncovalent docking results in terms of reliability based on the reported X-ray diffraction molecular interactions and crystal poses. The study established that by virtue of omitting the well-known OP hydrolysis, noncovalent mode suggested molecular interactions that were further from the catalytic triad and could not otherwise occur when the molecule is hydrolyzed as in the customized covalent docking mode. Moreover, the MM-GBSA concurred with the optimized covalent docking in eliminating such inaccurate molecular interactions. Additionally, the covalent docking mode confined the interactions and ligand poses to the catalytic site indicating relatively high accuracy and reliability. This study reports the optimized covalent docking panel that effectively confirmed the molecular mechanisms of OPs, as well as identifying the corresponding amino acid residues required to stabilize the aromatic, non-aromatic and hydrophobic OPs at the *Ag*AChE catalytic site in line with the reported X-ray diffraction studies. As such, the proposed manual customization of the Schrödinger's Glide covalent docking platform can be used to reliably predict molecular interactions between OPs and *Ag*AChE target.

Keywords: Molecular modeling; Covalent docking; Organophosphates; *Anopheles*; Acetylcholinesterase

1. Introduction

Acetylcholinesterase (AChE), a serine hydrolase located at the cholinergic brain synapses and neuromuscular junctions, attains normal neuronal impulse transmission by hydrolyzing the neurotransmitter, acetylcholine, forming acetate and choline [1]. This enzyme has been an effective target in malaria vector control using organophosphates (OPs) and carbamates, the largest group of insecticides. The OPs were the most frequently used World Health Organization (WHO) prequalified insecticides in a recent 10-year survey [2]. The OPs are considered more potent than carbamates due to the "aging" process that forms a true covalent bond resulting in the permanent deactivation of AChE enzyme [3, 4]. These phosphate ester compounds get hydrolyzed by AChE to a covalently bonded dialkylphosphate and release a leaving group, one of the singly bonded substituents with the weakest bond (Figure 1) [5]. The insecticidal activity of OPs is achieved by this irreversible inhibition of AChE leading to continuous cholinergic impulse transmission and subsequent death from neuronal hyperexcitation [6]. This mechanism of action is evident by the efficacy of malathion, an OP insecticide used in malaria vector control, that is known to exert 100% mortality to susceptible *Anopheles* populations at 5% (v/v) concentration [7].

Common malaria vectors are of the *Anopheles gambiae* complex [8]. The genes encoding the AChE of *An. gambiae* (*Ag*AChE) and corresponding amino acid sequences have been previously characterized [9]. The amino acid residues involved in the OP hydrolysis is a catalytic serine, Ser^{203} (human AChE numbering) that utilizes the adjacent His^{447} and Glu^{334} to enhance its nucleophilicity in the reaction [10]. This catalytic triad corresponds to Ser^{360}, His^{600} and Glu^{359} in *Ag*AChE [11]. This irreversible binding mechanism has been confirmed by X-ray crystallography studies [12-14]. Furthermore, the phosphyl adducts of aged AChE catalytic site serine have been detected by mass-spectrometry [15].

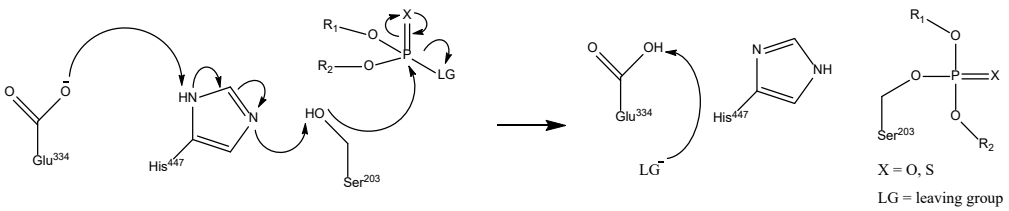


Figure 1. General OP hydrolysis by catalytic serine in the AChE catalytic site [5].

Molecular modelling is an efficient strategy that has been successfully used to study the interaction of a ligand and a target protein at a molecular level [16]. The molecular docking algorithms are generally important in predicting crystal poses and binding affinities of small molecules at the target site [17, 18]. The molecular modelling scoring functions are used to estimate and rank the binding affinities of ligand poses based on the strengths of the ligand-protein interaction [19, 20]. The molecular mechanics-based approach of the binding free energy determination, such as molecular mechanics-generalized born surface area (MM-GBSA), is regarded a more reliable method to predict binding affinities and ligand poses [21, 22]. While general docking assumes a protein is rigid and gives flexibility to only a ligand, MM-GBSA provides for some protein flexibility in noncovalent complexes [23, 24]. The reversible docking has been widely explored yet covalent docking is still scarce even with well-known irreversible inhibitors [25]. The known covalent reaction of OPs with AChE has not been displayed before in the available covalent docking panels of molecular modelling [25]. Most studies attribute this loop hole to either the lack of covalent docking systems [26], unavailable chemical reactions displaying OP hydrolysis in covalent docking panels [25] or limitations of the molecular modelling software [27]. As a result, OPs have been consistently assessed in noncovalent docking in molecular modelling studies [22, 25, 28]. While still performing the noncovalent docking, some studies opted for predicting a potential for covalent binding of OPs by assessing the binding pose and a distance between a phosphorus group of an OP and the catalytic serine residue [29-31]. The manual development of a covalent bonding feature by defining events during the reaction process has been a suggested solution to the limited exploration of covalent docking studies [27, 32, 33].

Moreover, the molecular docking can be performed when a crystal structure of the target protein is available [34]. Alternatively, homology modelling can be used to generate a three-dimensional structure of the target protein should its amino acid sequence be obtained [35]. The crystal structure for *Ag*AChE catalytic subunits has only recently been made available in Protein Data Bank (PDB), the main source of proteins for docking studies [19, 36]. As a result, there is no available data on OPs docking study on *Ag*AChE, but on other comparable targets including human AChE, though docked in a noncovalent manner [22, 37]. Schrödinger's Glide docking suite with added extra components accounting for solvation and repulsion contributions on top of various chemical forces in ligand-protein complex formation, is well-recognized for better binding affinity prediction, higher binding accuracy and consistency together with more reliable discrimination between active and inactive compounds [19, 26, 38-

40]. The Glide program has the noncovalent docking functions standard precision (SP) and extra precision (XP), as well as a covalent docking panel [22, 41, 42]. Notable is the Glide's covalent docking virtual screening workflow that has been proven highly accurate and fast in covalent docking studies [43].

This study reports the manual customization of the Glide covalent docking panel to define the reactive amino acid residue of the receptor, ligand reactive group, reaction sites on both a ligand and a receptor, formal charge and bonds created and/or broken during a reaction course. To validate the algorithm, a series of common OPs was docked in wild-type *Ag*AChE. Moreover, two OP metabolites, malaoxon (malathion's oxidative desulfuration metabolite by insect monooxygenases [7]) and methamidophos (acephate's hydrolysis metabolite by insect carboxyamidases [44]), known to be more potent than their parent drugs [45, 46], were also docked and their binding scores compared to that of their parent forms. The study goes further to explore the differences in binding crystal poses, molecular interactions and binding affinity predictions of OPs in the covalent docking versus in noncovalent docking. The two docking functions were also assessed for reliability by comparing their molecular interactions with those reported in X-ray diffraction studies. This was based on the reports that a best docking is one which regenerates the X-ray pose and interactions [18]. Carleso *et al*., (2019) stated that the comparison of Glide noncovalent and covalent modes can overcome docking challenges in the difficult-to-explore systems [47]. This research demonstrates for the first time, the covalently phosphorylated catalytic serine residue while releasing the leaving groups of OPs. Further, this is for the first time *Ag*AChE-OP complexes, in both noncovalent and covalent interactions, are reported and compared.

2. Material and methods

2.1. Target proteins preparation

Schrödinger Release 2018-2 docking suite, Maestro version 11.6, (Schrödinger LLC, New York) was used for all the docking studies. The *Ag*AChE protein (PDB: 5YDI) was retrieved from PDB [11, 36]. The 5YDI crystal structure (resolution: 3.45 Å) was in a ligand-free state. The protein was prepared with a protein preparation wizard tool for the docking studies [48]. The preparation comprised of removal of water molecules beyond 5 Å from the binding site and those forming less than 3 H-bonds to a protein or ligand. Removing these water molecules

unable to form all possible H-bonds has been reported to increase reliability in binding affinity prediction [18]. The non-protein hetero groups were also removed and completion of any missing hydrogens, side chains and loops was done. The protein was optimized to sample hydrogen orientations at pH 7.0 using PROPKA [22, 49, 50]. To satisfy the conditions of catalytic serine phosphorylation displayed in equation 1, the protonation states of His^{600} and Glu^{359} were reassessed after the optimization at pH 7.0 to ensure that they remain neutral and charged, respectively. Should their required protonation states be found to have been changed by optimization at pH 7.0, the new residue states were mutated back to their required original states and conformations [19, 50, 51]. The protein minimization by OPLS3e force field to fix all molecular overlaps and strains was then performed [49]. The restrained minimization was terminated when the average root mean square deviation (RMSD) of the non-hydrogen atoms was converged to 0.3 Å [22, 52].

2.2. Receptor grid generation

The prepared 5YDI had 2 chains (A and B). Unlike humans, mosquitoes are known to have two *ace* genes (*ace*-1 and *ace*-2) coding for AChE1 and AChE2 proteins, respectively [9, 53]. Since it is reported that the site of action is AChE1 [54, 55], the chains were split before generating the grid and only chain A protein was used for subsequent docking studies thereafter. The grid was generated using OPLS3e force field [49]. The amino acid residues selected to define the active site that enclosed a catalytic Ser^{360}, while incorporating a catalytic triad were Ala^{242}, Trp^{245}, Phe^{277}, Phe^{281}-Tyr- Ser-Gly^{284}, Ala^{361}-Gly^{362}, Tyr^{489}-Phe^{490}, Trp^{441}-Gly-Tyr-Leu-Gly-Ile-Cys-Glu^{448}, Pro^{450}, Tyr^{494}, Phe^{560}, Trp^{592}, Met^{599} and Gly^{601}.

2.3. Ligand preparation

The chemical structures of common OP insecticides [28, 56, 57] were obtained from PubChem database. The selected OPs were malathion, malaoxon, acephate, methamidophos, dimethoate, thiometon, phosmet, terbufos, ethion and azinphos-methyl (Figure 2) and all these are known to be effective AChE-inhibitor OP insecticides [58, 59]. The selected OPs structurally fall into three subgroups being aromatic, non-aromatic and hydrophobic OPs. Ligands were drawn using Maestro's 2D Sketcher and subsequently subjected to LigPrep (ligand preparation) module of Schrödinger suite [60]. A robust p*Ka* prediction tool, Epik, was

used to generate possible ionization and tautomeric states at pH 7.0 (+/- 2.0) [61-63] and each ligand could generate maximum 32 stereoisomers. The ligands were then minimized by the OPLS3e force field [49].

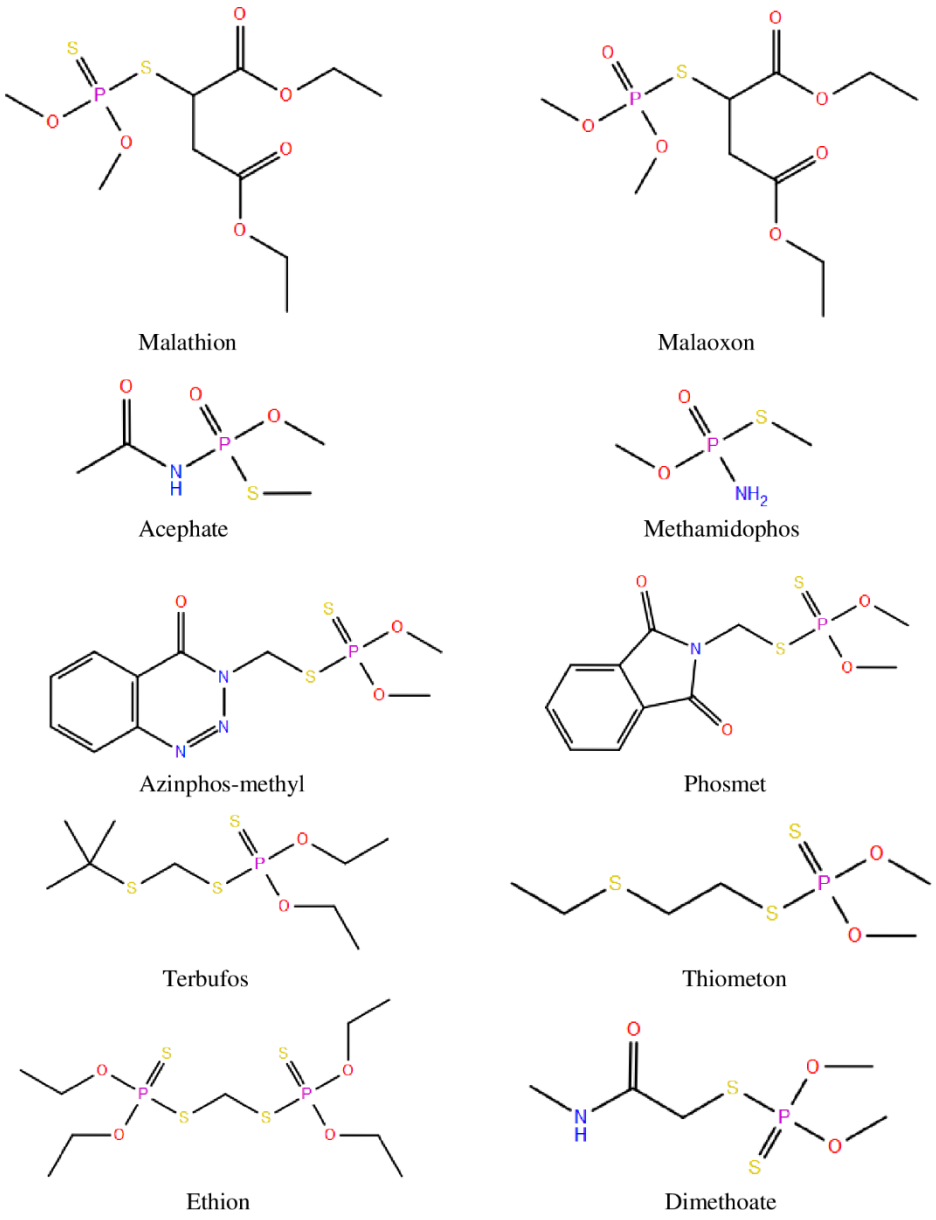


Figure 2. Chemical structures of ligand OPs.

2.4. Noncovalent docking of OPs against AgAChE

Determination of noncovalent interactions of OPs with *Ag*AChE was done through standard precision (SP) and extra precision (XP) modules. The ligand docking panel was displayed on the workspace and the previously generated 5YDI receptor grid retrieved, while the ligands were selected from the project table. The ligands could be flexible, and their nitrogen inversions

(pyramidal nitrogen atoms) and ring conformations were sampled during the docking process. Only amides were penalized for nonplanar conformations as a default setting. Lastly, Epik state penalties were applied to the docking scores. This is for adopting higher energy states for ligands that were ionized or tautomerized in the preparation stage [62, 63]. The SP docking format followed by XP, was then selected. The output poses per ligand was set to 1 best pose, while post-docking minimization was also performed. The SP and XP poses were compared to assess consistency in noncovalent docking, while prediction for covalent bond formation was also done. It is known that a pose that puts an electrophilic ligand atom close enough to the nucleophilic amino acid residue of the receptor resembles a potential for covalent bonding [27, 43]. In this study, this was defined as a pose at the catalytic triad placing a phosphorus atom of the OP closer to the catalytic serine residue [25]. Since XP is reported to be more rigorous and accurate than SP [18, 22], only XP complexes were kept and further evaluated for binding affinity profiles in the MM-GBSA panel.

2.5. MM-GBSA-based re-docking of OPs against AgAChE

The binding affinities of OP ligands to *Ag*AChE were assessed through MM-GBSA based re-docking. The previously docked noncovalent complexes were retrieved and the solvation model VSGB together with OPLS3e force field were used in the re-docking. Distance from ligand was changed from 0 Å to 5 Å to allow enough flexibility for the target protein [23, 24]. The binding affinities for OPs were compared.

2.6. Optimization of covalent docking of OPs against AgAChE

Firstly, the covalent docking reaction indicating the catalytic serine – OP covalent interaction was sought in the Glide's predefined (built-in) and custom covalent reactions available online at Schrödinger's covalent reactions repository (https://www.schrodinger.com/products/covdock). Following the negative results from a reaction's search, the customized Glide covalent docking reaction algorithm was generated to define an electrophilic phosphorus center of OPs, nucleophilic hydroxyl group of serine residue (Ser-OH) on the target protein, ligand attachment atom on a receptor (S), receptor attachment atom on a ligand (P), formal charge (0) and bonds created (1 bond between an oxygen atom of Ser-OH and phosphorus atom of phosphate group on OP) and/or broken (1 bond on a ligand

(P-S or P-N (in the case of acephate)) to liberate a leaving group) during a reaction process. The final customized Glide covalent docking algorithm was as follows:

RECEPTOR_SMARTS_PATTERN 2,[C,c]-[S,O;H1,-1]

LIGAND_SMARTS_PATTERN 1,[P]=[O,S]

CUSTOM_CHEMISTRY ('<1>', ('charge', 0, 1))

CUSTOM_CHEMISTRY ('<1>|<2>', ('bond', 1, (1, 2)))

CUSTOM_CHEMISTRY ('<2>[P,S]', ('bond', -1, (1, 2)))[1]

[1] *For acephate*:

CUSTOM_CHEMISTRY ('<2>[P,N]', ('bond', -1, (1, 2))) based on its distinct chemical structure.

The algorithm was stored in a cdock format and retrieved during the covalent docking study of OPs.

2.7. Covalent docking of OPs against AgAChE

The covalent docking studies were performed using the covalent docking module of the Schrödinger Release 2018-2 suite [42]. The covalent docking panel (CovDock) was used. The CovDock workflow has been reported to be highly accurate in pose prediction of covalent inhibitors [42]. The prepared OP ligands were selected from a project table, while a reactive receptor residue (Ser^{360}) was selected from a 5YDI protein on the workspace. The amino acid residues Phe^{560}, Trp^{245}, Tyr^{489} and Glu^{359} were selected to define the active site that enclosed a catalytic Ser^{360} while incorporating a catalytic triad to generate the binding site displayed in Figure 3.

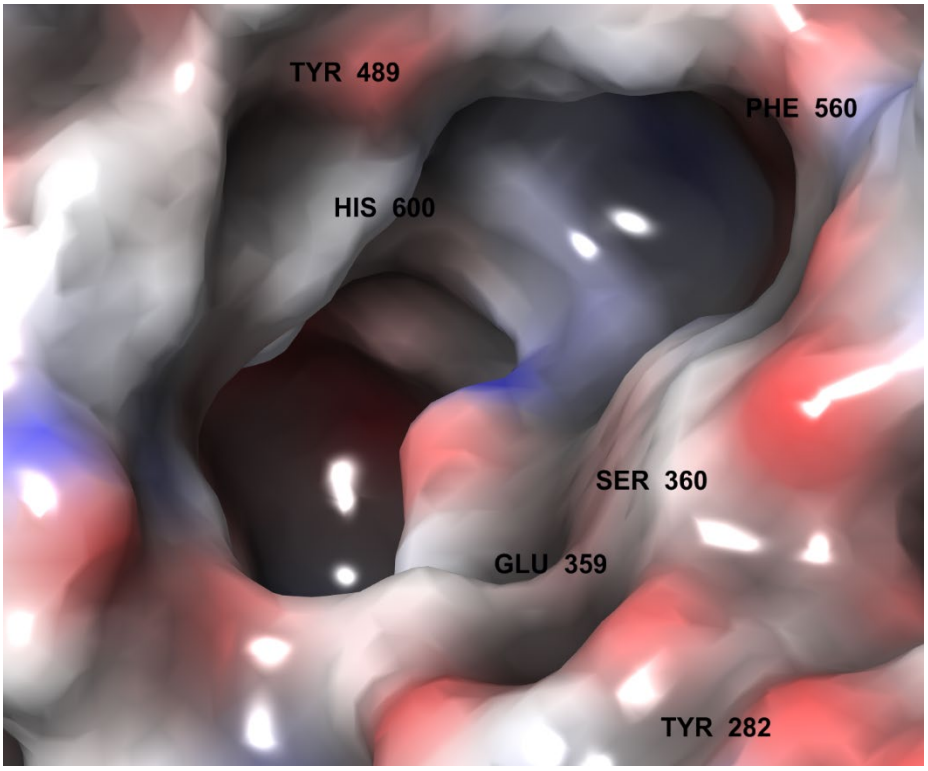


Figure 3. Generated binding site on the *Ag*AChE.

The box size enabled docking of ligands of ≤ 20 Å length. The customized covalent docking algorithm was then selected as the reaction type. No constraints were imposed on the ligand for docking and virtual screening mode was selected. The total energy 2.5 kcal/mol was set as the cutoff to retain poses for further refinement by default, while the maximum number of poses to retain for further refinement, was 200. The output poses per ligand reaction site was set to 1 best pose.

3. Results and discussion

3.1. Noncovalent docking of OPs against AgAChE

The noncovalent docking produced poses at the catalytic triad with the conformations enabling phosphate groups to be closer to the catalytic Ser^{360} residue. The OPs generated H-bonds between Ser^{360} and oxygen or sulfur atoms that were doubly bonded to the phosphorus atom (Figure 4-5 and Figure S1.0-S1.1 (SI)). Alternatively, this H-bond was formed using one of the two alkoxy groups attached to the phosphorus atom of the OPs as in the case of phosmet, terbufos, thiometon and ethion (Figure S1.2-S1.5 (SI)).

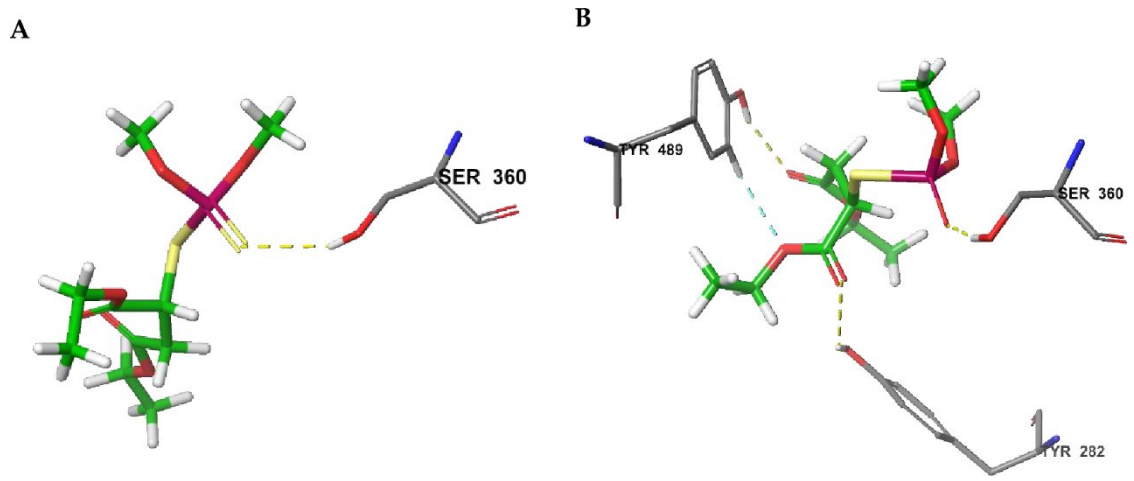


Figure 4. Malathion (A) and malaoxon (B) docked at *Ag*AChE with a noncovalent function.

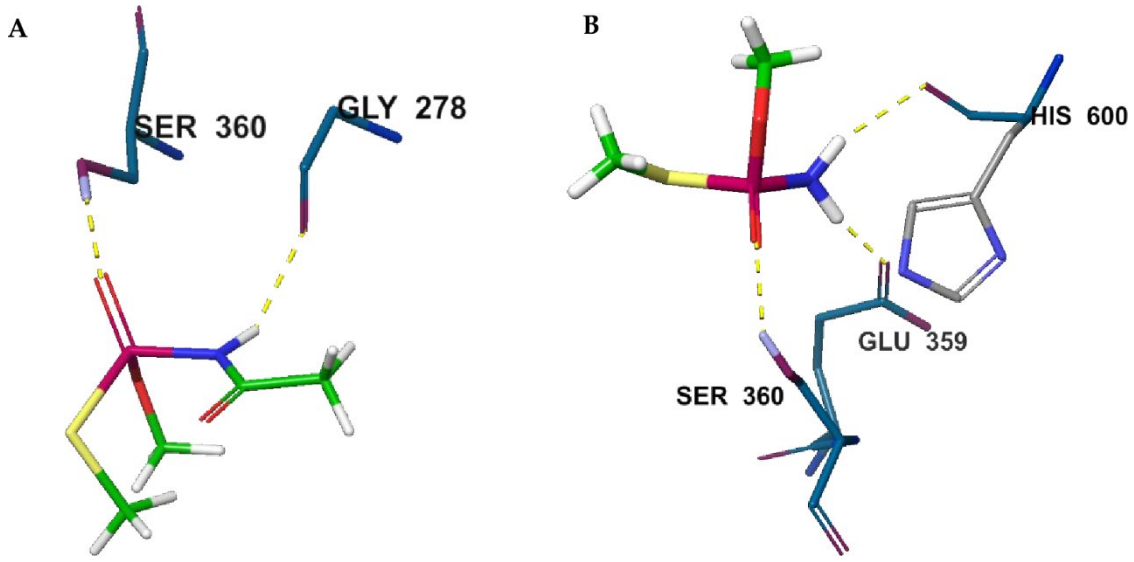


Figure 5. Acephate (A) and methamidophos (B) docked at *Ag*AChE with a noncovalent function.

While malathion formed only a Ser^{360} H-bond, its metabolite malaoxon, known to be relatively more potent [45], formed extra H-bonds with Tyr^{282} and Tyr^{489} residues. Moreover, methamidophos displayed a favourable and unique profile of interaction by forming H-bonds with the catalytic triad, Ser^{360}, Glu^{359} and His^{600} residues as opposed to its parent molecule, acephate, that interacted with only Ser^{360} and Gly^{278}. Additional bonds were either an aromatic π-stacking with Tyr^{282} residue or aromatic H-bond with Ser^{283} for the aromatic OPs such as phosmet and azinphos-methyl, respectively. However, azinphos-methyl formed extra H-bond with Tyr^{489} through its benzotriazine group. Dimethoate also formed the extra H-bond with Ser^{283} through its amide group. The relatively hydrophobic OPs, terbufos, thiometon and ethion could only form the Ser^{360} H-bond (Figure S1.3-S1.5 (SI)) suggesting a potential for none other

than a covalent bond in the polar binding site. The orientation of the leaving groups opposite to the catalytic serine observed in noncovalent docking has previously been reported as an indication of the possibility for elimination of the leaving group [13, 14]. However, the noncovalent binding scores generally could not show significant difference between the metabolites, malaoxon and methamidophos and their parent drugs, malathion and acephate, respectively (Table 1). Haider *et al*., (2020) suggested that MM-GBSA scoring and pose generation functions offer more accurate results than the noncovalent mode [64]. The supplementation of noncovalent docking with MM-GBSA scoring has shown efficiency in many studies [64-66]. Conversely, the setback with CovDock scoring system is that of not considering the covalent bond formation. This results in lower scores and may show only a little difference from the noncovalent docking scores [42, 47]. As a result, the OPs are ranked according to their MM-GBSA binding scores in Table 1.

3.2. MM-GBSA-based re-docking of OPs against AgAChE

The MM-GBSA module was more rigorous in identifying the amino acid residues involved in the binding of these OPs. For malaoxon, while noncovalent docking suggested two tyrosine amino acid residues Tyr^{282} and Tyr^{489} apart from the catalytic Ser^{360}, MM-GBSA rather indicated the catalytic site amino acid residues His^{600} and Tyr^{282} (Figure 6). This was also the case with acephate and azinphos-methyl where His^{600} and Tyr^{282}, respectively, were identified by MM-GBSA and not noncovalent mode, as some of the critical amino acid residues for their binding and correct ligand pose generation (Figure 7 and Figure S2.0). With His^{600} being a catalytic triad amino acid, this shows that MM-GBSA generated poses that were closer to the AChE binding site as previously reported [67]. Interestingly, this favourable interaction with OPs has been reported in the X-ray diffraction studies [12-14, 68]. The MM-GBSA study showed an increased binding affinity for methamidophos over acephate (Table 1) in agreement with the reported clinical data [46]. In a similar manner, malaoxon had a higher affinity score than malathion in correlation to the literature [45]. This complies with the literature that MM-GBSA is able to differentiate between closely related ligand structures and thereby generate accurate poses and affinity ranking [65, 66]

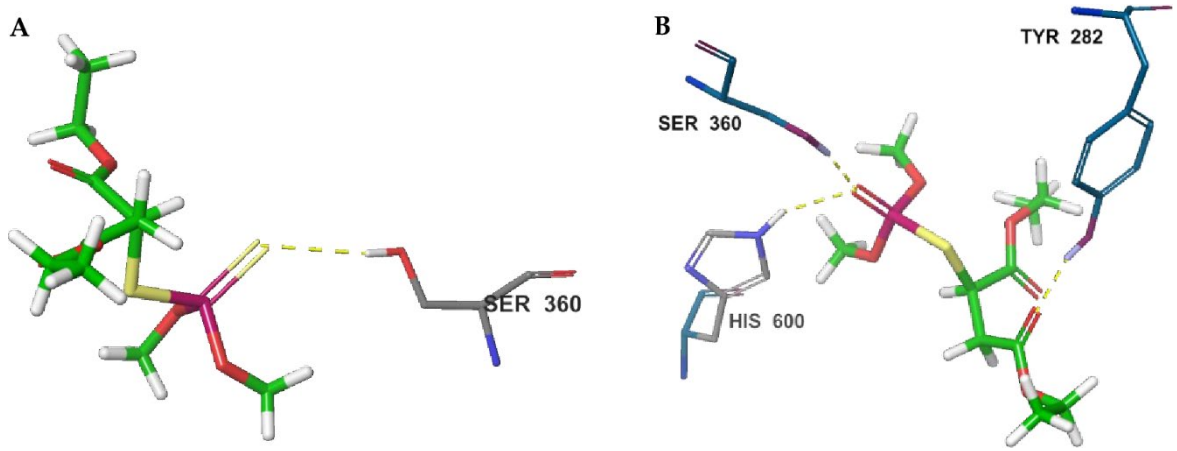


Figure 6. Malathion (A) and malaoxon (B) re-docked at *Ag*AChE with MM-GBSA method.

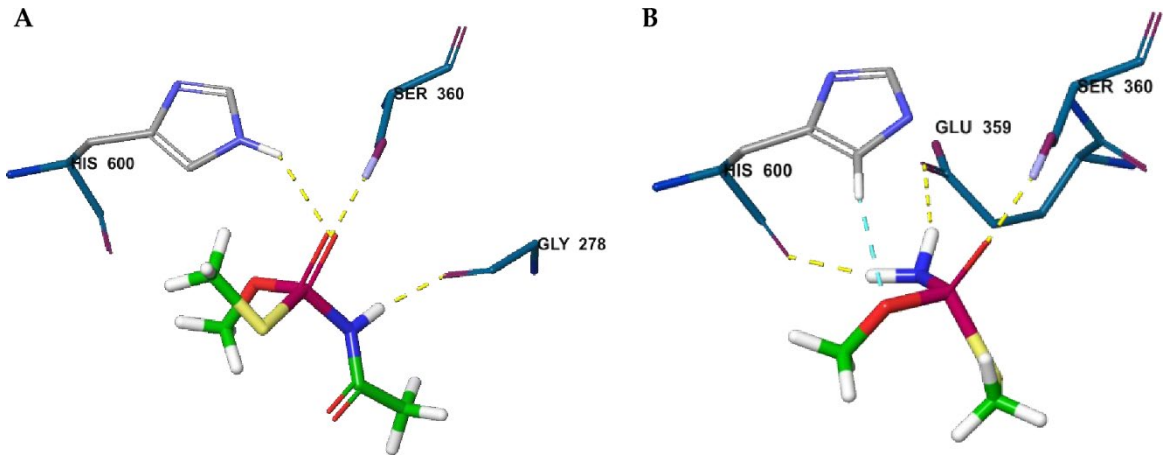


Figure 7. Acephate (A) and methamidophos (B) re-docked at *Ag*AChE with MM-GBSA method.

3.3. Optimization of covalent docking of OPs against AgAChE

The customized covalent docking reaction algorithm was successfully generated and utilized, where it produced the covalently phosphorylated catalytic serine residues and displayed the OP leaving groups in agreement with the literature and X-ray diffraction studies [5, 12-14]. The concept proved true for both sulfur and oxygen-based OPs (those bearing a P=S or P=O bond) having either sulfur- or nitrogen-based leaving groups.

3.4. Covalent docking of OPs against AgAChE

Using the newly customized covalent docking reaction algorithm, the OPs in this study were successfully covalently docked in *Ag*AChE catalytic site. The CovDock docking scores are

displayed in Table 1. The covalently bonded OPs displaying phosphorylated Ser^{360} residues and released leaving groups are displayed in Figure 8-9 below and in Figure S3.0-S3.5 (SI). While malathion formed the Ala^{361} and Gly^{280} H-bonds with the leaving group, these H-bonds were formed with a covalently bonded portion in malaoxon, acephate and methamidophos (Figure 8-9), as well as with dimethoate (Figure S3.1 (SI)). Additionally, malathion, acephate, methamidophos and azinphos-methyl formed extra H-bonds with the His^{600} residue. This type of bonding profile that involves Gly^{280}, Ala^{361} and His^{600}, while covalently bonded to Ser^{360} residue has previously been reported in X-ray diffraction with two of the most toxic chemical warfare OP nerve agents, namely sarin and soman. Similarly, this binding mode has been reported with di*iso*propyl phosphorofluoridate, a potent OP insecticide that was also developed as a warfare agent and is also used for its anticholinergic effects in chronic glaucoma [12, 69-72].

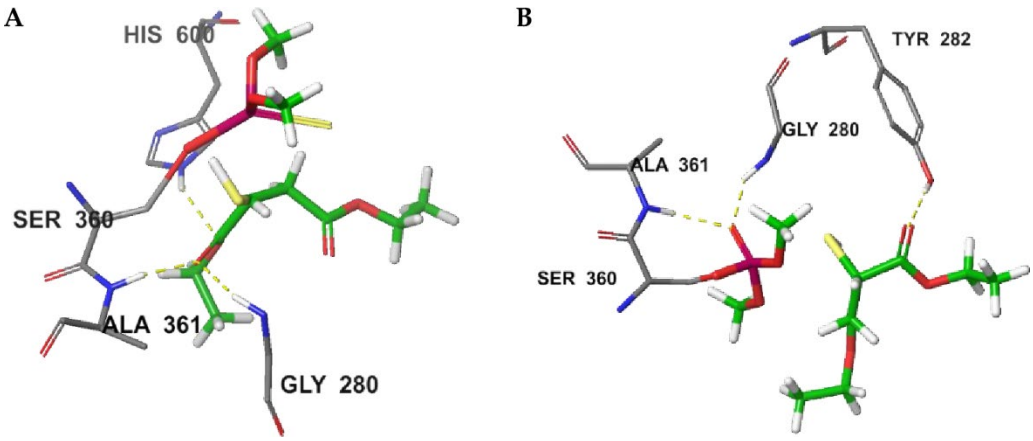


Figure 8. Malathion (A) and malaoxon (B) docked at *Ag*AChE with new covalent function.

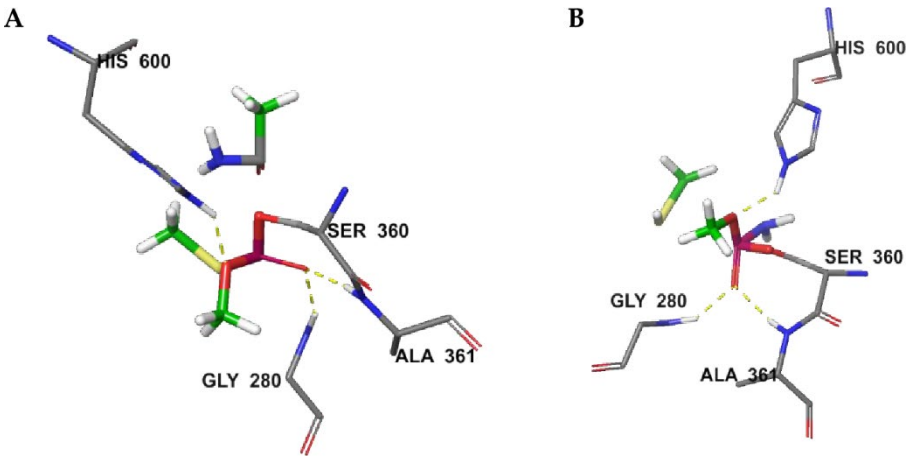


Figure 9. Acephate (A) and methamidophos (B) docked at *Ag*AChE with new covalent function.

Table 1. Glide docking scores for OPs bonded to 5YDI target *Ag*AChE protein.

Glide docking scores (kcal/mol) Wild-type *Ag*AChE (5YDI)			
Organophosphate	**Glide-dock XP**	**MM-GBSA**	**Optimized CovDock**
azinphos-methyl	-5.391	-67.55	-6.546
malaoxon	-5.856	-60.01	-7.035
dimethoate	-3.924	-49.14	-5.217
malathion	-5.42	-47.92	-5.184
ethion	-4.541	-44.87	-4.923
phosmet	-5.558	-43.29	-6.827
methamidophos	-4.027	-32.89	-6.12
thiometon	-3.704	-31.93	-4.393
terbufos	-5.459	-26.11	-4.802
acephate	-4.081	-23.45	-5.355

Unlike the noncovalent docking, the customized covalent docking generated poses that were closer to the catalytic site and interacted mainly with the catalytic site amino acid residues. This was evident with malathion, malaoxon, acephate, methamidophos, dimethoate, and azinphos-methyl that interacted with His^{600}, Ala^{361}, Gly^{280}, and Tyr^{282} residues in addition to the Ser^{360} covalent bond. While malathion interacted with only Ser^{360} in the noncovalent mode, it effectively interacted with Ser^{360}, His^{600}, Ala^{361} and Gly^{280} in the covalent docking platform (Table 2). Similarly, malaoxon was predicted to interact with Tyr^{489} and Tyr^{282} in noncovalent docking. However, Tyr^{489} was excluded in both MM-GBSA and CovDock modes that rather indicated increased interaction with catalytic site amino acid residues (Table 2). This was a similar case with azinphos-methyl where this tyrosine residue (Tyr^{489}) located far from the catalytic triad was excluded at MM-GBSA and CovDock stages. Sasmal *et al*., (2020) stated that high accuracy and reliable affinity prediction is obtained with poses generated closest to the binding site [67]. On the other hand, it was observed that due to their small size, acephate and methamidophos could effectively reach the catalytic triad and interact with the catalytic site amino acid residues throughout all the docking modes (Table 2). It is agreed that ligands with a similar scaffold display a similar binding conformation [73]. Nevertheless, it is evident from the molecular interactions (Table 2) and displayed conformations (Figure 4) that the noncovalent mode did not establish a similar pose even with molecules that differ by one atom

such as malathion and malaoxon. Kaus *et al.*, (2015) found out that the Glide noncovalent mode could only correctly predict the ligand poses for four of the twenty ligands with a similar scaffold [65]. Interestingly, the optimized covalent docking mode established similar binding modes for similar OPs such as malathion and malaoxon, acephate and methamidophos as well as phosmet and azinphos-methyl (Table 2, Figures 7, S2.0, and S2.2).

Table 2. Comparison of amino acid residues involved in the binding of OPs to 5YDI target *Ag*AChE protein in different docking methods.

	Amino acid residues		
Organophosphate	**Glide-dock XP**	**MM-GBSA**	**Optimized CovDock**
azinphos-methyl	Ser^{360}, Ser^{283}, Tyr^{489}	Ser^{360}, Tyr^{282}	Ser^{360}, Tyr^{282}, His^{600}
malaoxon	Ser^{360}, Tyr^{282}, Tyr^{489}	Ser^{360}, Tyr^{282}, His^{600}	Ser^{360}, Ala^{361}, Gly^{280}, Tyr^{282}
dimethoate	Ser^{360}, Ser^{283}	Ser^{360}, Ser^{283}	Ser^{360}, Ala^{361}, Gly^{280}
malathion	Ser^{360}	Ser^{360}	Ser^{360}, Ala^{361}, His^{600}, Gly^{280}
ethion	Ser^{360}	Ser^{360}	Ser^{360}
phosmet	Ser^{360}, Tyr^{282}	Ser^{360}, Tyr^{282}	Ser^{360}, Tyr^{282}
methamidophos	Ser^{360}, Glu^{359}, His^{600}	Ser^{360}, Glu^{359}, His^{600}	Ser^{360}, Ala^{361}, His^{600}, Gly^{280}
thiometon	Ser^{360}	Ser^{360}	Ser^{360}
terbufos	Ser^{360}	Ser^{360}	Ser^{360}
acephate	Ser^{360}, Gly^{278}	Ser^{360}, Gly^{278}, His^{600}	Ser^{360}, Ala^{361}, His^{600}, Gly^{280}

Moreover, critical analysis of the generated conformations also identified some differences between noncovalent and covalent modes. By virtue of not considering the OP hydrolysis, noncovalent mode may suggest intermolecular interactions that may not otherwise occur when the molecule is hydrolyzed. This was observed, for instance, in the case of azinphos-methyl, malaoxon and dimethoate where amino acid residues located further from the catalytic triad, namely Ser^{283} and Tyr^{489}, could form H-bonds with such OPs only in the noncovalent mode. In its original structure as retained in the noncovalent docking, dimethoate extended to Ser^{283} while it was however confined closer to the catalytic triad in the covalent mode (Figure 10).

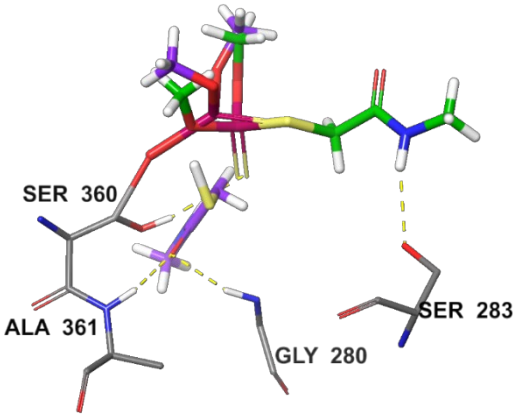


Figure 10. Superimposed dimethoate in noncovalent (green) and covalent (violet) binding states.

Similarly, in the covalent mode, malathion was held tightly to the catalytic triad and the leaving moiety was released closer to the catalytic serine hence interactions with Ala^{361} and Gly^{280} residues. Also, malaoxon was bound tightly to the catalytic serine allowing the orientation of its doubly bonded phosphoryl oxygen (P=O-) to face towards Ala^{361} and Gly^{280} residues. However, both malathion and malaoxon were relatively weakly bound by the catalytic serine in the noncovalent docking with their alkoxy carbonyl groups assuming the orientation facing away from the catalytic triad. This enabled the interaction with Tyr^{489} residue located far from the catalytic triad, in the case of malaoxon (Figure 11).

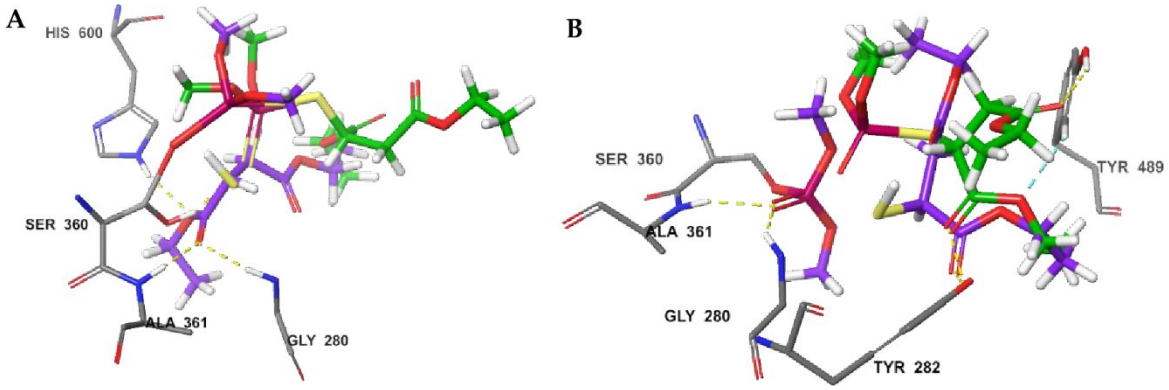


Figure 11. Superimposed malathion (A) and malaoxon (B) in noncovalent (green) and covalent (violet) binding states.

3.5 Catalytic site distortion observation

Several amino acid rearrangements occur upon aging [74]; where the catalytic histidine is reported to be mobile during the aging process [12, 13, 74]. In this study, histidine did not show evidence of rearrangement; however, the catalytic serine showed reorientation with larger ligands, such as malathion and malaoxon (Figure 12). This was not a case with smaller ligands, such as acephate and methamidophos (Figure 12), which is in agreement with the literature that smaller OPs do not cause distortion at the catalytic site upon dealkylation [13]. It was notable that with malathion and malaoxon, the distance between the catalytic serine and histidine had increased substantially (3.42 Å) from the non-aged form (3.19 Å) or that reported in the original protein (3.10 Å) [36].

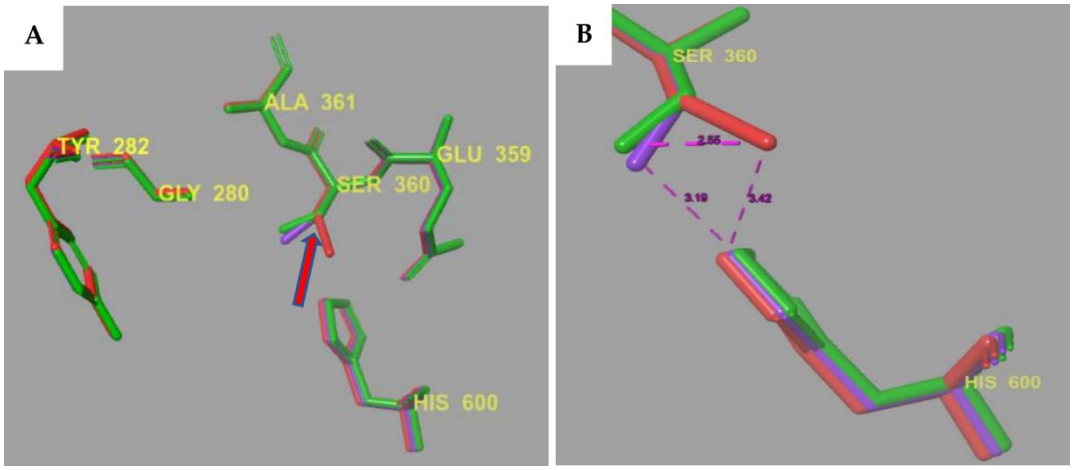


Figure 12. Superimposed *Ag*AChE active site amino acid residues non-aged (green) and aged by malathion or malaoxon (red) versus acephate or methamidophos (violet). **(A)** Shows the catalytic serine reorientation (arrow) in malathion or malaoxon phosphorylation (red) and **(B)** shows the reorientation distance from the non-aged catalytic serine (2.55 Å) and increased distance from histidine (3.42 Å). Ligands have been undisplayed to enhance visibility.

While all the subgroups could form a covalent bond with Ser^{360}, there was a noticeable difference in the bonding interactions between aromatic OPs, non-aromatic OPs and hydrophobic OPs with the target sites. The aromatic OPs (azinphos-methyl and phosmet) were stabilized by the aromatic interactions (π - π stacking) mainly from Tyr^{282}, the non-aromatic OPs (malaoxon, acephate, methamidophos and dimethoate) utilized Gly^{280}, Ala^{361} and His^{600} residues, while the hydrophobic OPs only formed a covalent bond. Due to smaller sizes, the elimination of the leaving groups in methamidophos and acephate, has been reported to induce

no distortion of the catalytic site [13]. This study has shown catalytic serine reorientation with malathion and maloxon (Figure 8); which has previously been reported with the high molecular weight ligands such as di*iso*propyl phosphorofluoridate [13]. The increase in distance between the catalytic serine and histidine at the malathion or malaoxon phosphorylated sites indicated a potential for steric effects with these bigger molecules. Additionally, the covalently bonded portions maintained an interaction with the His^{600} residue through their singly-bonded phosphoryl oxygen (P-O-) in these two latter OPs, which is a common characteristic seen in X-ray crystallographic complexes involving OPs and AChE targets [12-14, 68]. However, the X-ray crystallographic studies focus on the generated catalytic serine adducts rather than the dealkylated or deaminated leaving groups [12]; since aged OP-AChE complexes do not allow direct viewing of the leaving group. Nevertheless, its general topography is inferred from the aged active site [12, 75]. As a result, the reliability of the covalent docking mode utilized in this study could be better assessed based on the covalently bonded portions.

Furthermore, good correlation was obtained whereby noncovalent docking modes, MM-GBSA and CovDock, all suggested that malaoxon was among the three OPs with highest binding scores for the *Ag*AChE and was suggested as the best binding OP in the covalent docking panel (Table 1). Generally, the scores obtained in this study correlate to those previously obtained when docking related OPs to the human AChE with Glide XP and MM-GBSA [22].

Comparing the molecular interactions displayed by the malaoxon as opposed to malathion, malaoxon possesses an advantage with oxygen that has replaced sulfur in malathion. This oxygen is on the covalently bonded phosphate group and forms two favourable H-bonds with Gly^{280} and Ala^{361}. This is possibly due to oxygen being more electronegative than sulfur, hence a better H-bond acceptor [76]. This correlates to the literature that reports that the thiophosphoryl OPs are less potent AChE inhibitors compared to other phosphoryl derivatives [77]. The leaving group of malathion formed the other H-bond between its ester group and His^{600}, while malaoxon utilized Tyr^{282}. Together, formation of the Gly^{280} and Ala^{361} H-bonds with a covalently bonded group and Tyr^{282} H-bond with a leaving group, yielded a higher binding score for malaoxon as opposed to malathion. Similar to malathion, dimethoate has a sulfur-based phosphate group and accessible carbonyl group in the leaving moiety that displayed a similar binding profile with malathion and closer binding scores (Table 1).

On the other hand, oxygen-based phosphate groups (P=O), as in acephate and methamidophos, formed H-bonds with Gly^{280} and Ala^{361} with a covalently bonded phosphate group in a similar fashion to malaoxon. Methamidophos may be showing a higher score than acephate due to its lack of steric effects around the electrophilic phosphorus atom as the two insecticides display similar interactions within the *Ag*AChE catalytic site. All other assessed OPs could not form H-bonds with Gly^{280} and Ala^{361} presumably due to the lack of a P=O bond or an accessible carbonyl group in their leaving moieties. Azinphos-methyl and phosmet have carbonyl groups in their amides that are connected to large aromatic rings, facilitating aromatic π-interactions with the Tyr^{282} residue. The lack of a P=O bond and an accessible carbonyl in the leaving group resulted in OPs such as terbufos, thiometon and ethion, with generally hydrophobic leaving moieties, forming only a covalent bond with the catalytic serine residue. The importance of Tyr^{282} in the mechanism of action of the insecticides was evident by its ability to either form a H-bond with non-aromatic OPs that have H-bond acceptors of high electronegativity (malaoxon) or to establish aromatic interactions with aromatic OPs (azinphos-methyl and phosmet). This role of Tyr^{282} was also observed in the noncovalent docking module. The critical role of the aromatic amino acids in stabilizing OPs at the catalytic site has previously been supported by studies on human AChE [28]. This binding profile obtained with the aromatic OPs that utilizes Ser^{360}, Tyr^{282} and His^{600} has been previously reported for aromatic OP analogues in the AChE protein of *Musca domestica* (housefly insect) [78, 79].

Therefore, in this study noncovalent docking was coupled to covalent docking for the first time. The noncovalent docking generated poses at the catalytic triad with orientations that suggested a potential for covalent bond formations. However, the noncovalent mode generated poses that were relatively far from the catalytic triad and suggested the intermolecular interactions further from the binding site. This indicated low accuracy and less reliability of OP complexes generated through noncovalent options. On the other hand, in agreement to the literature [66], the MM-GBSA method produced ligand poses closer to the catalytic triad accompanied by increased interactions with the catalytic site amino acid residues. This method was rigorous in excluding amino acid residues that are distant from the catalytic triad such as Ser^{283} and Tyr^{489} that were otherwise suggested by the noncovalent panel. The MM-GBSA procedure showed a greater potential for covalent bond formation and interaction with the catalytic site amino acid residues. This was subsequently confirmed by the establishment of the predicted covalent bond in the covalent docking and favourable interactions with catalytic

triad and immediate amino acid residues. In addition, the hydrophobic OPs showed potential for only a covalent bond and indeed, in the covalent docking study, this was the case. The customized covalent model was in agreement with the MM-GBSA for exclusion of amino acid residues located further from the catalytic triad. However, this covalent docking mode generally established more catalytic site interactions than both MM-GBSA and noncovalent docking modes. Notably, with the covalent docking panel ending in two apparent molecules (covalently bonded dialkylphosphate and a leaving group), the intermolecular interactions generated with noncovalent docking that maintained the original OP structure were altered substantially in the covalent docking panel. A docking panel that generates ligand poses closer to the binding site is more reliable [67]. While noncovalent docking created poses and intermolecular interactions far from the catalytic triad, the customized covalent docking method generally confined poses and interactions closest to the catalytic triad. This docking system eliminated distant amino acid residues such as Ser^{283} and Tyr^{489} and maintained interactions with mainly the catalytic site residues such as Gly^{280}, Ala^{361} and His^{600} residues in correlation with the X-ray diffraction studies [12, 69, 70].

4. Conclusions

The reliable covalent docking for OPs in *Ag*AChE has been optimized through manual customization of the Schrödinger's Glide covalent docking reaction algorithm. The noncovalent docking module generated crystal poses and molecular interactions that were relatively distant from the catalytic site. Additionally, the noncovalent docking could not establish similar binding orientations for the structurally similar OPs showing non-reliability in the investigations of OPs binding mode. The MM-GBSA corrected the binding poses by eliminating interactions with amino acid residues distant from the catalytic triad and established intermolecular interactions that stabilized OPs in the orientations that are more appropriate for a covalent bond formation. Upon submission of the OPs to the newly customized covalent docking reaction algorithm, the required covalently phosphorylated catalytic serine residues and the hydrolyzed leaving groups were effectively generated. Furthermore, the covalent docking mode restricted the interactions and ligand poses to the catalytic site as well as establishing similar binding modes for OPs with similar scaffolds indicating relatively high accuracy and reliability. The binding crystal poses and molecular interactions displayed in the covalent docking studies were similar to those previously reported

in X-ray diffraction studies. This study also established that high molecular weight OPs such as malathion and malaoxon may have capacity to induce catalytic site distortion. Moreover, the amino acid residues playing important roles in the binding affinities of OPs and corresponding structural features have been identified. Generally, the aromatic OPs formed H-bonds with Ser^{360} and His^{600} whereby their aromatic rings were stabilized by the Tyr^{282} residue. The non-aromatic OPs on the other hand, utilized Ser^{360}, Gly^{280}, Ala^{361} and His^{600}, while the hydrophobic OPs only formed a covalent bond with the Ser^{360} residue. Therefore, in this study, a covalent docking reaction algorithm that effectively displays the molecular mechanism of OPs is proposed for further evaluation in covalent docking studies involving a larger library of OPs or OP analogues and a wide variety of targets such as G119S mutated *Anopheles* AChE (PDB: 6ARY) and human AChE (PDB: 5HF5).

Supplementary Materials: The following are available as supplementary data: Figure S1.0-S1.5: azinphos-methyl, dimethoate, phosmet, terbufos, thiometon and ethion, respectively, docked in noncovalent function, Figure S2.0-S2.5: azinphos-methyl, dimethoate, phosmet, terbufos, thiometon and ethion, respectively, re-docked with MM-GBSA method, Figure S3.0-S3.5: azinphos-methyl, dimethoate, phosmet, terbufos, thiometon and ethion, respectively, docked in a new covalent function.

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CHAPTER 4

The *in vitro* and *in silico* assessment of donepezil derivatives for the *Anopheles* acetylcholinesterase inhibition

While Chapter 3 presented mainly the *in silico* methods, this chapter investigated the *in silico* and *in vitro* acetylcholinesterase inhibition assays, coupled to the *in vivo* evaluation of *Anopheles* larval toxicity. The toxicity assay was performed on *Artemia franciscana*. The *in silico* assays were also expanded to include homology modelling while the *in vitro* assays were conducted through modified Ellman assay. The *in vivo* assays on *Anopheles* larva and *Artemia* nauplii were conducted with standard World Health Organization (WHO) assays. The paper related to this chapter has been published in PLoS One. The supplementary information related to this paper is available in Appendix B.

Interestingly, this chapter disproved our hypothesis that donepezil derivatives lacking activity against mammal acetylcholinesterase target may be potent against *Anopheles* target. The derivatives tested in this study were largely inactive as *Anopheles* AChE inhibitors. It was also noted that these were also mostly inactive against the four stages of *Anopheles* life cycle. For this reason, the focus of this objective was shifted towards the essential oil constituents (terpenoids) as potential *Anopheles AChE* inhibitors hence the following chapters (5-7) are based on assessment of terpenoids.

Chapter type: Published ISI paper (Appendix C.1)

Title of manuscript: The *in silico* and *in vitro* analysis of donepezil derivatives for *Anopheles* acetylcholinesterase inhibition

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Conceptualization	✓						✓
Investigation	✓	✓	✓				
Method	✓						
Data analysis	✓						
Writing – original draft	✓						
Writing – review and editing	✓		✓	✓	✓	✓	✓
Visualization	✓						
Supervision				✓	✓	✓	✓
Funding acquisition				✓	✓	✓	✓

Abstract: Current studies on *Anopheles* anticholinesterase insecticides are focusing on identifying agents with high selectivity towards *Anopheles* over mammalian targets. Acetylcholinesterase (AChE) from electric eel is often used as the bioequivalent enzyme to study ligands designed for activity and inhibition in human. In this study, previously identified derivatives of a potent AChE, donepezil, that have exhibited low activity on electric eel AChE were assessed for potential AChE-based larvicidal effects on four African malaria vectors; *An. funestus*, *An. arabiensis*, *An. gambiae* and *An. coluzzii*. This led to the identification of four larvicidal agents with a lead molecule, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2** showing selectivity for *An. arabiensis* as a larvicidal AChE agent. Differential activities of this molecule on *An. arabiensis* and electric eel AChE targets were studied through molecular modelling. Homology modelling was used to generate a three-dimensional structure of the *An. arabiensis* AChE for this binding assay. The conformation of this molecule and corresponding interactions with the AChE catalytic site was markedly different between the two targets. Assessment of the differences between the AChE binding sites from electric eel, human and *Anopheles* revealed that the electric eel and human AChE proteins were very similar. In contrast, *Anopheles* AChE had a smaller cysteine residue in place of bulky phenylalanine group at the entrance to the catalytic site, and a smaller aspartic acid residue at the base of the active

site gorge, in place of the bulky tyrosine residues. Results from this study suggest that this difference affects the ligand orientation and corresponding interactions at the catalytic site. The lead molecule **2** also formed more favourable interactions with *An. arabiensis* AChE model than other *Anopheles* AChE targets, possibly explaining the observed selectivity among other assessed *Anopheles* species. This study suggests that 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2** may be a lead compound for designing novel insecticides against *Anopheles* vectors with reduced toxic potential on humans.

Keywords: Malaria; *Anopheles*; Acetylcholinesterase; Molecular modelling

1. Introduction

Donepezil (1-benzyl-4-((5,6-dimethoxy-1-indanon)-2-yl)methylpiperidine) shown in Fig 1 is a known potent human acetylcholinesterase (AChE) inhibitor used clinically in the management of symptoms associated with mild to severe Alzheimer's disease [1, 2]. The derivatisation of donepezil has been pursued to produce more active AChE agents against the human target with several studies have shown evidence of derivatives with high potency [3-7]. Particularly, van Greunen *et al.* [4] reported a potent derivative by converting the methyl linker (part A; Fig 1) between the piperidine ring and indanone group to an ester linker. This lead compound displayed a 50% inhibitory concentration (IC_{50}) value (0.03 ± 0.07 µM) similar to that of donepezil (0.05 ± 0.06 µM) when screened *in vitro* against AChE from *Electrophorus electricus* (electric eel) [4]. The AChEs from the electric eel and human have been shown to display similar activities, kinetics and inhibition profiles, as a result, electric eel AChE is used as a less expensive alternative to human AChE in bioassays [8, 9]. In a follow-up study, van Greunen and colleagues [3] synthesized and assessed various analogues of this lead compound for improved AChE activity. These new analogues featured the substitution of the ester linker found between the indanone and piperidine ring systems in their previous hit, by an amide that is more stable against rapid metabolism. In addition, the indanone (part B; Fig 1) was replaced with various aryl and aromatic groups [10]. Though at least two analogues were considerably active (IC_{50} <10 µM), none were as active as donepezil against electric eel AChE, and some were biologically inactive (IC_{50} >100 µM) [3].

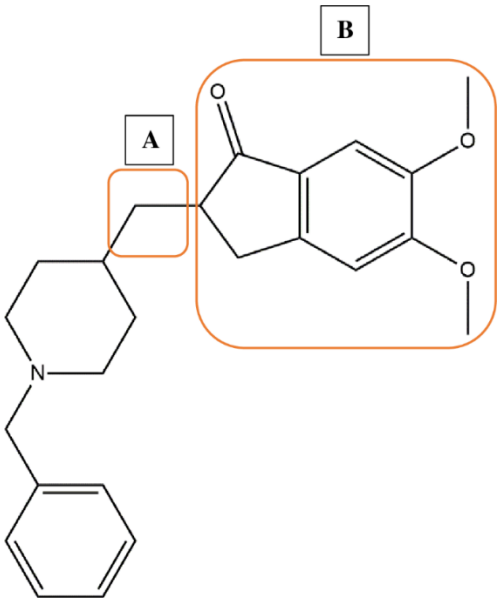


Fig 1: Chemical structure of donepezil showing two common sides of derivatization: A) methyl linker and B) indanone moiety.

Current studies on *Anopheles* AChEs are aimed at achieving high selectivity towards *Anopheles* over mammalian targets to reduce human toxicity [11-14]. Utilizing the molecular differences between human and insect AChE binding sites, these studies target conserved amino acid residues in *Anopheles* and compounds with selectivity index more than 100-fold towards *Anopheles* AChE have thus far been reported [11, 12]. The current study assessed the donepezil derivatives prepared by van Greunen *et al.* [3] for potential *Anopheles* AChE inhibition and rationalised their binding profiles through molecular docking. Interestingly, the parent drug, donepezil, has been proven to be active against insect AChE [15, 16], but is known to be approximately 40 times more selective to human AChE than the corresponding *Anopheles* target [16].

2. Materials and Methods

This study received the animal research ethics waiver (Waiver Number: 07-11-2017-O) from Wits Animal Research Ethics Committee. Nine donepezil derivatives (Fig 2) with low activity (LC_{50} values >50 μM) against electric eel AChE from a previous study [3] were used for this research. Acetylthiocholine iodide, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dimethylsulfoxide (DMSO), Triton X-100, potassium dichromate, propoxur and sodium phosphate buffer (dibasic) (Na_2HPO_4) were bought from Sigma Aldrich (South Africa) with a purity >90%.

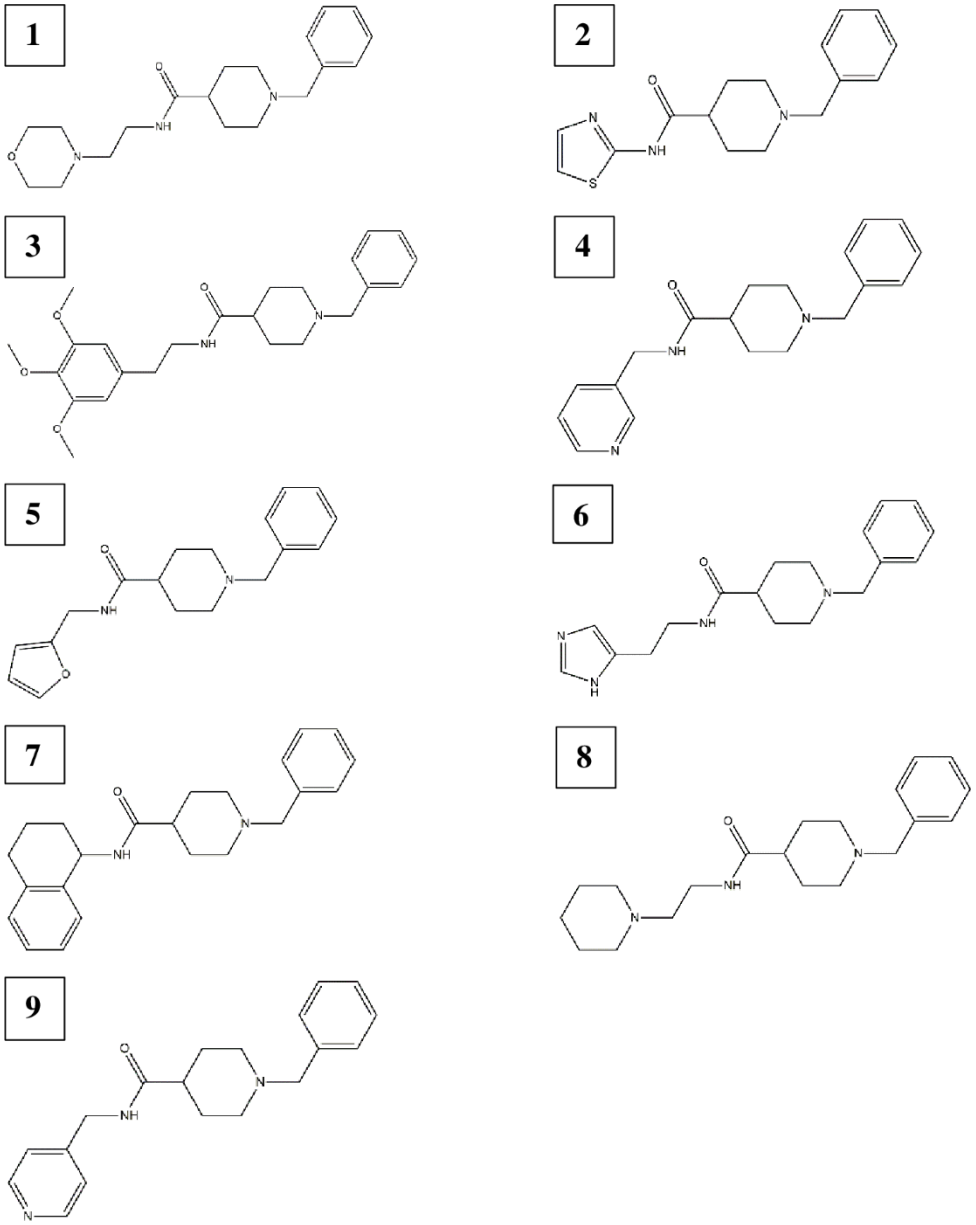


Fig 2: Chemical structures of the nine donepezil derivatives screened. 1-benzyl-*N*-(2-morpholinoethyl) piperidine-4-carboxamide **1**, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2**, 1-benzyl-*N*-(3,4,5-trimethoxyphenethyl) piperidine-4-carboxamide **3**, 1-benzyl-*N*-(pyridine-3-ylmethyl) piperidine-4-carboxamide **4**, 1-benzyl-*N*-(furan-2-ylmethyl) piperidine-4-carboxamide **5**, *N*-[2-(1H-imidazol-4-yl)ethyl]-1-benzylpiperidine-4- carboxamide **6**, 1-benzyl-*N*-(1,2,3,4-tetrahydro-naphthalen-1-yl) piperidine-4-carboxamide **7**, 1-benzyl-*N*-(2-(piperidin-1-yl)ethyl)piperidine-4-carboxamide **8**, and 1-benzyl-*N*-(pyridine-4-ylmethyl) piperidine-4-carboxamide **9** [3].

2.1. Anopheles spp. rearing

The laboratory-reared colonies of *An. funestus* (FUMOZ), *An. arabiensis* (KWAG), *An. gambiae* (COGS) and *An. coluzzii* (G3) were used for larvicidal and AChE screening assays. These are common *Anopheles* species responsible for malaria transmission in Africa [17-20].

These colonies were maintained under standard insectary conditions as reported by Hunt *et al.* [21] and Zengenene *et al.* [22]. FUMOZ was first collected from Mozambique in 2000 and has a low-level intensity of pyrethroid and carbamate resistance [23-25]. KWAG was collected from KwaZulu-Natal (South Africa) in 2005 and has shown resistance to pyrethroids and the organochloride, dichloro-diphenyl-trichloroethane (DDT) [19, 26]. On the other hand, COGS was collected in 2009 from Congo and displays resistance to multiple insecticides such as pyrethroids, organochlorides and carbamates [27, 28].

2.2. Larvicidal assay

The larval toxicity of novel donepezil analogues was assessed using the World Health Organization (WHO) bioassay for testing mosquito larvicides [29]. Briefly, batches of 20 third-instar larvae of each colony were transferred into the test cups into which 250 µL of a specific donepezil derivative was added. The incubation mixture was performed in a total volume of 250 mL of deionized water under 27°C and humidity ≥78%. The test compounds were dissolved in DMSO and assessed for larvicidal activity at 10 times increasing concentrations from 0.0005 µM to 500 µM. Propoxur, a standard larvicide and an AChE agent [30, 31], was used as a positive control, while DMSO was employed for the negative vehicle control. Larval mortality was recorded in 24, 48 and 72 hours and larvae were fed with protein dog food at day 0 and after every mortality counting [32].

2.3. Brine shrimp lethality assay

Artificial seawater was prepared by dissolving 32 g of Tropic Marine® Sea salt in 1L of deionized water. The seawater was poured into an inverted plastic bottle after which the brine shrimp eggs were added for hatching. Regular airflow was supplied to the seawater to continually disperse the eggs and oxygenate the water. Moreover, a concentrated light was supplied from a lamp (220-240 V, 15W) to provide warmth to optimize hatching conditions for the 24 h incubation time [33]. Following this, the cytotoxicity potential of the donepezil derivatives was evaluated by the brine shrimp lethality assay using *Artemia franciscana* [34]. The same concentrations used in the larvicidal assessment were also used for the toxicity evaluation. Inside 48-well plates, 50 µL of the test compound was incubated with 30 – 50 nauplii in 450 µL of the seawater. The wells were then observed under the stereo microscope (Olympus) at 10X magnification for dead nauplii and the induced mortality was recorded after 24 h. Where mortality was observed, the morphological changes were observed at 10X magnification using the stereo microscope mounted with a Dino-Eye camera. For the negative

control, DMSO was used in place of the test compound, while potassium dichromate was used as a positive control [35, 36].

2.4. AChE assay

The evaluation of AChE activity of the donepezil derivatives was conducted using the modified Ellman assay [11, 37]. Mosquitoes from the four *Anopheles* colonies were separately homogenized in Na_2HPO_4, 1% Triton X-100 (pH 8.0) and used as an enzyme source. Protein content was assessed using the standard Lowry protein assay [38]. The incubation mixture in a 96-well plate consisted of 20 µl of the enzyme in 132 µL of the assay buffer (Na_2HPO_4, 1% Triton X-100; pH 8.0). Twenty (20) µL of the donepezil derivatives at concentrations ranging from 0.0005 µM to 500 µM dissolved in DMSO were added. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), was freshly prepared in sodium phosphate buffer (dibasic; pH 7.0) and 8 µL (0.2 mM) added to the incubation mixture. To initiate the reaction, 20 µL (1 mM) of the AChE substrate, acetylthiocholine iodide was added making a total volume of 200 µL in each well and the absorbance readings were obtained using the UV-Visible spectrophotometer at 412 nm. Propoxur was kept as a positive control on the basis of having both AChE and larvicidal activities [31], while DMSO was used as a negative control. DMSO is known to exhibit AChE inhibition at concentrations above 1% [39], as such the highest DMSO concentration used in this study was 0.4%. For screening against electric eel AChE, donepezil was used as a positive control due to its known potent inhibitory activity against this target [3, 4]. However, the insecticide propoxur was also assessed against this target to determine AChE selectivity between electric eel and *Anopheles* and subsequent comparison with the test compounds.

2.5. *In silico* studies

2.5.1. Homology modelling

As a crystal structure of *An. arabiensis* was not available, it was elected to employ the use of homology modelling [40]. This approach involved four successive steps: (i) target amino acid sequence identification, (ii) template identification, (iii) sequence alignment between target and template, and (iv) model building and optimization [41, 42]. Homology modelling is considered the most accurate *in silico* approach to generate 3D models of proteins [42], however, certain minimum requirements had to be met. To produce a reliable protein structure, an existing sequence that matched at least 30% of the target sequence should be used as a template. These are known to display similar structures and interaction mechanisms [43-45].

Moreover, for the 30% identity cut-off, the length of the aligned sequences between the two should be >100 amino acid residues [46]. Sequence identity without taking into account the length of aligned amino acid residues, has been shown to result in less accurate models [47]. The amino acid sequence of *An. arabiensis* was retrieved from UniProt Knowledge Base (Accession number: A0A182HKN4). This accession number was submitted to the SWISS-MODEL database server for a reference 3D structure search and model building [48]. *An. gambiae* AChE (PDB: 5YDI) was selected as a template [49]. UCSF Chimera v1.16 was used for model optimization and visualization [50, 51]. Moreover, the correctness of the model was checked through ProQ webserver [52], and the model was validated with Verify3D and MolProbity [53, 54]

2.5.2. Molecular docking

Schrödinger Release 2018-2 molecular docking package, Maestro version 12.9, (Schrödinger LLC, New York) was used for ligand binding assessments [55]. The generated *An. arabiensis* AChE 3D structure and PDB sourced related proteins for comparative studies were prepared through Maestro's protein preparation function. This included the AChE proteins from *An. gambiae* (PDB: 5YDI) [49]), electric eel (PDB: 1EVE [56]) and human (PDB: 4EY7 [57]). The preparation included optimization of H-bonds and removal of non-hetero groups and non-essential water molecules before minimization by OPLS3e force field [58]. The receptor grid was also generated using the OPLS3e force field to define the binding site [55]. Similarly, the ligands were prepared for docking using the LigPrep tool in which they were allowed to generate possible stereoisomers as well as ionization and tautomeric states at pH 7.0 (±2.0) [59, 60]. Finally, the extra precision mode was used for assessing the binding profiles of the prepared ligands to the receptor sites [61, 62].

2.5. Statistical Analyses

All *in vitro* and *in vivo* assays were performed in triplicate and five replicates were made per assay. The 50% lethal concentration (LC_{50}) values were calculated from the probit analysis method using the SPSS Statistics v28 package (International Business Machines Corporation, NY, USA) and the IC_{50} values were determined by non-linear regression analysis using GraphPad Prism 9 (GraphPad Software, CA, USA) [30, 36].

3. Results and Discussion

3.1. Larvicidal activity

Only derivatives **1**, **2**, **5** and **8** (Fig 2) showed larvicidal activities (Table 1). Specifically, derivatives **1**, **5** and **8** were active against *An. funestus* with lower LC_{50} values (2.65, 2.96 and 0.80 µM) compared to the positive control, propoxur (9.90 µM) (Table 1). On the other hand, derivative **2** was selective to *An. arabiensis*. This derivative was about 10-fold (LC_{50}: 0.88 µM) more potent than propoxur (LC_{50}: 8.77 µM) for *An. arabiensis* larval toxicity (Table 1). As a result, only derivatives **1**, **2**, **5** and **8** were selected for further analysis in the AChE inhibition assay.

Table 1: Larvicidal activities of the assessed compounds.

	***Anopheles* colonies** **Larvicidal LC_{50} (95% confidence interval range) (µM)** **[Chi square (X^2); degree of freedom (df); *p* value; intercept; standard error (SE)]**			
Donepezil derivative	***An. funestus***	***An. arabiensis***	***An. gambiae***	***An. coluzzii***
1	2.65 (1.72 – 4.12) [X^2: 4.76; df: 5; $p = 0.45$[a]; intercept: -0.27; SE: 0.06]	> 100	> 100	> 100
2	> 100	0.88 (0.35 – 2.27) [X^2: 17.56; df: 5; $p = 0.004$[b]; intercept: 0.05; SE: 0.07]	> 100	> 100
3	> 100	> 100	> 100	> 100
4	> 100	> 100	> 100	> 100
5	2.96 (1.94 – 4.57) [X^2: 5.43; df: 5; $p = 0.37$[a]; intercept: -0.31; SE: 0.06]	> 100	> 100	> 100
6	> 100	> 100	> 100	> 100
7	> 100	> 100	> 100	> 100
8	0.80 (0.56 – 1.16) [X^2: 3.58; df: 5; $p = 0.61$[a]; intercept: -0.78; SE: 0.07]	> 100	> 100	> 100
9	> 100	> 100	> 100	> 100
Propoxur	9.90 (2.95 – 41.41) [X^2: 26.24; df: 5; $p < 0.001$[b]; intercept: -0.78; SE: 0.08]	8.77 (2.81 - 32.71) [X^2: 23.95; df: 5; $p < 0.001$[b]; intercept: -0.75; SE: 0.08]	63.99 (38.23 – 116.29) [X^2: 3.96; df: 5; $p = 0.56$[a]; intercept: -1.11; SE: 0.09]	62.68 (37.72 – 112.78) [X^2: 3.58; df: 5; $p = 0.61$[a]; intercept: -1.12; SE: 0.09]

[a] Since the significance level was greater than 0.15, a heterogeneity factor was not used in the calculation of confidence interval limits.

[b] Since the significance level was less than 0.15, a heterogeneity factor was used in the calculation of confidence interval limits.

3.2. Brine shrimp lethality

None of the derivatives induced artemicidal effects (100% viability at 500 µM). This suggests relative safety of these novel compounds compared to the positive control, potassium dichromate that attained the LC_{50} value of 0.004 (0.002 – 0.007) µM (X^2: 8.10; df: 5; $p = 0.151$; intercept: 1.30; SE: 0.09) in agreement with a previous assessment [63]. This was a favourable outcome for the donepezil derivatives **1**, **2**, **5** and **8** which had potent larvicidal effects (Table 1), as it suggests that when used as larvicides, these derivatives would potentially be nontoxic to other aquatic lives.

3.3. AChE inhibition

Though the derivatives **1**, **5** and **8** showed potent larvicidal activity against *An. funestus*, none of these derivatives displayed AChE activities against this colony (Table 2). This suggests that these derivatives exert larvicidal activity through a different mechanism other than AChE inhibition. Surprisingly, derivatives **1**, **5**, **8** and the positive control, propoxur, showed moderate to low activity against the *in vitro An. gambiae* AChE target. This was in discordance to the *in vivo* larvicidal data where these molecules were inactive. It is common to have discrepancies between *in vitro* and *in vivo* data [64-66] and it has also been shown to occur with insecticide-resistant *Anopheles* larvae [67]. In fact, *An. gambiae* larvae have been shown to possess pharmacokinetic barriers such as thickened cuticle that play a role in preventing compound penetration at effective concentrations [67, 68].

Table 2: Comparison of the *Anopheles* AChE inhibitory potential of derivatives **1**, **2**, **5** and **8**.

	***Anopheles* AChE inhibition**			
	IC_{50} ± SE (95% confidence interval range) (µM)			
Donepezil derivative	***An. funestus***	***An. arabiensis***	***An. gambiae***	***An. coluzzii***
1	> 100	> 100	48.85 ± 10.49 (25.77 – 71.93)	> 100
2	> 100	6.05 ± 2.21 (2.78 – 13.16)	> 100	> 100
5	> 100	> 100	31.42 ± 7.56 (14.78 – 48.06)	> 100
8	> 100	> 100	28.13 ± 6.89 (12.96 – 43.31)	> 100
Propoxur	0.89 ± 0.20 (0.45 – 1.33)	0.78 ± 0.16 (0.42 – 1.13)	26.08 ± 6.21 (12.42 – 39.74)	25.04 ± 6.14 (11.53 – 38.55)

Nevertheless, derivative **2** is known to have activity against electric eel AChE [3] and displayed an IC_{50} value of 55.70 ± 12.02 µM (Table 3). Interestingly, **2** showed potent AChE activity specifically against *An. arabiensis* (IC_{50} = 6.05 ± 2.21 µM; Table 2 (log-dose response curve shown in Fig S1)), for which it also displayed larvicidal effects (Table 1). The calculated selectivity index (SI = IC_{50} electric eel AChE / IC_{50} *An. arabiensis* AChE) between the two AChE targets was 9.2. With the selectivity index less than 10, it may not be considered selective for *An. arabiensis* over electric eel AChE [69, 70]. However, in comparison to propoxur, which was essentially non-selective between *An. arabiensis* (IC_{50} = 0.78 ± 0.16 µM; Table 2) and electric eel (IC_{50} = 1.28 ± 0.35 µM; Table 3) in agreement with previous studies [13, 71], derivative **2** showed better potential for *Anopheles* selectivity. Furthermore, with good correlation between the larvicidal and AChE activity of derivative **2** consistently against *An. arabiensis*, presents it as a potential lead molecule for further derivatization into more potent and selective analogues. For this reason, the donepezil derivative **2**, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide, was selected as a lead molecule and assessed further for its AChE binding profile.

Table 3: Comparison of the electric eel AChE inhibitory potential of derivatives **1**, **2**, **5** and **8**.

Donepezil derivative	***Electric eel* AChE inhibition IC_{50} ± SE (95% confidence interval range) (µM)**
1	>100
2	55.70 ± 12.02 (29.24 – 82.16)
5	>100
8	88.29 ± 19.90 (44.49 – 132.10)
Propoxur	1.28 ± 0.35 (0.25 – 2.03)

4. *In silico* studies

4.1. Homology modelling

The 3D AChE model for *An. arabiensis* was successfully built from the *An. gambiae* (PDB: 5YDI) template. Sequence identity between the two models was 100% with more than 500 aligned amino acid residues (range 162 – 698) translating into a target structural coverage of

0.73. Global model quality estimate (GMQE) and model quality estimation QMEANDisCo Global were used to estimate the overall model quality [72, 73]. The GMQE score was 0.71, while the QMEANDisCo Global achieved 0.92 ± 0.05 indicating that the final model was analogous to the experimental crystal structures. The quality of the model was assessed by comparing it to the reference and non-redundant 3D structures from PDB (Figs 3A and 3B). Similarly, quaternary structure quality estimation (*QSQE*) assessed the accuracy of the generated target 3D structure in terms of the inter-chain contacts in accordance with the template and resulting alignment. A value above 0.7 indicates a reliable model [48, 74] and the final model in this study reached a score of 0.74.

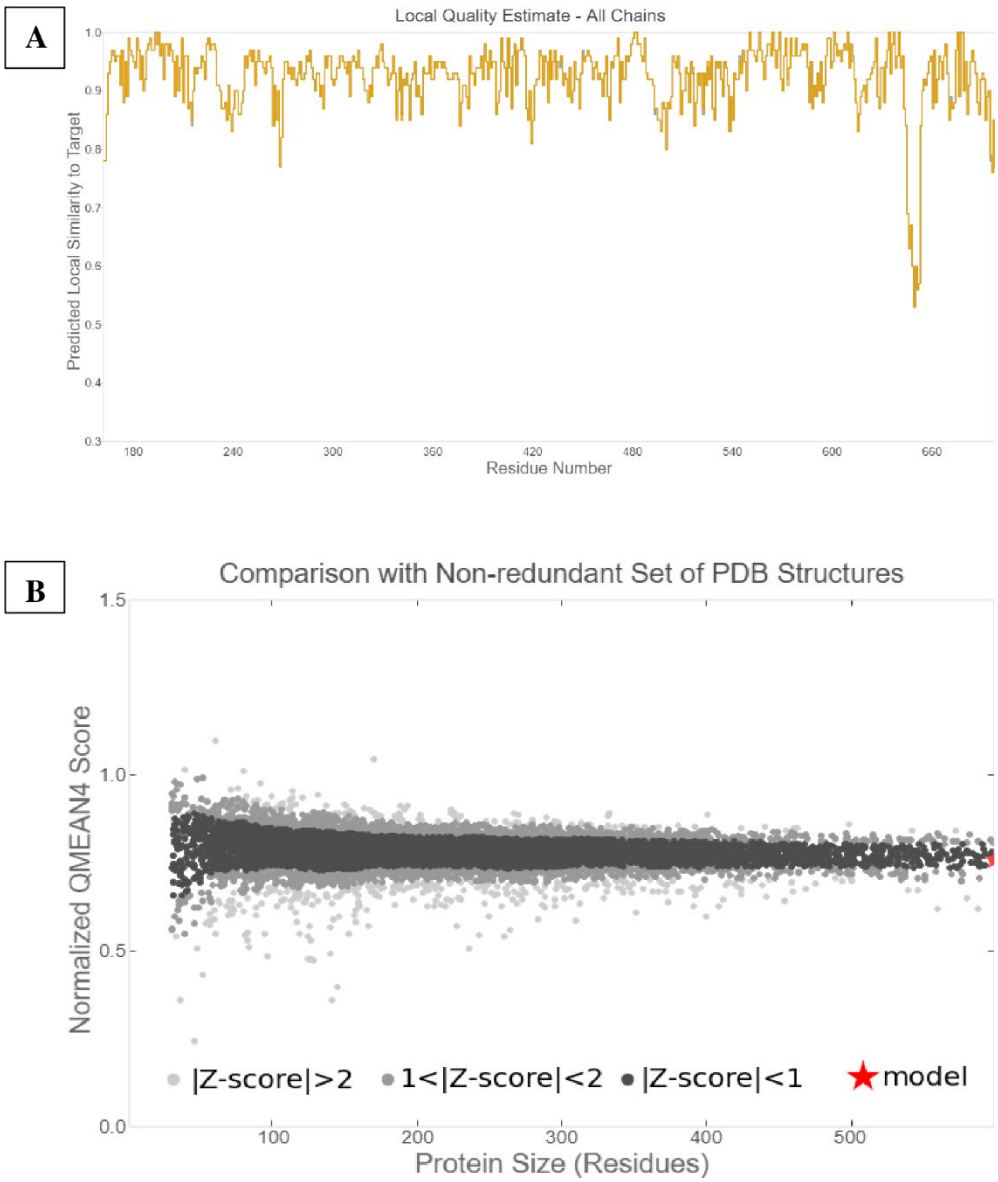
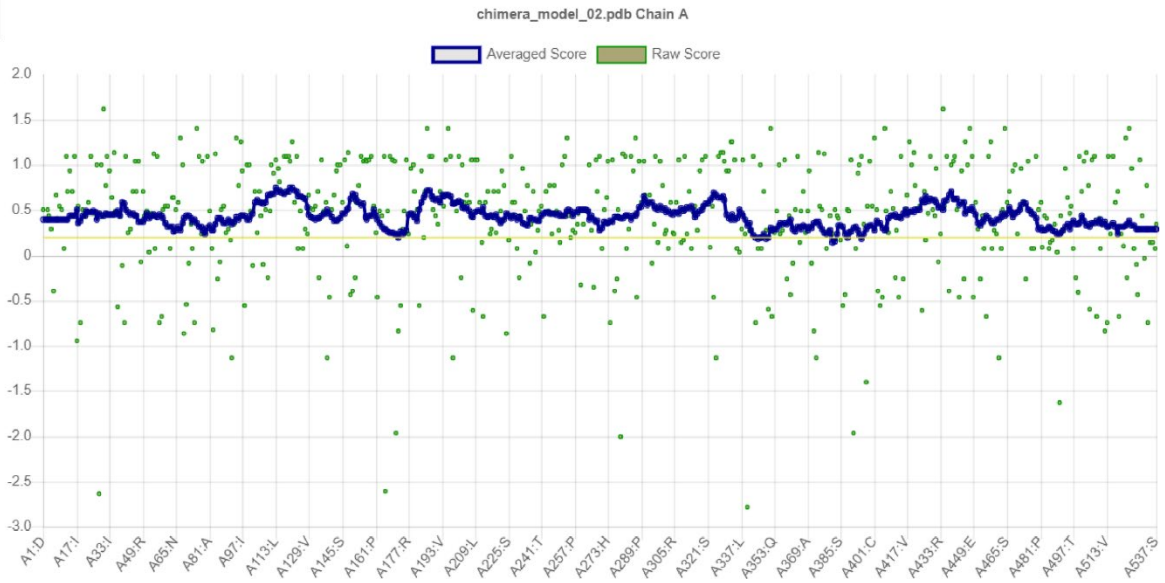


Fig 3: Quality of the *An. arabiensis* homology model. A) Shows the quality estimate of the generated model based on its similarity to the template and B) shows its comparison to non-redundant 3D structures.

The assessment of the correctness of the model through ProQ showed an LGscore of 11.077. This program analysed relative frequencies of intramolecular atomic interactions within the model where the LGscore >4 indicated an extremely good model [52]. In addition, the Verify3D suggested a model PASS with 97.11% of the amino acid residues a 3D/1D score of ≥0.2 (Fig 4A). This tool assessed how compatible a generated model was with its amino acid sequences. This is assessed based on the optimum environment for each amino acid residue such as the area of the residue buried deep in the protein, and hence inaccessible to the solvent, the area of the side-chains made of polar atoms, as well as the quality of the local secondary structure [53]. A favourable MolProbity score of 1.36 and 98th percentile was obtained for the model (Fig 4B) confirming the validity of the model [54]. The Ramachandran plot (Fig 4C) reported a score of 95.14% for amino acid residues in favoured regions, 99.8% in allowed regions and 0.0% for both Ramachandran outliers and Cβ deviations. This suggested a valid model with a stable backbone [75].

A



B

PROBITY

Analysis output: all-atom contacts and geometry for model_02_-_Chain_A1FH.pdb

Duke Biochemistry
Duke University School of Medicine

Summary statistics

All-Atom Contacts	Clashscore, all atoms:	2.39		99th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	3	0.66%	Goal: <0.3%
	Favored rotamers	424	93.39%	Goal: >98%
	Ramachandran outliers	0	0.00%	Goal: <0.05%
	Ramachandran favored	509	95.14%	Goal: >98%
	Rama distribution Z-score	-2.69 ± 0.31		Goal: abs(Z score) < 2
	MolProbity score^	1.36		98th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 4374	0.00%	Goal: 0%
	Bad angles:	30 / 5970	0.50%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	2 / 40	5.00%	Expected: ≤1 per chain, or ≤5%
Low-resolution Criteria	CaBLAM outliers	15	2.8%	Goal: <1.0%
	CA Geometry outliers	5	0.94%	Goal: <0.5%
Additional validations	Chiral volume outliers	0/636		
	Waters with clashes	0/0	0.00%	See UnDowser table for details

In the two column results, the left column gives the raw count, right column gives the percentage.
* 100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clashscore the comparative set of structures was selected in 2004, for MolProbity score in 2006.
^ MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.
Key to table colors and cutoffs here:

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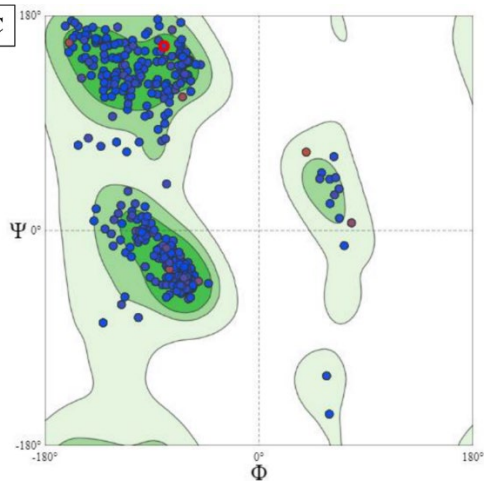


Fig 4: Validity of the model displayed by Verify3D tool (A) and scored by MolProbity (B) and the corresponding Ramachandran plot (C).

The new model was aligned with the existing *An. gambiae* AChE 3D structure (PDB: 5YDI) and visualized in UCSF Chimera. This program showed 100% alignment of the AChE catalytic sites of the modelled *An. arabiensis* and reference *An. gambiae* (Fig 5)**.**

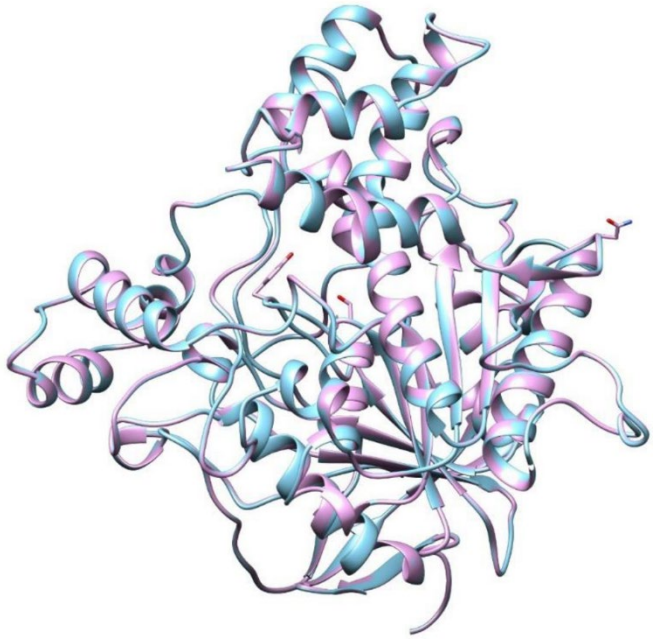


Fig 5: Alignment of the catalytic sites of the generated *An. arabiensis* AChE (sky blue) and reference *An. gambiae* (plum).

4.2. Molecular modelling

Assessment of molecular interactions with the AChE catalytic sites of electric eel (Fig 6A) and a built model of *An. arabiensis* (Fig 6B) showed some similar interactions with the targets. The thiazole group was bound by arginine residues (Arg^{289} (electric eel)/Arg^{233} (*An. arabiensis*), however with an additional aromatic stabilization by Tyr^{493} in the *An. arabiensis* model (Fig 6B). Several amino acid residues were involved in the interaction with the *N*-benzylpiperidine moiety. For the electric eel, these included two catalytic triad residues Glu^{199} through hydrogen bonding and stabilization of the *N*-benzylpiperidine ring through aromatic pi-pi interaction with His^{440}. This ring was held on the other side by another pi-pi interaction with Phe^{330}, while Trp^{84} and Tyr^{334} formed pi-cation interactions with the ionized nitrogen of *N*-benzylpiperidine. Similarly, this nitrogen was involved in pi-cation interactions with Trp^{245} and Tyr^{489} along with Glu^{359} in the *An. arabiensis* model, however, with no stabilization of the *N*-benzylpiperidine ring (Fig 6B). This caused the derivative to adopt a different orientation of the *N*-benzylpiperidine group when contrasted against the conformation observed in the electric eel AChE site. This possibly caused the observed lower binding score. To gain a better

understanding, the differences in the binding sites of *Anopheles*, electric eel and human AChEs from PDB were assessed.

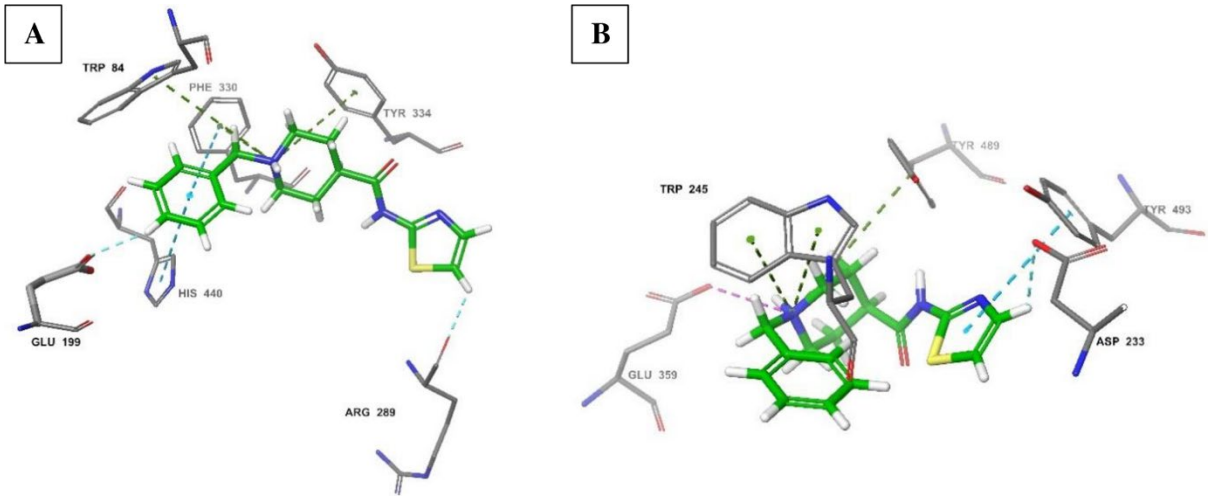


Fig 6: Molecular interactions of derivative **2** with electric eel AChE (A; Score: -11.8) and *An. arabiensis* (B; Score: -7.5).

A comparison of electric eel, human and *Anopheles* AChE catalytic sites was performed by superimposing amino acid residues that represent the entrance to the catalytic site and the catalytic site amino acids through the Glide's Quick Align function. PDB 1EVE was used for electric eel [56], PDB 4EY7 for human [57], and PDB 5YDI for the *Anopheles* (*An. gambiae*) AChE target [49]. The key observable difference between *Anopheles* AChE and the two other proteins was the replacement of a larger phenylalanine (Phe^{288} (human)/Phe^{295} (electric eel)) with a smaller cysteine residue (Cys^{447}) at the entrance of the catalytic site. Similarly, a smaller aspartic acid residue (Asp^{602}) was identified at the base of the *Anopheles* AChE catalytic site, in place of the bulky tyrosine residues (Tyr^{442} (human)/Tyr^{449} (electric eel)) (Fig 7) in agreement with the reported literature [76].

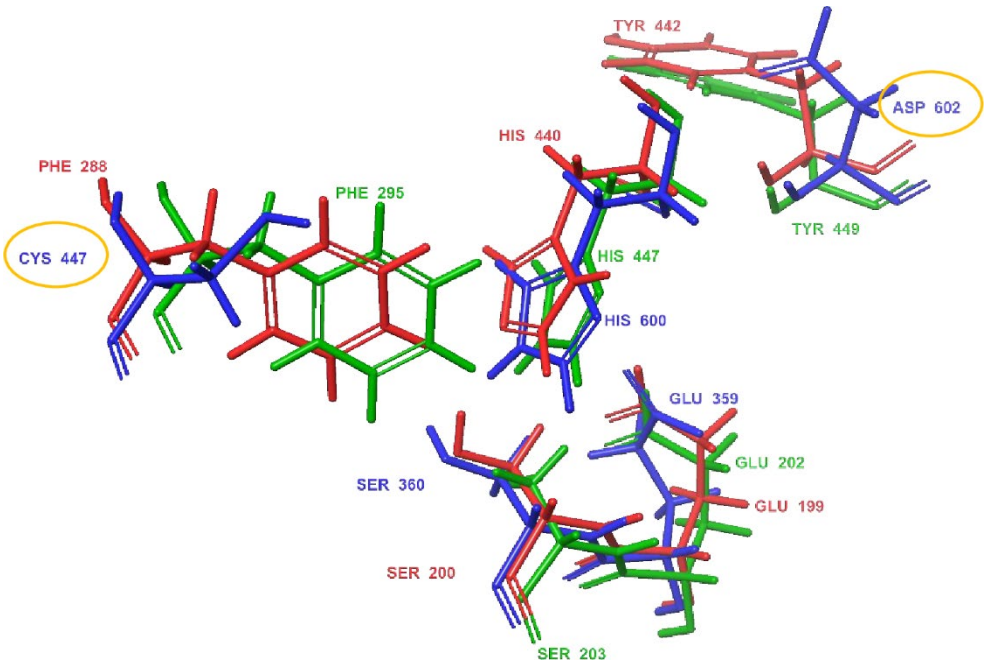


Fig 7: Superimposed amino acid residues showing the catalytic site entrance and the catalytic triad of electric eel (red), human (green) and *Anopheles* (blue) AChE. The distinct Cys^{447} at the *Anopheles* AChE catalytic entrance and Asp^{602} at the catalytic site base are shown by circles.

To gain further insights into the influence of these differences in ligand binding, the potential differences between the binding poses of the known human and electric eel AChE inhibitor, donepezil [2, 3, 5], when bound to these three AChE proteins were assessed (Fig 8). Donepezil displayed a similar binding pose and interactions with both electric eel and human AChE targets (Figs 8A and 8B). At both sites, donepezil was stabilized by aromatic interactions through the corresponding tryptophan residues. The donepezil indanone aromatic ring interacted with Trp^{279} and Trp^{286} in electric eel and human AChEs, respectively; while its *N*-benzylpiperidine ring established pi-pi stacking with Trp^{84} in electric eel and Trp^{86} in human target. Additionally, the catalytic site histidine residues (His^{440}; electric eel and His^{447}; human) also established the aromatic pi-pi interactions with the *N*-benzylpiperidine ring. However, in the human target, Glu^{202} was also involved in this interaction. The phenylalanine residues played a critical role in maintaining the crystal pose of donepezil through hydrogen bonding. This was achieved through Phe^{288} residue in electric eel and the comparable Phe^{295} in human AChE. Likewise, the pi-cation interactions between the nitrogen atom of *N*-benzylpiperidine and aromatic amino acids stabilized donepezil in the binding pose. In electric eel AChE, these interactions were generated from Phe^{330}, Phe^{331} and Tyr^{334}, while only Trp^{86} established this interaction in the corresponding human target (Figs 8A and 8B). In support of the binding similarity observed in this study, the binding potency of donepezil against electric eel and

human AChE has also been reported to be similar (IC_{50} 0.035 µM and 0.030 µM, respectively) [77, 78].

On the other hand, donepezil generated different interactions with the *Anopheles* AChE from those obtained in electric eel and human targets (Fig 8C). There was no stabilization of the indanone moiety. However, the *N*-benzylpiperidine group was stabilized by Trp^{245} residues that are comparable to Trp^{279} and Trp^{286} in electric eel and human targets, respectively. The pi-cation interaction with the *N*-benzylpiperidine portion was established by the Trp^{245} and Tyr^{493} residues. This caused the aromatic ring of the *N*-benzylpiperidine to face down towards Gly^{278} residue and away from the catalytic triad amino acid histidine (His^{359}; *Anopheles*) that played a key role in the binding of donepezil in electric eel (His^{440}) and human (His^{447}) AChEs. For the first time, this study shows that donepezil adopts a different binding pose in the *Anopheles* target, lending support to previous studies that showed significant differences in selectivity for donepezil between human and *Anopheles* AChEs [16].

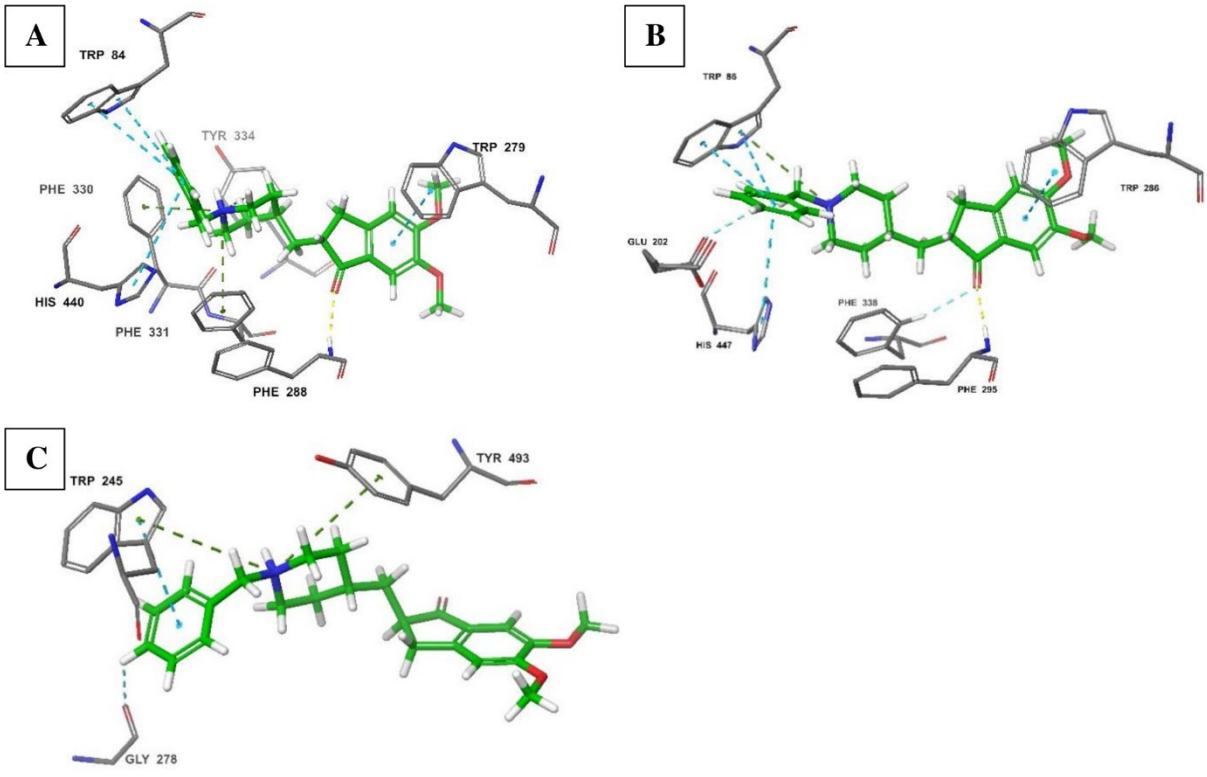


Fig 8: Comparison of the molecular interactions of donepezil with the AChE catalytic sites of electric eel (A; PDB: 1EVE; score: -15.0), human (B; PDB: 4EY7; score: -16.2) and *An. gambiae* (C; PDB: 5YDI; score: -10.2).

Finally, as derivative **2** displayed selective inhibition of AChE for *An. arabiensis* over those from *An. gambiae, An. coluzzii* and *An. funestus,* the binding pose difference across the

assessed *Anopheles* colonies was assessed. However, the only available crystal structures from PDB repository were from *An. gambiae* [49, 79]. Therefore, additional models were generated from *An. coluzzi* (UniProt accession number: A0A6E8V9T9) and *An. funestus* AChE amino acid sequences (UniProt accession number: A0A182RZ85). Using SWISS-MODEL, the template for *An. coluzzi* was identified as *An. gambiae* (PDB: 5YDH) with GMQE score of 0.81, GSQE score of 0.79, and 100% sequence identity with amino acid coverage from 162 to 699. The final QMEANDisCo Global was 0.93 ± 0.05. On the other hand, the best template identified for *An. funestus* was *An. gambiae* (PDB: 5YDI) with GMQE and GSQE scores of 0.80 and 0.74, respectively. This obtained 98.01% amino acid sequence identity with coverage from 160 to 696 and QMEANDisCo Global was 0.92 ± 0.05. The Ramachandran plots for *An. coluzzi* and *An. funestus* models obtained from the Ramachandran plot server [80] are reported in Figs S2 and S3, respectively. Further, the local quality estimate results of the models and comparisons to non-redundant experimental crystal structures (obtained from SWISS-MODEL) are displayed in Figs S4 and S5 for *An. coluzzi* and *An. funestus*, respectively; followed by their validity verifications through Verify3D (Figs S6 and S7, respectively). As a result, a comparison of the binding profile differences was performed using three new AChE models for *An. arabiensis*, *An. coluzzi,* and *An. funestus*, as well as two PDB sourced *An. gambiae* AChE targets; wild-type (PDB: 5YDI) [49] and resistant (PDB: 6ARY) through target site mutation (G280S) [79].

The observed stabilization of thiazole ring by Tyr^{493} in the *An. arabiensis* model (Fig 9A) could not be attained in the wild-type *An. gambiae* (Fig 9B). Similarly, the pi-cation stabilization by Tyr^{489} and Glu^{359} were also lost in the wild-type *An. gambiae* site. Instead, the interaction with the wild-type *An. gambiae* AChE showed aromatic hydrogen bonding between the *N*-benzylpiperidine ring and amino acid residues Gly^{278} and Ser^{283}. The *N*-benzylpiperidine nitrogen atom was then involved in pi-cation interaction with aromatic amino acids, Trp^{245} and Tyr^{493}, while Tyr^{282} established the hydrogen interaction with an oxygen group of the amide linker (Fig 9B). The generated crystal pose at this site was clearly different from that obtained with the *An. arabiensis* model. At the mutated target site of *An. gambiae* target (Fig 9C), the orientation of derivative **2** was clearly the opposite of that generated in *An. arabiensis* model and this displayed comparatively fewer intermolecular interactions. At this target, Glu^{359} was involved in hydrogen bonding with the thiazole group and the *N*-benzylpiperidine moiety was stabilized by Tyr^{493} and Asp^{233}, in direct contrast to that observed with the *An. arabiensis* model

where these Tyr^{493} and Asp^{233} stabilized the thiazole group. The Tyr^{489} held the molecule in space by establishing a hydrogen bond with the amide linker.

Least interactions were obtained against *An. coluzzii* target (Fig 9D). While the conformation of derivative **2** was similar to that obtained against mutant *An. gambiae* AChE (Fig 9C), where it only retained two interactions composed of Tyr^{489} H-bond and Asp^{233} pi-cation bond. Therefore, at both *An. gambiae* and *An. coluzzii* targets, derivative **2** assumed the orientation that was directly opposing that at *An. arabiensis* AChE catalytic site. Finally, at the *An. funestus* AChE site (Fig 9E), derivative **2** regenerated the crystal pose similar to that obtained at wild-type *An. gambiae* target (Fig 9B) with similar binding scores (-9.1 and -9.2 kcal/mol, respectively). In a similar fashion to wild-type *An. gambiae* AChE and different from *An. arabiensis* target, the pi-cation interaction with a tyrosine residue, Tyr^{491}, was established and supplemented by the linker H-bond with Tyr^{280} and Phe^{447}. At this target however, there was no further stabilization of the *N*-benzylpiperidine portion, but more H-bonds with the thiazole group from Gly^{443} and Glu^{446} residues (Fig 9E).

In general, derivative **2** generated a unique crystal pose against *An. arabiensis* model which may explain biological activity exclusively against this *Anopheles* species. On the other hand, this compound assumed a comparable pose between two targets, *An. funestus* wild-type and *An. gambiae* AChEs and established another conformation that was characteristic of *An. coluzzii* and mutant *An. gambiae* AChE.

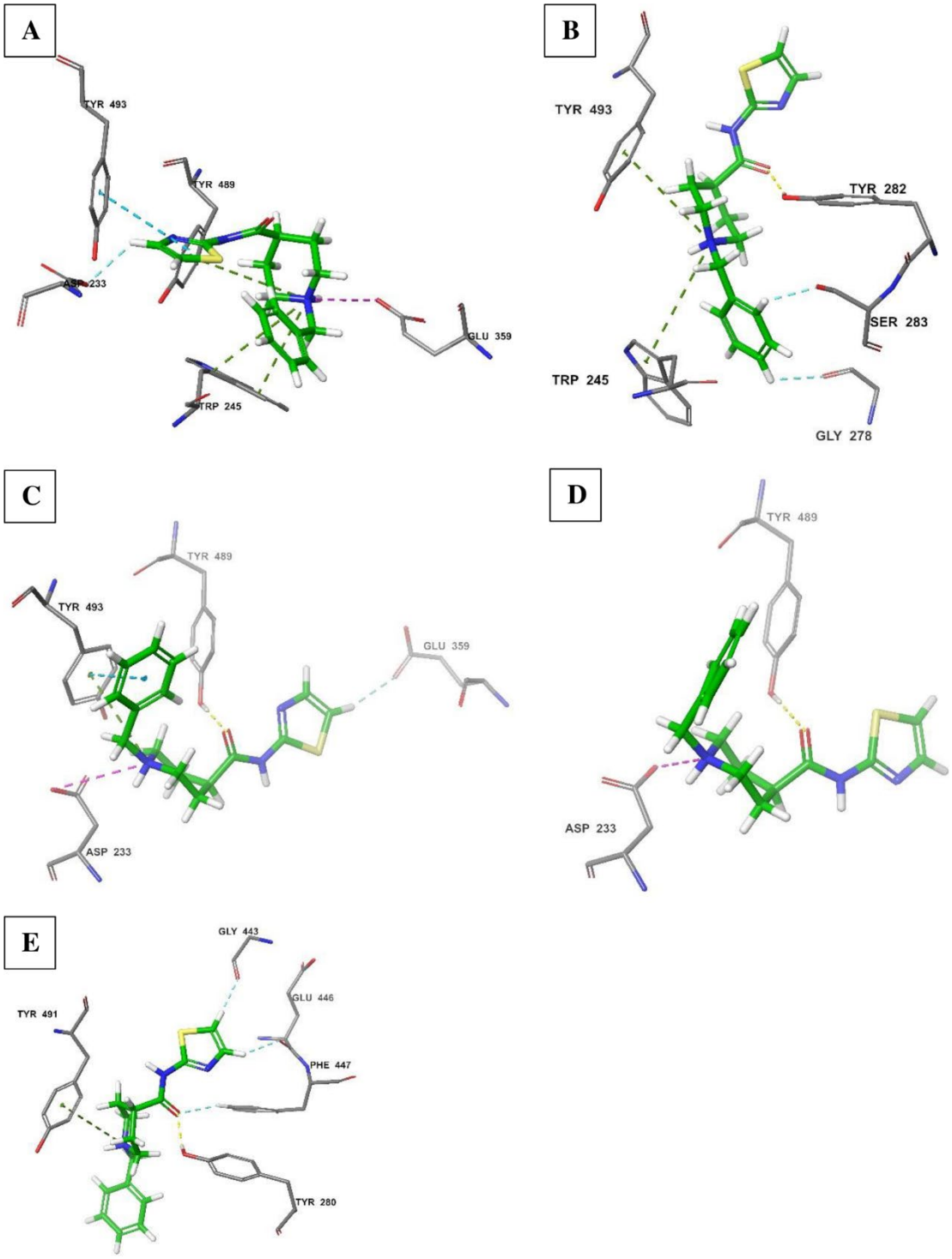


Fig 9: Comparison of the molecular interactions of derivative **2** with the AChE catalytic sites of *An. arabiensis* model (A; score: -7.5), wild-type *An. gambiae* (B; score: -9.2), target site mutated *An. gambiae* (C; score: -7.8), *An. coluzzii* model (D; score: -9.0) and *An. funestus* model (E; score: -9.1).

As expected, this study showed great similarity between the AChEs from electric eel and human, in support of previous literature [4, 77, 78]. Interestingly, there were clear differences in ligand binding between either the electric eel or human AChE and *Anopheles* AChE, these

differences potentially arising as a result of molecular differences around the catalytic site. In line with previous reports [76], the conserved Cys^{447} at the entrance to the catalytic site and Asp^{602} at the base of the active site were identified in this study. This molecular difference may bring about differences in ligand orientation, as well as interactions it establishes with surrounding amino acid residues. A smaller cysteine residue at the catalytic site entrance may create more volume available for a ligand to assume a different orientation as seen with a control, donepezil, and derivative **2**.

5. Conclusions

The current study identified a lead compound, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2**, as an AChE-based larvicidal agent against *An. arabiensis* KWAG. Derivative **2** displayed more than 9-fold higher potency towards *An. arabiensis* AChE over that of electric eel AChE. Through molecular docking, this study has highlighted the close similarity between electric eel and human AChE, as well as their marked difference from *Anopheles* AChE. The differences in the binding of derivative **2** with electric eel and *An. arabiensis* AChE, respectively, were investigated at a molecular level through molecular modelling. Homology modelling was employed to generate a 3D AChE structure of *An. arabiensis* after which it was used for comparative binding assessments. The conformation of the molecule and interactions with the protein amino acid residues was noticeably different between the two targets. This informed our study to assess the molecular differences between the AChE binding sites from electric eel, human and *Anopheles*. A critical distinction observed in *Anopheles* as opposed to the two mammal proteins, is a smaller cysteine residue in place of bulky phenylalanine groups at the entrance to the catalytic site and a smaller aspartic acid replacing larger tyrosine residues at the base of the catalytic site, in correlation with previous X-ray diffraction studies [76]. The influence of these amino acid residues on ligand binding and crystal pose generation needs to be further investigated.

Further, the selectivity of derivative **2** to *An. arabiensis* amongst other *Anopheles* species was evaluated by comparing binding profiles between the *An. arabiensis* model and *An. gambiae* AChE targets (wild-type and resistant (mutant)) from the PDB repository, as well as those of *An. coluzzii* and *An. funestus* generated through homology modelling. The molecule was better stabilized by a mixture of hydrogen bonds and pi-pi stacking in the *An. arabiensis* model than in the corresponding *Anopheles* targets. In conclusion, this study suggests that 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide presents as a lead compound for the design and

synthesis of novel insecticides against the African malaria vector, *An. arabiensis*. Further derivatizations of this molecule may generate molecules with high potency against a wide range of *Anopheles* species and increased selectivity to *Anopheles* over mammal AChE.

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CHAPTER 5

The *in vitro* and *in silico* assessment of select terpenoids for the *Anopheles* acetylcholinesterase inhibition

Based on the review (Chapter 2) that showed a potential for essential oil constituents to exert *Anopheles* acetylcholinesterase inhibition, this chapter established the *in vitro* acetylcholinesterase assay with essential oil constituents (terpenoids). This is coupled with the *in silico* method using the techniques optimized in Chapters 3 and 4 (homology modelling, molecular modelling and molecular mechanics simulations) to elucidate the insecticidal mechanism of terpenoids at a molecular level. This chapter is presented in the form of a published paper in Parasitology International.

Chapter type: Published ISI paper (Appendix D.1)

Title of manuscript: *In vitro* and *in silico* analysis of the *Anopheles* anticholinesterase activity of terpenoids

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Method	✓		
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Writing – original draft	✓		
Writing – revision and editing	✓	✓	✓
Visualization	✓		
Supervision		✓	✓
Funding acquisition		✓	✓

Abstract: Insecticides are continuously losing effectiveness in the malaria vector programs due to increasing resistance against them. This signals an urgent need for novel insecticides against the malaria vectors. *Anopheles gambiae, An. coluzzii*, *An. arabiensis*, and *An. funestus* are major vectors in the high malaria endemic African region. Previous studies have identified essential oils as potential sources of cost-effective bioinsecticides. Various terpenoid classes form the main chemical constituent repository of essential oils, many of which have been shown to possess insecticidal effects against *Anopheles* species. The current study aimed to assess the bioactivity of terpenoids including four sesquiterpene alcohols, farnesol, (-)-α-bisabolol, *cis*-nerolidol, and *trans*-nerolidol; a phenylpropanoid methyleugenol, and a monoterpene (*R*)-(+)-limonene using the *in vivo* larvicidal screening assay against the four *Anopheles* species. The mechanism of action was investigated through *in vitro* acetylcholinesterase inhibition assay and further assessed at the molecular level through molecular modelling and molecular mechanics simulations. All six terpenoids showed potent larvicidal activity against the four *Anopheles* species. In the screening for larvicidal selectivity using *Artemia* model, farnesol, (-)-α-bisabolol, methyleugenol, and (*R*)-(+)-limonene were more selective against *Anopheles* in comparison to other aquatic organisms. Insights into the mechanism of action revealed that the six terpenoids are strong AChE inhibitors against *An. funestus* and *An. arabiensis* while there was a moderate inhibitory activity against *An. gambiae* AChE but very weak activity against *An. coluzzii.* In correlation to the *in vitro* and *in vivo* data, the *in silico* techniques also identified farnesol as the most active terpenoid with high affinity towards *Anopheles* AChE while (*R*)-(+)-limonene was identified as a weak, nonspecific AChE inhibitor. Interestingly, farnesol established a favourable hydrogen bonding interaction with a conserved amino acid residue, Cys^{447}, at the entrance to the active site gorge. Other terpenoids, (-)-α-bisabolol and methyleugenol, displayed a strong interaction with the catalytic Ser^{360} and

adjacent amino acid residues; but sparing the mutable Gly^{280} residue that confers resistance to the current anticholinesterase insecticides. As a result, this study identified farnesol, (-)-α-bisabolol, and methyleugenol as selective bioinsecticidal agents with potent *Anopheles* AChE inhibition. These terpenoids present as natural compounds for further development as anticholinesterase bioinsecticides.

Keywords: *Anopheles*, acetylcholinesterase, *in silico*, *in vitro*, terpenoids

1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) is a serine hydrolase enzyme belonging to the family of cholinesterases. It is located at the neuromuscular junctions and cholinergic brain synapses where it functions to maintain normal neuronal impulse transmission through hydrolysis of a neurotransmitter, acetylcholine. Particularly in insects, the cholinergic system is localized at the central nervous system [1, 2]. Specialized insects, namely the female *Anopheles* mosquitoes, transmit malaria infections globally. More than 400 *Anopheles* species have been identified and about 30 these are the malaria vectors [3]. *Anopheles gambiae* s.l complex constitutes main African malaria vectors. The member species in *An. gambiae* complex include *An. gambiae* (formerly *An. gambiae* S-molecular form)*, An. coluzzii* (previously *An. gambiae* M-molecular form) and *An. arabiensis* [4]. On the other hand, *An. funestus* group includes the *An. funestus* s.s., another major vectors in Sub-Saharan Africa [5]. Generally, these four species, *An. gambiae, An. coluzzii*, *An. arabiensis*, and *An. funestus*, are the dominant malaria vectors in Africa, as reported in recent World Health Organization (WHO) malaria report [6].

Malaria vector control programs mainly use insecticides to advance towards a goal of zero malaria transmissions [7, 8]. Insecticides have unique modes of action and acetylcholinesterase inhibitors are one of these. Insecticide classes such as organophosphates and carbamates specifically inhibit AChE. Unfortunately, insecticide resistance against these two classes has already been reported [6, 9]. Resistance mechanisms have been attributed to target site mutation, as well as metabolic resistance resulting from overexpressed metabolic enzyme genes [10-12]. AChE target site mutation, known as G119S (substitution of glycine by a serine residue at position 199; *Torpedo californica* numbering), is the main diagnostic factor of organophosphate and carbamate cross-resistance [13]. Another challenge with the current anticholinesterase insecticides is low selectivity index between insect and mammal targets [14]. Given these, there is an urgent need to discover novel acetylcholinesterase inhibitors with better selectivity and able to cause mortality in insecticide resistant vector populations. AChE remains a target site in *Anopheles* mosquitoes since the cholinergic system, including the AChE enzyme, is an essential component of the insect nervous system [15]. Therefore, current research continues to focus on the identification of novel agents that have a high selectivity for all variants of *Anopheles* AChE compared to mammalian AChE [16-18].

Essential oils are potential natural sources of cost-effective and eco-friendly bioinsecticides [19, 20]. Main constituents in essential oils are, amongst others, terpenoids such as

sesquiterpenes, monoterpenes, and diterpenes as well as volatile phenolic compounds known as phenylpropanoids [21-23]. The sesquiterpene alcohols, farnesol and (-)-α-bisabolol, are main essential oil constituents in numerous aromatic plants including *Rosa x. damascene* Mill., *Polianthes tuberosa* L., *Matricaria chamomilla* L., *Cymbopogon nardus*, and *C. citratus* [24, 25]. These essential oils have shown insecticidal activities from various studies. For example, *C. nardus* (citronella) and *C. citratus* (lemongrass) essential oils have shown toxic effects on *An. gambiae* adult mosquitoes [26]. The geometric isomers, cis-nerolidol and trans-nerolidol, are main constituents in many *Piper* species including *P. nigrum, P. umbellatum, P. capense*, and *P. claussenianum* (Miq.) [27, 28]. Nerolidol has previously shown larvicidal activity against *An. sinensis* larvae [29]. (*R*)-(+)-Limonene is a monoterpene obtained as a major constituent in aromatic plants such as *Citrus limon, Baccharis dracunculifolia* De Candole (1836) and *Schinus terebinthifolius* Raddi [30-33]. The achiral form of limonene has previously shown potent larvicidal activity against *An. sinensis* [29]. Similarly, the phenylpropanoid, methyleugenol, is a major phenolic constituent of many plants, including *C. nardus, Ocimum basilicum*, and *Colocasia antiquorum* [34, 35]. The essential oil of O. basilicum has exhibited the larvicidal activity against *An. subpictus* while that of *C. nardus* possesses larvicidal effects against *An. gambiae* [26, 35].

Jankowska et al. [36] highlighted that some essential oils exhibit insect neurotoxic effects marked by rapid paralysis and death. This is consistent with neurotoxicity caused by AChE inhibition and indeed various essential oils and their constituents have been reported to possesses insect anticholinesterase activity [37-39]. The *in vitro* insect anticholinesterase activity can reliably be assessed by using an insect homogenate [37, 40-42]. For imporved activity profile, it is of paramount importance to evaluate the binding profiles of newly identified *Anopheles* AChE inhibitors in comparison to the current ones. Molecular modelling technology is well suited for this action to identify the binding modes, intermolecular interactions, and binding poses of compounds at the target site [43, 44]. Terpenes consist of lipophilic cyclic or acyclic hydrocarbons that may result in hydrophobic interactions with target site [45, 46]. Even though hydrogen bonding is recognized as a marker of molecular recognition and specificity at the binding site [47, 48]; van der Waals interactions play a critical role in the binding of organic molecules, however, these interactions are often neglected in molecular studies [48-50]. The protein-ligand hydrophobic interactions are better assessed through the solvent-accessible (SA) surface area method combined with generalized Born (GB) or Poisson-Boltzmann (PB) models. These include

implicit solvation approaches such as molecular mechanics-GBSA (MM-GBSA) simulation [51, 52]. In the Schrödinger's docking suite, the solvation model VSGB in MM-GBSA simulation considers the hydrophobic portions of the ligand and its interaction with the active site cavity of the protein by approximating the solvation and de-solvation energy [51, 53-55]. As a result, the Schrödinger's Maestro docking program will be used to assess the binding profiles of these terpenoids as well as assessment of their affinities through MM-GBSA simulation.

The current study reports on the *in vivo* larvicidal activity of terpenoids including four sesquiterpene alcohols (farnesol, (-)-α-bisabolol, *cis*-nerolidol, and *trans*-nerolidol), a monoterpene ((*R*)-(+)-limonene) and a phenylpropanoid (methyleugenol) against the laboratory strains of *An. gambiae, An. coluzzii*, *An. arabiensis*, and *An. funestus.* The mechanism of action of the terpenoids was assessed through the *in vitro* acetylcholinesterase inhibition against the same strains. Following confirmation of AChE inhibition as the possible larvicidal mechanism of action, the anticholinesterase activity of these essential oil constituents (EOCs) was further investigated through *in silico* molecular modelling and molecular mechanics simulation studies.

2. Methods and materials

Acetylcholinesterase from *Electrophorus electricus* (electric eel) was bought from Sigma-Aldrich (South Africa). Propoxur, acetylthiocholine iodide, Triton X-100, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dimethylsulfoxide (DMSO), sodium phosphate buffer (dibasic) (Na_2HPO_4), potassium dichromate and all terpenoids were bought from Sigma-Aldrich with a purity > 90%.

2.1. Anopheles strains

This study obtained the animal research ethics waiver (Waiver Number: 07-11-2017-O) from Wits Animal Research Ethics Committee. Four *Anopheles* strains used in this study, *An. gambiae* (COGS)*, An. coluzzii* (G3), *An. arabiensis* (KWAG), and *An. funestus* (FUMOZ), were maintained under the typical insectary conditions as described by Hunt et al. [56] and Zengenene and colleagues [57]. *An. gambiae* COGS is a laboratory resistant strain and was first established from Republic of the Congo in 2009 and is resistant to multiple insecticide classes including pyrethroids, organochlorines, and carbamates but susceptibility to organophosphates [58]. *An. coluzzii* G3 strain was colonized from The Gambia in 1970's [59-

61] and has also shown resistance to pyrethroids and DDT [62]. The *An. arabiensis* KWAG was first colonized in 2005 from Kwazulu-Natal, South Africa, and *An. funestus* FUMOZ was established from Mozambique in 2000 [56, 63]. Both KWAG and FUMOZ colonies are known to be resistant to pyrethroids and carbamates [63-67]; with KWAG showing resistance to an organochlorine, dichloro-diphenyl-trichloroethane (DDT) [68, 69].

2.2. Larvicidal assay

The larval toxicity of these EOCs was evaluated using the World Health Organization (WHO) larvicidal bioassay [70]. Twenty (20) third instar larvae of each *Anopheles* species were transferred into the test cups containing deionized water. A specific test compound or positive control (250 µL) was then added, and the incubation mixture made up to 25 mL total volume. All test compounds and the positive control were dissolved in DMSO and evaluated for larvicidal activity at seven concentrations ranging from 0.0005 µM to 500 µM (10-fold serial dilutions). Propoxur, a known larvicide and anticholinesterase agent, was used as a positive control and DMSO was used for the negative control test [71, 72]. The experiments were run in triplicate under 27 °C and humidity ≥78% simulating the insectary conditions. Larval mortality was documented at 24, 48 and 72 h. The larvae were fed with protein crushed dog biscuits mixed with brewer's yeast (3:1) at day 0 and after every mortality counting to prevent mortality related to starvation. The nutritional content of this meal includes fibre, protein, fat, vitamins and trace elements required for larval development [73]. The 50% lethal concentration (LC_{50}) values were determined by probit analysis method using the SPSS Statistics v27 software [71].

2.3. Artemia lethality assay

The cytotoxicity potential of the EOCs was assessed through the *Artemia* lethality assay using *Artemia franciscana* [74]. The *Artemia* eggs were hatched in artificial sea water (32 g/L Tropic Marine® Sea Salt in deionized water) supplied with constant aeration to disperse the eggs under an optimal room temperature for 24 h [75]. The same concentrations as used in the larvicidal assessment were used for the *Artemia* toxicity evaluation for direct comparison. Fifty microlitres of each test compound dissolved in DMSO was incubated in a 48-well plate with 30 – 50 nauplii in 450 µL of the sea water. The wells were observed under the stereo microscope for any dead nauplii at the beginning of the experiment and the EOC-induced mortality was recorded over 24 h. For consistent comparison, propoxur was used as a positive

control and DMSO as a negative control. The experiment was performed in triplicate and LC_{50} values were calculated with the probit analysis in SPSS Statistics v27 package [76].

2.4. AChE assay

The anticholinesterase activity of the EOCs was assessed using the modified Ellman assay [77, 78]. The *Anopheles* mosquitoes from four species were separately homogenized in assay buffer (dibasic sodium phosphate buffer, 1% Triton X-100 (pH 8.0)) and the homogenate used as an enzyme source. Protein content was determined using the standard Lowry protein assay and the mosquito homogenate standardized to 0.8 ug protein/ml for each experiment [79]. The incubation mixture in the 96-well plate was composed of the mosquito homogenate (20 µl) in 132 µL of assay buffer. For selectivity analysis, AChE from electric eel (0.02 U/mL) was used in place of the *Anopheles* homogenate. To this latter incubation mixture, 20 µL of test compounds (0.0005 µM to 500 µM) were added along with 8 µL (0.2 mM) of the freshly prepared Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB in Na_2HPO_4 (pH 7.0). To initiate the enzymatic reaction, 20µL (1mM) of acetylthiocholine iodide (AChE substrate) was added and the absorbance measured at 412 nm with a UV-Visible plate spectrophotometer every 30 seconds. Propoxur was kept as a positive control since it is an anticholinesterase insecticide [72], while DMSO was used in the negative control wells. To note, DMSO is known to exhibit AChE inhibition at concentrations above 1% [80], as such, 0.4% was the highest DMSO concentration attained in the final incubation mixtures. The 50% AChE inhibitory concentration (IC_{50}) values were calculated with GraphPad Prism 9 (GraphPad Software, CA, USA).

2.5. Molecular docking and molecular mechanics simulations

2.5.1. Extra-precision docking

Schrödinger Release 2021-3 molecular modelling software, Maestro version 12.9, (Schrödinger LLC, New York) was used to assess the AChE target site binding profiles of terpenoids [43]. This study intended to also evaluate the differences in binding poses of the EOCs across the four *Anopheles* species, however, this was hindered by the availability of only *An. gambiae* crystal protein structure [81, 82].

The *An. gambiae* AChE 3D structure was sourced from PDB (PDB: 5YDI) and prepared by the Maestro's protein preparation function. This preparation included optimization of hydrogen bonds and removal of non-hetero groups as well as non-essential water molecules before

minimization under the OPLS3e force field [83]. The receptor grid was generated using the same force field to define the binding site [43]. On the other hand, the EOCs were prepared with the LigPrep tool (Figure 1) and were allowed to generate their possible stereoisomers as well as the tautomeric and ionization states at pH 7.0 (±2.0) [84, 85]. The scaling factor for van der Waals interactions was set to 0.5Å at a partial cut-off charge of 0.15, to ensure scaling of only nonpolar hydrogens [49]. Finally, the prepared ligands and receptors were used for molecular docking under the rigorous extra precision mode [86, 87].

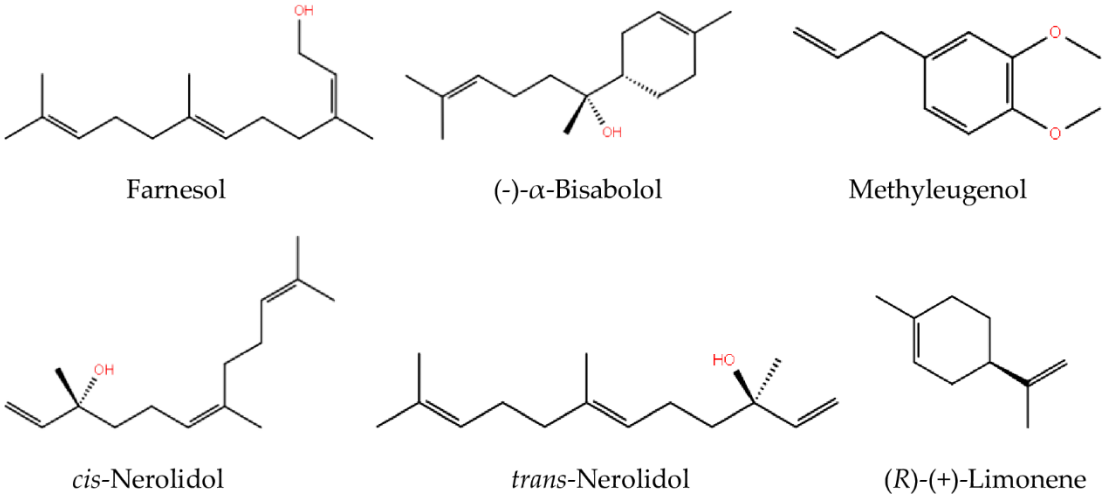


Figure 1. Chemical structures of the terpenoids.

2.5.2. Molecular mechanics simulations

The binding affinities of the terpenoids were evaluated through the MM-GBSA simulations. The previously docked extra precision complexes were retrieved for redocking under the MM-GBSA conditions. The same hydrophobic interaction settings used in the extra precision docking mode were adopted; however, the permitted distance between the ligand and receptor was changed from 0 Å to 5 Å to allow receptor flexibility during the simulation. The effective solvation model VSGB along with the OPLS3e force field were used in the current molecular mechanics simulation [88, 89].

3. Statistical analyses

The *in vivo* larvicidal and artemicidal assays as well as *in vitro* anticholinesterase studies were performed in triplicate. The 50% and 95% lethal concentrations (LC_{50} and LC_{95}) values were calculated from the probit analysis method using the SPSS Statistics v28 package (International Business Machines Corporation, NY, USA) and the IC_{50} values were determined by non-linear regression analysis using GraphPad Prism 9 (GraphPad Software, CA, USA) [71].

4. Results and discussion

4.1. Larvicidal activity

To identify terpenoids with larvicidal effects, the compounds were screened against four *Anopheles* species to assess their activity differences; and to assess cytotoxicity against other aquatic organisms, they were subsequently screened for artemicidal activity. Based on the LC_{50} values against *An. funestus*, farnesol and (-)-α-bisabolol were about time times more active than a positive control, propoxur, while *cis*-nerolidol and *trans*-nerolidol were five times more active than propoxur (Table 1). The phenylpropanoid methyleugenol and a monoterpene (*R*)-(+)-limonene, were only about two times more active than propoxur (Table 1). For *An. arabiensis*, two sesquiterpene alcohols, farnesol and (-)-α-bisabolol, were still the most active and about 150 times more active than propoxur. The monoterpene (*R*)-(+)-limonene displayed the lowest activity (LC_{50} = 6.32 µM) but still significantly more active than propoxur (LC_{50} = 8.77 µM) (unpaired *t*-test: P = 0.0008). Though the terpenoids potency was relatively low in *An. gambiae*, a similar trend was observed where farnesol and (-)-α-bisabolol were most active (mean LC_{50} ~ 7 µM) followed by nerolidol geometric isomers (mean LC_{50} ~ 10 µM) and methyleugenol (LC_{50} = 21.16 µM) while (*R*)-(+)-limonene was rather inactive (LC_{50} > 100 µM). Farnesol and (-)-α-bisabolol were still the most active terpenoids against *An. coluzzii* with LC_{50} values less than 1 µM while *cis*-nerolidol, *trans*-nerolidol and methyleugenol attained similar potency with mean LC_{50} ~ 2 µM (one-way ANOVA: P = 0.4419). The standard carbamate, propoxur, showed low potency against *An. gambiae* and *An. coluzzii* with LC_{50} values of 63.99 and 62.68 µM, respectively. In terms of the LC_{95} values, farnesol and (-)-α-bisabolol were still the most active terpenoids attaining lethality values less than 100 µM against all *Anopheles* species except for *An. gambiae* (Table 1). In fact, all terpenoids attained LC_{95} values in the millimolar range (> 1000 µM) against *An. gambiae*. (*R*)-(+)-Limonene was still suggested the lowest active terpenoid whereby it attained LC_{95} values in the millimolar range; along with the positive control, propoxur.

In general, *An. arabiensis* was more susceptible to the assessed terpenoids (Table 1). Taking into account the toxicity against *A. franciscana*, it was noted that farnesol and (-)-α-bisabolol were five to ten times more selective to *An. arabiensis* than *Artemia* (LC_{50} on *Artemia*/LC_{50} on *Anopheles* [90]). Interestingly, with relatively high LC_{50} against *Artemia* (42.79 µM), methyleugenol was highly selective (selectivity index more than 10 [90]) towards *Anopheles* species including *An. funestus*, *An. arabiensis*, and *An. coluzzii*. The monoterpene (*R*)-(+)-

limonene was inactive against *Artemia* (LC_{50} > 100 µM) though it was active against only two *Anopheles* species, *An. funestus* and *An. arabiensis* with LC_{50} ranging from 5 – 7 µM. Although a positive control, propoxur, was also relatively active against *An. funestus* and *An. arabiensis* (LC_{50} range: 8 - 10 µM), it was found to be equally toxic to *Artemia* (LC_{50}: 9.08 µM). This carbamate has previously been reported to exhibit toxicity at LC_{50} range of 3.7 – 6.6 µg/mL (17.68 – 31.54 µM) against non-target aquatic species [91]. Moreover, using *A. salina*, carbamates have previously been shown to be highly toxic against this *Artemia* species with LC_{50} values as low as 1.74 µM [92].

Table 1: Comparison of the larvicidal and artemicidal activities of the assessed terpenoids.

	Larvicidal *Anopheles* species				**Artemicidal *Artemia* species**
	LC_{50} (µM) (95% CI)				**LC_{50} (µM) (95% CI)**
	LC_{95} (µM) (95% CI)				**LC_{95} (µM) (95% CI)**
Compound	***An. funestus***	***An. arabiensis***	***An. gambiae***	***An. coluzzii***	***A. franciscana***
Farnesol	1.15 (0.39 – 3.60)	0.05 (0.03 – 0.07)	6.52 (2.84 – 16.37)	0.23 (0.16 – 0.34)	0.37 (0.13 – 1.03)
	85.21 (18.92 – 1555.01)	8.41 (4.30 – 19.40)	>1000	17.85 (9.81 – 37.54)	226.17 (44.38 – 3127.27)
(-)-α-Bisabolol	1.42 (0.43 – 5.08)	0.06 (0.04 - 0.10)	7.18 (3.18 – 17.62)	0.26 (0.18 – 0.37)	0.33 (0.12 – 0.94)
	89.02 (18.16 – 2490.30)	15.00 (7.46 - 35.80)	>1000	22.14 (12.04 – 47.12)	205.39 (39.44 – 3026.37)
cis-Nerolidol	2.37 (1.26 – 4.57)	1.21 (0.50 – 3.01)	10.29 (3.41 – 37.46)	2.08 (0.78 – 5.90)	1.26 (0.24 – 8.38)
	400.39 (137.93 – 1801.45)	279.94 (69.05 – 2544.15)	>1000	277.04 (62.26 – 3727.60)	512.45 (47.07 – 120317.60)
trans-Nerolidol	2.11 (1.13 – 4.00)	1.43 (0.50 – 4.38)	10.87 (3.88 – 36.07)	2.15 (0.82 – 6.01)	1.31 (0.28 – 7.11)
	311.33 (110.51 – 1337.96)	576.73 (104.42 – 10391.25)	>1000	365.47 (80.70 – 4800.78)	512.70 (54.85 – 59841.34)
Methyleugenol	4.35 (1.26 – 17.50)	2.22 (0.72 – 7.59)	21.16 (7.18 – 84.95)	2.02 (0.92 – 4.57)	42.79 (18.56 – 120.01)
	617.12 (99.28 – 25529.99)	690.51 (114.63 – 17233.40)	>1000	560.78 (152.03 – 3954.66)	>1000
(*R*)-(+)-Limonene	5.41 (2.57 – 12.20)	6.32 (2.35 – 19.24)	>100	>100	>100
	>1000	>1000	>1000	>1000	>1000
Propoxur	9.90 (2.96 -41.41)	8.77 (2.81 – 32.71)	63.99 (38.23 – 116.29)	62.68 (37.72 – 112.76)	9.08 (2.04 – 64.67)
	>1000	>1000	>1000	>1000	>1000

CI = confidence intervals

4.2. AChE inhibition activity

The terpenoids mechanism of action was elucidated by screening them for *Anopheles* AChE inhibition. To assess the selectivity potential, the terpenoids were further screened against electric eel AChE as a comparable enzyme to human AChE [93]. In correlation to the larvicidal activities, the terpenoids were generally more inhibitory against AChE from *An. arabiensis* and *An. funestus* than *An. gambiae* and *An. coluzzii* (Table 2). Farnesol was highly active against *An. funestus* (IC_{50} = 0.127 μM); however, it was the only terpenoid that also possessed inhibitory activity against electric eel AChE. It was nonetheless approximately ten times more selective towards *An. funestus*. The selectivity indices between electric eel and other *Anopheles* colonies, especially *An. gambiae* and *An. coluzzii* were relatively low for farnesol, indicating non-selectivity [90]. In agreement to the literature, propoxur was non-selective between the *Anopheles* and electric eel targets [16, 94]. The nerolidol isomers were equally active against *An. funestus* AChE (unpaired *t*-test: $P = 0.0805$) and more active than (-)-α-bisabolol (one-way ANOVA: $P = 0.0035$) and methyleugenol (one-way ANOVA: $P = 0.0005$), while (*R*)-(+)-limonene was the least active and approximately ten times less active than propoxur. Propoxur was statistically more active against *An. arabiensis* than *An. funestus* AChE (unpaired *t*-test: $P = 0.0108$), which is in line with sensitivity pattern observed for these strains (Table 1). Moreover, all terpenoids were similarly (IC_{50} range: 0.5 - 0.7 μM) potent against *An. arabiensis* in comparison to the IC_{50} values ranging from 3 – 7.5 μM against *An. gambiae* (Table 2); which were generally ten times higher than those obtained in the screening against *An. arabiensis* AChE.

Notably, (*R*)-(+)-limonene showed potential activity against *An. gambiae* AChE as an *in vitro* target (Table 2) which did not correlate with the *in vivo* larvicidal data (Table 1). This is similar to the positive control (propoxur) that showed significantly higher activity against the extracted target than the whole organism (unpaired *t*-test: $P < 0.0001$) for both *An. gambiae* and *An. coluzzii*. This suggests the involvement of pharmacokinetic barriers in the whole larvae, of which decreased cuticle penetration has previously been shown to play a role [95]. Propoxur has formerly been reported as inactive (IC_{50} > 100 μM) against AChE from the *An. gambiae* and *An. coluzzii* larvae [96]. Perrier et al. [97] reported that propoxur was inactive even in the physiological studies with isolated *An. gambiae* neurons. *An. coluzzii* AChE proved to be the least sensitive to the assessed terpenoids where none of them attained an IC_{50} value < 50 μM.

This therefore rules out AChE inhibition as the mechanism of action for the observed larvicidal activities against the utilized *An. coluzzii* G3 strain.

Table 2: Comparison of the *Anopheles* and electric eel anticholinesterase activity of the identified terpenoids.

	AChE inhibition IC_{50} ± SD (µM)				
Compound	***An. funestus***	***An. arabiensis***	***An. gambiae***	***An. coluzzii***	***E. electricus***
Farnesol	0.127 ± 0.001	0.710 ± 0.007	7.431 ± 0.195	69.055 ± 0.652	1.156 ± 0.059
(-)-α-Bisabolol	0.586 ± 0.137	0.721 ± 0.013	5.220 ± 0.517	61.120 ± 1.182	>100
cis-Nerolidol	0.244 ± 0.021	0.639 ± 0.004	3.534 ± 0.410	>100	>100
trans-Nerolidol	0.273 ± 0.005	0.537 ± 0.025	4.303 ± 0.404	>100	>100
Methyleugenol	0.488 ± 0.064	0.606 ± 0.058	6.883 ± 0.115	>100	>100
(*R*)-(+)-limonene	7.487 ± 0.161	0.622 ± 0.096	3.256 ± 0.004	>100	>100
Propoxur	0.893 ± 0.044	0.778 ± 0.004	28.060 ± 1.778	23.325 ± 4.379	1.283 ± 0.046

4.3. In silico investigation of the receptor binding of terpenoids

To further elucidate the mechanism of action, the binding conformations of the terpenoids against the AChE targets were evaluated through molecular docking and molecular mechanics simulations. Since large structural portions of terpenoids are nonpolar (Figure 2), the optimized non-polar interactions through MM-GBSA simulation were taken into consideration. However, since van der Waals interactions are naturally weak, and their energies are mainly considerable in combination [50, 98, 99], only the amino acid residues forming three or more of these interactions with the ligand after being optimized with MM-GBSA re-docking were considered and reported in Table 3 and Table 4. Lastly, due to MM-GBSA being more accurate in pose prediction and affinity ranking [100], only crystal poses from this molecular mechanics simulation were reported.

In correlation to the *in vivo* and *in vitro* results, farnesol was still the most active terpenoid as suggested by the highest binding score and target interaction through various amino acid residues against *Anopheles* AChE (Table 3). The initial (extra precision) docking of farnesol suggested a similar binding score against *Anopheles* (-6.893 kcal/mol; Table 3) and electric eel (-6.518 kcal/mol; Table 4). However, in a more accurate molecular mechanics simulation, the results correlated with the *in vitro* data showing the binding affinity higher for *Anopheles* (-48.82 kcal/mol; Table 3) that the corresponding electric eel AChE (-41.53kcal/mol; Table 4). Moreover, farnesol established H-bonds with Gly^{445}, Cys^{447} and Glu^{448} in the *Anopheles* target (Figure 2A) while it could only form H-bonds with aromatic amino acid residues Phe^{288} and Phe^{331} in electric eel (Figure 2B). Interestingly, farnesol preferentially interacted with a conserved amino acid residue Cys^{447} that is a current target in the design of *Anopheles* selective anticholinesterases [16, 101, 102].

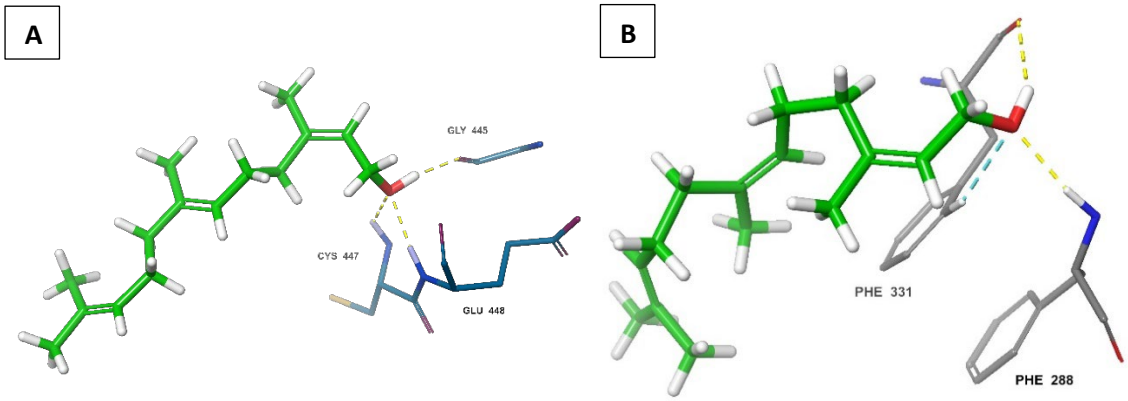


Figure 2. Differences in farnesol poses against *Anopheles* AChE (A) and electric eel AChE (B).

Interestingly, the positive control, propoxur, generated a similar crystal pose against *Anopheles* and electric eel AChEs in support of the literature, where it is nonselective between these two targets (Figure 3A and 3B) [16, 94]. There was a correlation of binding scores at each docking level whereby the initial docking scores were -5.683 kcal/mol (*Anopheles*) and -5.809 kcal/mol (electric eel) and the subsequent MM-GBSA attained -32.03 kcal/mol (*Anopheles*) and -38.16 kcal/mol (electric eel). This covalent inhibitor was stabilized by aromatic pi-pi interaction through its aromatic ring and the phenylalanine residue (Phe^{490}; *Anopheles*/Phe^{331}; electric eel) while held tightly by the catalytic serine (Ser^{360}; *Anopheles*/Ser^{200}; electric eel). The H-bonds were formed with amino acid residues adjacent to the catalytic serine including the Ala^{361}, His^{600} and Gly^{280} (Figure 3A). The latter amino acid is of great concern due to its involvement in mutation and resistance in *Anopheles* AChE [82]. This is a similar binding profile that has been reported for organophosphates where they also interact with Gly^{280} [43]. This supports the idea that substitution of this smaller amino acid with a relatively larger serine residue (G280S) in resistant strains, confers steric hindrance to the optimal binding of organophosphates and carbamates [16, 103]. In the electric eel target, propoxur established the comparable H-bonds with Ala^{201}, Gly^{118}, and Gly^{119}, as well as aromatic H-bonding with Tyr^{121} (Figure 3B).

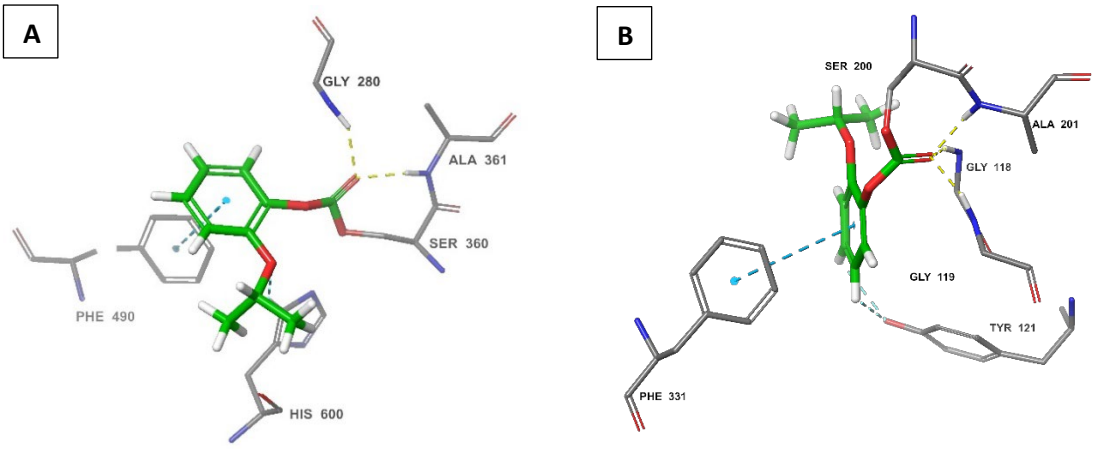


Figure 3. Comparison of propoxur binding pose against *Anopheles* (A) and electric eel (B).

(-)-α-Bisabolol established only two H-bonds with critical amino acids including the catalytic Ser^{360} and adjacent Gly^{278} utilizing it hydroxyl group (Figure 4A). The nerolidol geometric isomers established same interactions with the target site amino acids (Figures 4B and 4C). Notably, they formed a H-bond with a mutable Gly^{280} among others that included Gly^{278} and the catalytic $serine^{360}$. The influence of mutated Gly^{280} to Ser^{280} on the binding and effectiveness of these isomers should be directly evaluated by the biological and *in silico* studies. In comparison, the geometric isomers of nerolidol obtained similar scores (-5.572 kcal/mol and -5.518 kcal/mol for *cis*-nerolidol and *trans*-nerolidol, respectively) in both extra precision docking and MM-GBSA re-docking (-40.41 kcal/mol and -42.74 kcal/mol for *cis*-nerolidol and *trans*-nerolidol, respectively). Finally, methyleugenol was stabilized by pi-pi stacking through its aromatic ring by Trp^{245}, Phe^{490} and His^{600} as it formed H-bonds with both oxygen atoms from its methoxy groups and catalytic Ser^{360} residue (Figure 4D).

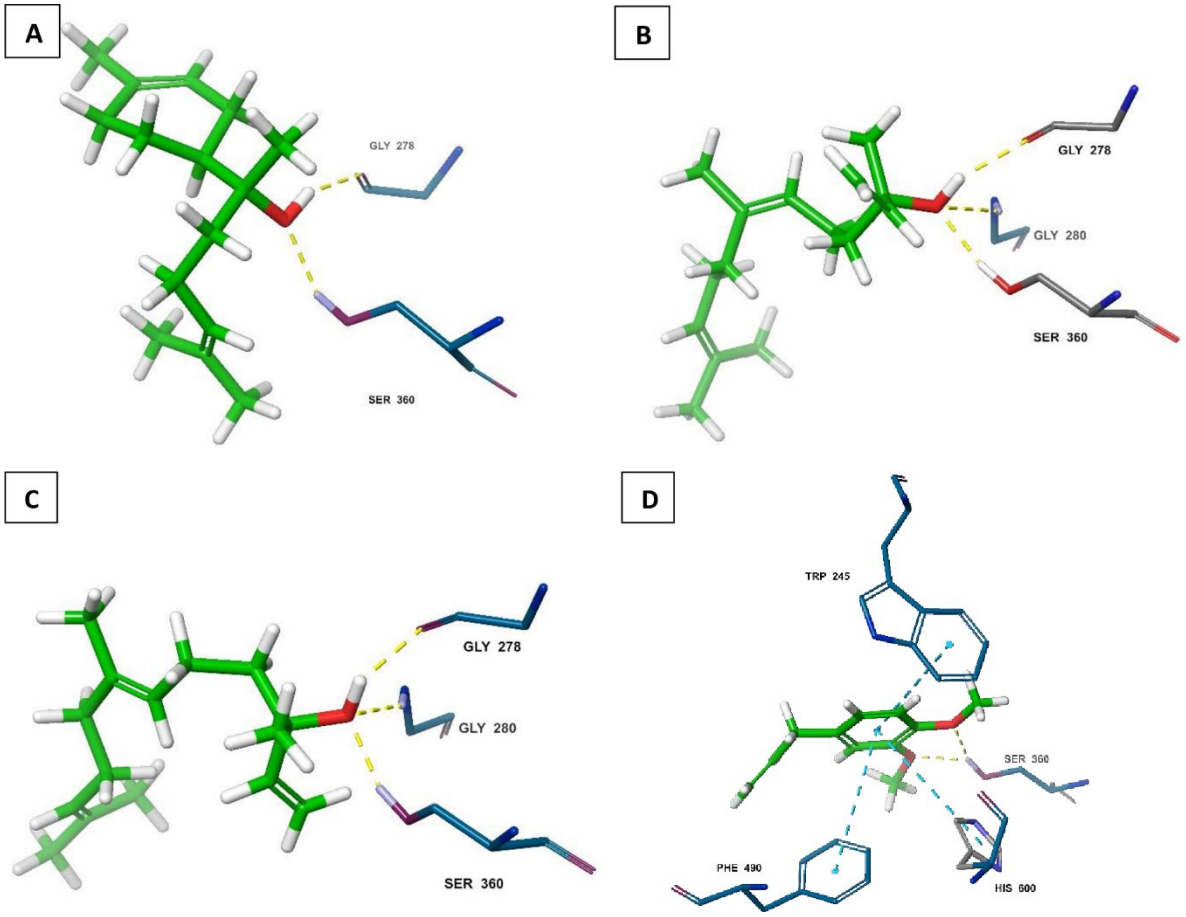


Figure 4. Crystal poses of (-)-α-bisabolol (A), *cis*-nerolidol (B), *trans*-nerolidol (C) and methyleugenol (D) in the *Anopheles* AChE binding site.

The nonpolar monoterpene, (*R*)-(+)-limonene, could not form any H-bonds and obtained the lowest affinity as per the MM-GBSA prediction (-25.03 kcal/mol) (Table 3). This supported the *in vivo* results (Table 1), whilst conflicting with the *in vitro* data that indicated that (*R*)-(+)-limonene was a highly potent AChE inhibitor against *An. gambiae* (Table 2).

Table 3: The binding scores and amino acid interactions of terpenoids and *Anopheles* AChE.

	***An. gambiae* AChE (PDB: 5YDI)**			
	Docking score (kcal/mol)		**Amino acid residues**	
Compound	**Glide docking**	**MM-GBSA simulation**	**H-bonds and aromatic bonds**	**Nonpolar bonds**
Farnesol	-6.893	-48.82	Gly^{445}, Cys^{447}, Glu^{448}	Trp^{245}, Tyr^{282}, Phe^{449}, Tyr^{493}, Tyr^{489}, Phe^{490}
(-)-α-Bisabolol	-5.483	-30.36	Ser^{360}, Gly^{278}	Trp^{245}, Phe^{449}, Phe^{490}
cis-Nerolidol	-5.572	-40.41	Gly^{278}, Gly^{280}, Ser^{360}	Trp^{245}, Tyr^{282}, Phe^{449}, Tyr^{489}, Tyr^{493}, Phe^{490}
trans-Nerolidol	-5.518	-42.74	Gly^{278}, Gly^{280}, Ser^{360}	Trp^{245}, Tyr^{282}, Tyr^{489}, Tyr^{493}, Phe^{449}, Phe^{490}
Methyleugenol	-4.944	-38.59	Trp^{245}, Ser^{360}, Phe^{490}, His^{600}	Trp^{245}, Tyr^{282}, Phe^{449}, Tyr^{489}, Phe^{490}
(*R*)-(+)-limonene	-4.32	-25.03	-	Phe^{449}, Tyr^{489}, Tyr^{493}, Tyr^{494}, Phe^{490}
Propoxur	-5.683	-32.03	Gly^{280}, Ser^{360}, Ala^{361}, Phe^{490}, His^{600}	Trp^{245}, Tyr^{282}, Phe^{449}, Tyr^{489}, Tyr^{493}

Table 4: The comparison of the docking scores and amino acid interactions of farnesol and propoxur against electric eel AChE.

	***E. electricus* AChE (PDB: 1EVE)**			
	Docking score (kcal/mol)		**Amino acid residues**	
Compound	**Glide docking**	**MM-GBSA simulation**	**H-bonds and aromatic bonds**	**Nonpolar bonds**
Farnesol	-6.518	-41.53	Phe^{288}, Phe^{331}	Tyr^{84}, Tyr^{121}, Phe^{290}, Phe^{330}, Tyr^{334}
Propoxur	-5.809	-38.16	Gly^{118}, Gly^{119}, Ser^{200}, Ala^{201}, Tyr^{121}	Trp^{70}, Trp^{279}, Phe^{330}, Phe^{331}, Tyr^{334}

Generally, the terpenoids interacted with the catalytic active site through H-bonds and established nonpolar interactions with aromatic amino acid residues localized at the entrance to the active site gorge. A free cysteine, Cys^{447}, is a conserved nucleophilic amino acid residue at the entrance to the active site gorge [82] and farnesol formed a H-bond with this amino acid. The aromaticity of this entrance is caused by Phe^{449}, Tyr^{493}, and Tyr^{489}, which were the common amino acids involved in the terpenoid van der Waals interactions. It has been reported that these aromatic amino acids can establish the aromatic and van der Waals interactions, useful in ligand trafficking [82]. The (*R*)-(+)-limonene chemical structure has no aromatic ring or polar groups (Figure 1), hence it could only interact with the target through van der Waals

interactions. As a result, this monoterpene assumed a preferred position at the entrance to the active site gorge and formed similar van der Waals interactions as other terpenoids, as indicated in Table 3. The superimposition of (*R*)-(+)-limonene with other terpenoids is displayed in Figure 5 showing that it assumed a similar binding pose but was lodged in the entrance due to higher capacity for nonpolar interactions at this site. Highly nonpolar molecules commonly display nonspecific binding and often show exaggerated high potencies in *in vitro* assays [104, 105]. This study suggests that the observed high potency of (*R*)-(+)-limonene (Table 2) in the *in vitro* screening and inconsistency with the *in vivo* (Table 1) and *in silico* (Table 3) findings is due to nonspecific binding at the entrance to the AChE active site that may block access to this site by the substrate.

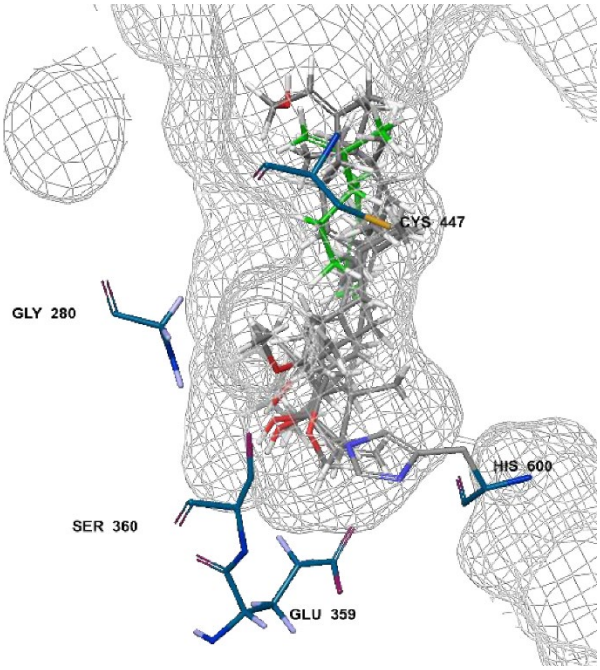


Figure 5: Superimposition of terpenoid poses on the active site of *Anopheles* AChE, with (*R*)-(+)-limonene lodged at the active site entrance (green).

5. Conclusions

This study identified terpenoids for potential application as anticholinesterase bioinsecticides. In this work, the *in vitro* AChE inhibition of terpenoids was integrated to the *in vivo* larvicidal effects, and their mode of action was further elucidated at the molecular level through the *in silico* models. Farnesol, (-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, (*R*)-(+)-limonene, and methyleugenol proved to be potent larvicidal agents against major African malaria vectors *An. funestus*, *An. arabiensis*, *An. gambiae*, and *An. coluzzii*. Two sesquiterpene alcohols, farnesol and (-)-α-bisabolol, were the most active followed by *cis*-nerolidol and *trans*-nerolidol while

methyleugenol and (*R*)-(+)-limonene were the least active agents. Interestingly, farnesol, (-)-α-bisabolol, methyleugenol, and (*R*)-(+)-limonene were more selective to *Anopheles* than *Artemia* in comparison to a positive control, propoxur, or the nerolidol isomers. The evaluation of these terpenoids against AChE samples extracted from the four *Anopheles* species indicated them as strong AChE inhibitors against *An. funestus* and *An. arabiensis* with moderate inhibitory activity against *An. gambiae* but very weak activity against *An. coluzzii.* This eliminated AChE inhibition as a possible mode of action for the observed terpenoid-induced *An. coluzzii* larval toxicity. Further elucidation of the mechanism of action using molecular modelling and molecular mechanics simulations technology identified farnesol as the most active terpenoid, in correlation to the *in vitro* and *in vivo* results. In general, the terpenoids interacted with the catalytic site through hydrogen bonding and with the entrance to the active site through van der Waals interactions, utilizing the polar environment of the active site and high density of the aromatic amino acid residues localized at the entrance, respectively. Most fascinating was the favourable hydrogen bonding interaction of farnesol and a conserved amino acid residue, Cys^{447}, at the entrance to the active site gorge. Farnesol generated completely different binding poses between *Anopheles* and electric eel AChE targets where a higher binding score and more H-bonds were formed in the *Anopheles* target in support of the *in vitro* and *in vivo* data. Another highly potent terpenoid, (-)-α-bisabolol, formed H-bonds with the catalytic Ser^{360} and adjacent Gly^{278} which differed to the nerolidol isomers that in addition, established the undesirable interaction with the mutable Gly^{280} residue that is capable of conferring resistance to the AChE inhibitors. The phenylpropanoid methyleugenol also displayed a strong interaction with the *Anopheles* AChE where its aromatic ring attracted aromatic stabilization from Trp^{245}, Phe^{490} and His^{600} placing its methoxy groups in a best position to interact with the catalytic Ser^{360} residue. Therefore, this study has identified farnesol, (-)-α-bisabolol, and methyleugenol as potent inhibitors of AChE in the *in vitro* and *in silico* studies with their biological activity proven in the *in vivo* larvicidal assay. These three EOCs present as terpenoids of interest for further development as anticholinesterase bioinsecticides.

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CHAPTER 6

The activity of terpenoids against multiple stages of *Anopheles* life cycle

Following the confirmation of *Anopheles* acetylcholinesterase inhibitory potential of terpenoids in Chapter 5, this chapter presents the proof-of-concept manuscript that exhibits the extrapolation of the *in vitro* data to the *in vivo* models. The manuscript shows the toxic effects of terpenoids against different life stages of *Anopheles* including egg, larva, pupa, and adult. This manuscript has been submitted to Experimental Parasitology.

Chapter type: Submitted manuscript (review stage)

Title of manuscript: Bioactivity of select essential oil constituents against life stages of *Anopheles arabiensis* (Diptera: Culicidae)

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Supervision		✓	✓
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Abstract: Malaria is transmitted by infected female *Anopheles* mosquitoes, and the African region bears a great burden of malaria infections and deaths. *Anopheles arabiensis* is a main malaria vector in arid African countries and like other anophelines, its life cycle comprises of three aquatic stages; egg, larva, and pupa, followed by a free flying adult stage. Current vector control interventions using synthetic insecticides target these stages using adulticides or larvicides. With escalating insecticide resistance against almost all conventional insecticides, identification of agents that simultaneously act at multiple stages of *Anopheles* life cycle presents a cost-effective opportunity. A further cost-effective approach would be the discovery of such insecticides from natural origin. Interestingly, essential oils present as potential sources of cost-effective and eco-friendly bioinsecticides. This study aimed to identify essential oil constituents (EOCs) with potential toxic effects against multiple stages of *An. arabiensis* life cycle. Five EOCs were assessed for inhibition of *Anopheles* egg hatching and ability to kill larvae, pupae and adult mosquitoes of *An. arabiensis* species. One of these EOCs, namely methyleugenol, exhibited potent *Anopheles* egg hatchability inhibition. On the other hand, all five EOCs exhibited potent larvicidal activity, while the two sesquiterpene alcohols, (-)-α-bisabolol and farnesol, also possessed pupicidal effects. Finally, all EOCs showed moderate lethality against adult mosquitoes. This study reports for the first time, methyleugenol, (-)-α-bisabolol and farnesol as potent bioinsecticides against early life stages of *An. arabiensis*. This synchronized activity against *Anopheles* aquatic stages shows a prospect to integrate EOCs into existing adulticide-based vector control interventions.

Keywords: Malaria, larvicides, adulticides, methyleugenol, nerolidol, (-)-α-bisabolol, farnesol

1. Introduction

Anopheles mosquitoes play a critical role as malaria vectors. There are more than 400 *Anopheles* species of which about 30 are responsible for malaria transmission (WHO 2021b). In 2020, malaria cases were estimated at 241 million globally of which 95% were from the African region. Additionally, 627 000 malaria-related deaths were recorded worldwide of which 96% were from Africa (WHO 2021a). *Anopheles gambiae*, *An. coluzzii* and *An. arabiensis* are the main African malaria vectors belonging to the *An. gambiae* complex (Dahan-Moss et al. 2020). *Anopheles arabiensis* is the main malaria vector in arid African countries like South Africa, Sudan, Ethiopia and Eritrea (Dandalo et al. 2017; Malcolm et al. 2007; Nyanjom et al. 2003; Orondo et al. 2021).

The life cycle of an *Anopheles* mosquito starts with the eggs being oviposited into water and in a few days hatch into larvae that develop into pupae, and from which the adults emerge two days later (WHO 2021b). Malaria vectors are mainly controlled by the use of insecticides. The small sizes of these insects provide a high surface area for the penetration and systemic distribution of the insecticides. Additionally, this minute size provides short pathways to the insect's nervous system making it a target for the majority of the insecticides (Lewis 1980). The aim of indoor residual spraying and long-lasting insecticide treated nets are to kill the adult female before the parasite development is completed and prevent the transmission of the disease. Insecticide classes used for vector control include pyrethroids, organochlorides, carbamates, organophosphates, and neonicotinoids (WHO 2019). However, insecticide resistance has rendered many interventions ineffective. Malaria vectors already displayed resistance to pyrethroids, carbamates, organophosphates, and the organochlorine, *dichloro-diphenyl-trichloroethane (*DDT) (Balkew et al. 2010; Brooke et al. 2015; Buxton et al. 2020; Casimiro et al. 2006; Messenger et al. 2017; Pinda et al. 2020).

Larviciding is another vector control intervention used and involves the application of specific chemical or microbial insecticides into the aquatic habitats of *Anopheles* mosquitoes (WHO 2013). This process aims at killing mosquito larvae and pupae (Choi et al. 2019). However, effective application of larviciding, especially on a large-scale, remains an operational challenge (Berlin et al. 2020; WHO 2019, 2021a). Chemical larvicides may include insect growth regulators such as diflubenzuron, methoprene, and pyriproxyfen or insecticidal agents, specifically the organophosphates temephos and fenthion (Antonio-Nkondjio et al. 2018; Choi et al. 2019; Thevasagayam et al. 1979). Acting on the earlier stages, insect growth regulators

prevent reproduction, ability of eggs to hatch, and the molting process from one stage to the next (Choi et al. 2019). Bacterial larvicides include *Bacillus thuringiensis* var. *israelensis, Bacillus sphaericus and* spinosyns from *Saccharopolyspora* species (Choi et al. 2019; Derua et al. 2019). Integrating larviciding with other control interventions have resulted in reduced malaria transmissions (Derua et al. 2019; Fillinger et al. 2009; Maheu-Giroux and Castro 2013). With increasing resistance to the insecticides targeting the adult mosquitoes stages, identification of additional larvicides is critical (Milugo et al. 2021). Koella *et al*. (Koella et al. 2009) suggested that a combination of an early stage-acting insecticide such as a larvicide and a late stage-acting (pupa or adult stage) insecticide may counteract the evolution of resistance.

Various essential oils have exhibited inhibitory activity against eggs (Rajkumar et al. 2011; Siriporn and Mayura 2012), larvae (Amer and Mehlhorn 2006; Dantanko and Malann 2020; Rajkumar et al. 2011), pupae (Gokulakrishnan et al. 2013; Narasimman et al. 2022) and adults (Damtie and Mekonnen 2021; Kweka et al. 2011) of *Anopheles* mosquitoes. Essential oils have been recommended as potential sources of eco-friendly and cost-effective bioinsecticides (Isman 2020a, b; Pavela 2015; Wing 2021). Moreover, two structurally related sesquiterpene alcohols, farnesol and (-)-α-bisabolol, are major phytoconstituents in the essential oils of various aromatic plants such as *Rosa x. damascene* Mill., *Matricaria chamomilla* L., *Polianthes tuberosa* L., *Cymbopogon citratus*, and *C. nardus* (Jung et al. 2018; Sarkic and Stappen 2018). The essential oils of *C. citratus* (lemongrass) and *C. nardus* (citronella) have shown adulticidal effects against *An. gambiae* (Norris et al. 2015). The geometric isomers of nerolidol, *cis*-nerolidol and *trans*-nerolidol, are some of the sesquiterpene alcohols that present as main constituents in the essential oils from *Piper* species; *P. claussenianum* (Miq.), *P. umbellatum*, *P. nigrum* and *P. capense* (Marques et al. 2011; Tchoumbougnang et al. 2009). Nerolidol has previously exhibited inhibitory effects against *An. sinensis* larvae (Wang et al. 2016). Likewise, a phenylpropanoid methyleugenol has been identified in essential oils of various plants including *Colocasia antiquorum, C. nardus,* and *Ocimum basilicum* (Govindarajan et al. 2013; Tan and Nishida 2012). These essential oils have a record of potential insecticidal activity against *Anopheles* species. For example, the essential oil of *C. nardus* displayed adulticidal effects against *An. gambiae,* while that of *O. basilicum* (basil) has shown larvicidal activity against *An. subpictus* (Govindarajan et al. 2013; Norris et al. 2015).

On account of increasing resistance against standard insecticides, the discovery of bioinsecticides that have toxic effects against multiple stages of *Anopheles* life cycle would

present a sustainable option for malaria vector control (Mpofu et al. 2016). The introduction of an agent that targets several points in the mosquito growth cycle would create the opportunity for a cost-effective intervention. Using *An. arabiensis*, here we present for the first time, the integrated biological activity of select EOCs, *cis*-nerolidol, *trans*-nerolidol, (-)-α-bisabolol, farnesol, and methyleugenol, across four stages of the *Anopheles* life cycle.

2. Materials and methods

The current study obtained the animal research ethics waiver from Wits Animal Research Ethics Committee (Waiver Number: 07-11-2017-O). The laboratory-reared colony of *An. arabiensis*, KWAG, established in 2005 from KwaZulu-Natal, South Africa (Mouatcho et al. 2009), was maintained under standard insectary environmental conditions (Hunt et al. 2005; Zengenene et al. 2021). All essential oil constituents (EOCs) (Figure 1), dimethylsulfoxide (DMSO; catalog number: 34869), and propoxur (catalog number: 45644) were sourced from Sigma-Aldrich (South Africa). Acetone (catalog number: A949-1) was purchased from Fisher Scientific (South Africa). All solvents were HPLC grade, and all essential oil-based test compounds and positive controls were of ≥90% purity.

2.1. Preparation of the test compounds and test conditions

The EOCs *cis*-nerolidol, *trans*-nerolidol, (-)-α-bisabolol, farnesol, and methyleugenol were dissolved in DMSO for all the insecticidal assays. The EOCs were initially evaluated in the high-throughput screening at 100 µM after which, if ≥50% bioactivity was obtained, the 50% lethality concentration (LC_{50} value) was determined using seven concentrations ranging from 0.0005 to 500 µM, unless otherwise mentioned. Based on its known ovicidal, larvicidal, pupicidal and adulticidal activities, a standard carbamate insecticide, propoxur, was used as the positive control as it has been determined to be inhibitory across all the *Anopheles* stages (Dohutia et al. 2015; Hoffmann et al. 2008; Patil et al. 1996; Sanil and Shetty 2012; Zhang et al. 2019). Tween®-20 and DMSO were used as the negative controls. All assays in this study were conducted under controlled conditions maintained at 27 °C, ≥75% relative humidity and a 12-hr light/dark photoperiod (Brooke et al. 2001).

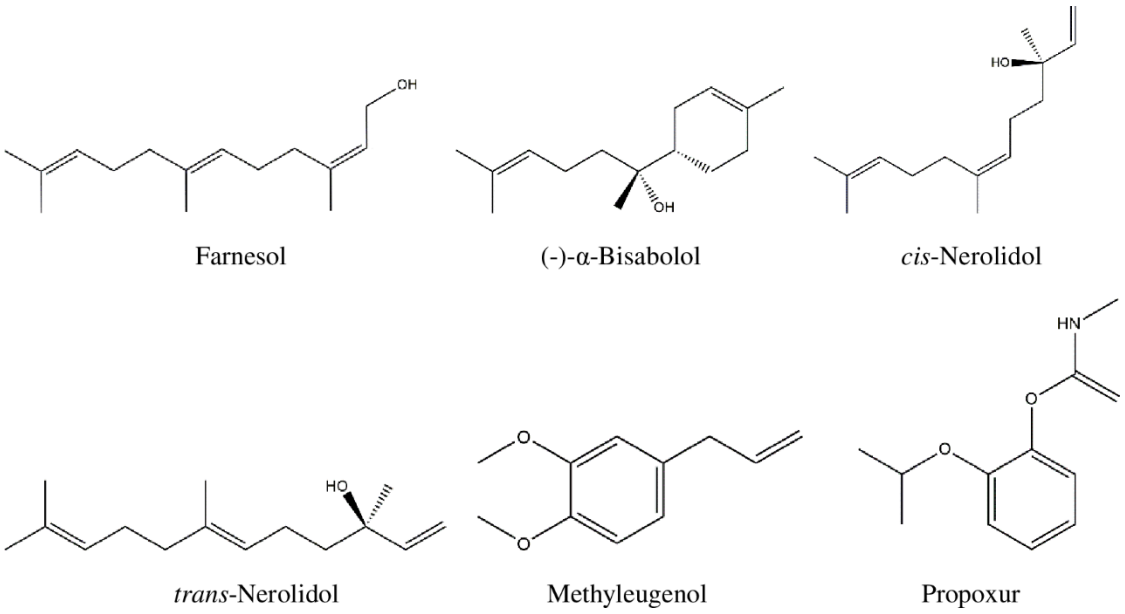


Fig 1: Chemical structures of the EOCs and a positive control, propoxur, investigated for insecticidal activity (Basera et al. 2019; Faehnrich et al. 2014; Marques and Kaplan 2011; Raymond et al. 2017; Whiteaker and Prather 2003).

2.2. Egg hatchability assay

The inhibition of egg viability was assessed according to the method described by Prajapati et al. (2005) and Ateyim et al. (2022). Briefly, 20 *An. arabiensis* eggs were incubated with 250 µL of test compound in 25 mL deionized water. The plates were inspected daily for emerging first-instar larvae. Ebrahimi et al. (2014) showed that non-agitation of the egg incubation mixture lead to delayed hatching and may pose a risk for unreliable data in laboratory studies. As a result, the incubation mixtures were slowly and non-vigorously agitated daily for five minutes (Ebrahimi et al. 2014). Good care was taken to ensure no spillage or egg adherence to the walls of the test cups. Number of eggs hatched was recorded at 24, 48 and 72 hrs post exposure and percent egg hatching calculated. The experiment was conducted in five replicates.

2.3. Larvicidal assay

The larvicidal activity of the test compounds was assessed according to the World Health Organization (WHO) bioassay protocol for evaluating mosquito larvicides (WHO 2005). Batches of 20 third instar *An. arabiensis* larvae were transferred into the test cups after which 250 µL of a test compound was added. These were incubated in a total volume of 25 mL deionized water. Larval mortality was recorded 24, 48 and 72 hrs post exposure. The larvae were fed with crushed dog biscuits and brewer's yeast mixture (3:1) throughout the duration of the experiment. This meal has a rich nutritional content of protein, fibre, fat, trace elements

(selenium and phosphorus) and vitamins (C and E) important for larval development (Zengenene et al. 2022). The experiment was done in five replicates.

2.4. Pupicidal assay

Evaluation for pupicidal activity was done using the method described by Gokulakrishnan et al. (2013) and Panneerselvam et al. (2012) informed by WHO guidelines (WHO 2005). Batches of 20 newly emerged pupae (1-2 hrs old) (Yugi et al. 2017) were collected with a dropper into a clear test cup and incubated with 250 µL of test compounds at various concentrations in 25 mL deionized water. Each test cup was covered with mosquito netting to prevent any possible emerged adults from escaping. Pupal mortality and adult emergence were recorded in 24 hrs. Five replicates were conducted.

2.5. Adulticidal assay

The adulticidal activity was determined using the WHO standard bioassay (WHO 2016). Batches of 25-30 non-blood fed female mosquitoes aged 2-5 days were evaluated in WHO exposure tubes. To assess the baseline susceptibility to a positive control, the mosquitoes were initially assessed with WHO 0.1% (w/v) propoxur test papers. For the assessment of the EOCs, Whatman Grade 1 (12 × 15 cm^2) filter paper was impregnated with 2 mL of the test compound diluted in acetone at 2-fold increasing concentrations ranging from 0.03 to 2% (w/v) (Bossou et al. 2013; Oliver et al. 2010; Wangrawa et al. 2018). Following the 1-hour exposure, mosquitoes were transferred into the holding tubes where knockdown (mosquitoes unable to fly) was recorded, and 10% (w/v) sugar solution was provided. The control group was exposed to the WHO organophosphate and carbamate (OP/C) oil control paper, as well as acetone. The final mortality was then recorded in 24 hours. The experiment was conducted in replicates of four and each test compound was assessed against no less than 100 mosquitoes.

2.6. Statistical analysis

Data was analysed by GraphPad Prism 9 (GraphPad Software, CA, USA) to obtain the 50% inhibitory concentrations (IC_{50}) from non-linear regression analysis. Additionally, probit analysis was used to acquire the lethal concentrations for 50% and 99% mortality (LC_{50} and LC_{99}, respectively) with 95% confidence intervals (CI), using the SPSS Statistics 28.0 software (International Business Machines Corporation, NY, USA). The statistical values such as p-

value, t-value, and degrees of freedom (df) between the data were obtained from t-test and one-way analysis of variance (ANOVA) (Siriporn and Mayura 2012).

3. Results

3.1. Egg hatching inhibition

The EOCs were screened for potential inhibition of *Anopheles* egg hatching; where the emergence of first-instar larvae was used as a marker of successful egg hatching and viability. Of the five assessed test compounds (Table 1), only methyleugenol inhibited *Anopheles* egg hatching at IC_{50} values (0.51 µM) lower than the positive control, propoxur (5.13 µM) (Figure 2).

Table 1: Concentration-based comparison of the *Anopheles* egg hatching inhibitory activities of the select EOCs compared to propoxur.

		Mean egg hatching inhibition (%)							
Compound	**Negative control (DMSO)**	**Concentration ± SD (µM)**							**IC_{50} ± SD** (95% CI limits)
		0.0005	**0.005**	**0.05**	**0.5**	**5**	**50**	**500**	
trans-Nerolidol	0.0 ± 0.0	1.3 ± 0.6	5.3 ± 0.6	13.7 ± 0.6	23.0 ± 1.0	39.0 ± 1.0	54.7 ± 0.6	56.0 ± 1.0	45.58 ± 2.18 (1.55 – 89.22)
cis-Nerolidol	0.0 ± 0.0	1.3 ± 0.6	4.3 ± 1.2	13.0 ± 1.0	23.0 ± 1.0	39.0 ± 1.0	54.3 ± 0.6	54.7 ± 0.6	46.28 ± 1.73 (0.31 – 92.37)
Farnesol	0.0 ± 0.0	1.7 ± 0.6	5.0 ± 1.0	13.7 ± 1.5	24.3 ± 0.6	38.7 ± 1.5	54.0 ± 1.0	59.0 ± 1.0	50.94 ± 6.07 (4.57 – 94.00)
(-)-α-Bisabolol	0.0 ± 0.0	2.3 ± 1.5	6.0 ± 2.0	13.7 ± 0.6	25.7 ± 1.2	39.7 ± 0.6	54.7 ± 0.6	55.7 ± 0.6	50.47 ± 2.38 (0.28 – 100.60)
Methyleugenol	0.0 ± 0.0	7.2 ± 1.2	10.3 ± 1.5	24.3 ± 0.6	53.3 ± 1.5	83.0 ± 2.6	97.0 ± 1.0	100 ± 0.0	0.51 ± 0.03 (0.37 – 0.64)
Propoxur	0.0 ± 0.0	6.0 ± 2.0	9.3 ± 1.5	12.7 ± 1.5	24.7 ± 0.6	43.3 1.5	77.3 1.2	81.0 1.0	5.13 ± 0.62 (3.42 – 6.77)

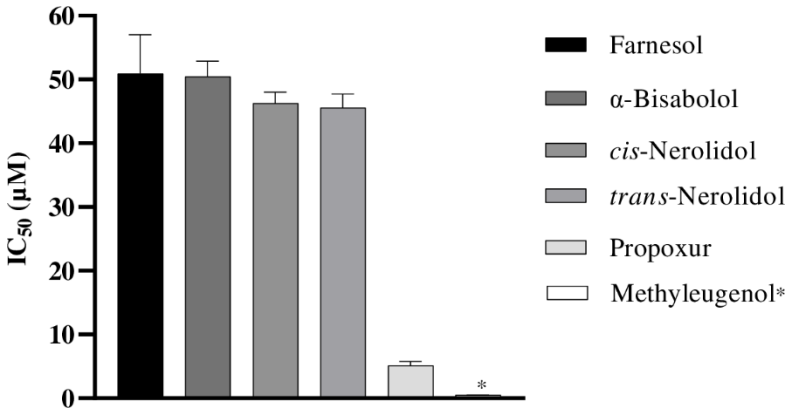


Fig 2: The IC_{50}-based comparison of the egg hatching inhibitory potential of EOCs.

In order to rationalize the potent inhibitory activity of methyleugenol and a positive control, propoxur, their chemical structures were assessed for similarities. Interestingly, methyleugenol which was the most active compound against *Anopheles* egg hatching, had remarkable structural similarity to propoxur where both had an 1,2-dimethoxybenze moiety (Figure 3).

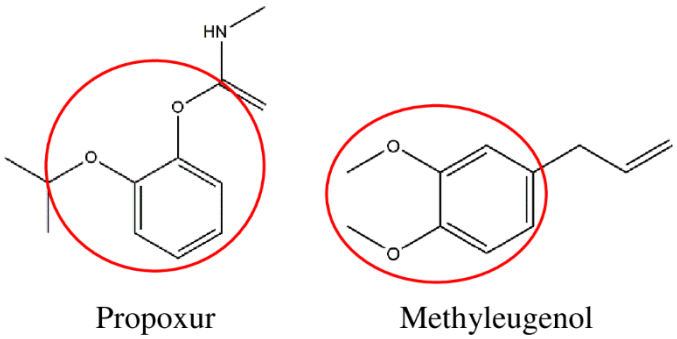


Fig 3: The structural comparison of methyleugenol and propoxur, a standard insecticide. These molecules share a 1,2-dimethoxybenze moiety (circled).

3.2. Larvicidal activity

Following assessment of the EOCs activity against the *Anopheles* egg stage, the sensitivity of the next stage of *Anopheles* life cycle, larva, were assessed. Unlike the egg stage where only one EOC, methyleugenol, showed considerable activity, all the assessed test compounds exhibited the larvicidal activity (Table 2). Most active were farnesol and (-)-α-bisabolol with similar LC_{50} values of 0.05 and 0.07 µM, respectively (paired t-test: $P = 0.1835$; t-value: 2.00; df: 2) that were significantly lower than that of propoxur (7.71 µM) (one-way ANOVA: $P < 0.0001$). The two geometric isomers, *cis*-nerolidol and *trans*-nerolidol, showed similar LC_{50} values (1.14 and 1.16, respectively; paired t-test: $P = 0.2952$; t-value: 2.00; df: 1) that were lower than that of propoxur (one-way ANOVA: $P < 0.0001$). The phenylpropanoid

methyleugenol had the LC_{50} of 2.37 µM and was still more active than the positive control, propoxur (one-way ANOVA: P <0.0001). All EOCs attained LC_{50} values less than 10 µM indicating that the *Anopheles* larvae were sensitive to these lipophilic EOCs (Indrayanto et al. 2021).

Table 2: Concentration-dependent larval lethality of the assessed EOCs compared to propoxur.

		Mean mortality ± SD (µM)							**Lethal concentrations**	
Compound	**Negative control (DMSO)**	**Concentration (µM)** **0.0005**	**0.005**	**0.05**	**0.5**	**5**	**50**	**500**	**LC_{50}** (95% CI limits)	**LC_{99}** (95% CI limits)
cis-Nerolidol	0.0 ± 0.0	2.0 ± 2.7	6.0 ± 2.2	14.0 ± 2.7	41.0 ± 2.7	57.0± 2.7	87.0 ± 2.7	100.0 ± 0.0	1.14 (0.49 – 2.72)	2611.42 (457.09 – 41920.92)
trans-Nerolidol	0.0 ± 0.0	3.0 ± 2.7	7.0 ± 2.7	24.0 ± 4.2	39.0 ± 4.2	55.0 ± 3.5	84.0 ± 4.2	100.0 ± 0.0	1.16 (0.47 – 2.98)	6636.48 (935.71 – 154249.22)
Farnesol	0.0 ± 0.0	6.0 ± 2.2	27.0 ± 2.7	55.0± 3.5	77.0 ± 2.7	87.0 ± 2.7	100.0 ± 0.0	100.0 ± 0.0	0.05 (0.03– 0.07)	94.31 (37.95 – 299.62)
(-)-α-Bisabolol	0.0 ± 0.0	6.0 ± 2.2	24.0 ± 4.2	53.0 ± 4.5	67.0 ± 2.7	86.0 ± 4.2	100 ± 0.0	100.0 ± 0.0	0.07 (0.03 – 0.13)	168.10 (38.30 – 1542.51)
Methyleugenol	0.0 ± 0.0	1.0 ± 2.2	7.0 ± 2.7	16.0 ± 2.2	28.0 ± 2.7	46 ± 2.2	82.0 ± 2.7	100 ± 0.0	2.37 (0.83 – 7.50)	7524.50 (837.74 – 389901.55)
Propoxur	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 2.7	7.0 ± 2.7	15.0 ± 3.5	37.0 ± 2.7	67.0 ± 2.7	100 ± 0.0	7.71 (2.54 – 27.55)	8404.41 (915.68 – 765319.58)

3.3. Pupicidal activity

Pupae are known to be a difficult stage for possible vector control implementation as the pupae are enclosed in a hard case and it is a non-feeding stage (Ha et al. 2017). However, this study showed the potential of developing pupicidal bioinsecticides. All assessed EOCs exhibited lower LC_{50} values when compared to a positive control, propoxur. Five sesquiterpene alcohols,

(-)-α-bisabolol (LC_{50} = 0.66 µM), farnesol (LC_{50} = 0.78), *trans*-nerolidol (LC_{50} = 3.06), and *cis*-nerolidol (LC_{50} = 3.95) possessed potent pupicidal activities compared to propoxur (LC_{50} = 39.09 µM) (Table 3). Methyleugenol was the least active EOC with a LC_{50} value of 18.80 µM, however, still more active than propoxur (Table 3).

Table 3: The EOC-induced pupal sensitivity to standardized concentrations compared to propoxur.

		Mean mortality (%)							**Lethal concentrations**	
Compound	**Negative control (DMSO)**	**Concentration ± SD (µM)**							**LC_{50}** (95% CI limits)	**LC_{99}** (95% CI limits)
		0.0005	**0.005**	**0.05**	**0.5**	**5**	**50**	**500**		
trans-Nerolidol	0.0 ± 0.0	3.0 ± 2.7	7.0 ± 2.7	16.0 ± 2.2	33 .0± 2.7	56 .0± 2.2	81 .0 ± 2.2	82 .0 ± 2.7	3.06 (1.85 – 5.16)	98519.33 (25559.16 – 551883.64)
cis-Nerolidol	0.0 ± 0.0	4.0 ± 2.2	8.0 ± 2.7	13.0 ± 2.7	31.0 ± 4.2	52.0 ± 2.7	78.0 ± 2.7	82.0 ± 2.7	3.95 (2.36 – 6.82)	179338.48 (42877.77 – 1125971.45)
Farnesol	0.0 ± 0.0	4.0 ± 2.2	10.0 ± 3.5	20.0 ± 3.5	42.0 ± 2.7	61.0 ± 2.2	91.0 ± 2.2	100.0 ± 0.0	0.78 (0.33 – 1.91)	3056.75 (489.73 - 55276.87)
(-)-α-Bisabolol	0.0 ± 0.0	3.0 ± 2.7	12.0 ± 2.7	22.0 ± 2.7	42.0 ± 2.7	62.0 ± 2.7	94.0 ± 4.2	100.0 ± 0.0	0.66 (0.26 – 1.72)	2189.03 (323.71 – 50709.38)
Methyleugenol	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 2.7	9.0 ± 4.2	20.0 ± 3.5	43.0 ± 2.7	66.0 ± 4.2	69.0 ± 2.2	18.80 (7.85 – 53.56)	517994.53 (47631.50 – 23523323.65)
Propoxur	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 2.7	9.0 ± 4.2	19.0 ± 4.2	34.0 ± 2.2	52.0 ± 2.7	69.0 ± 2.2	39.09 (21.66 – 77.60)	2032171.16 (342509.65 – 22318051.80)

Notably, farnesol and (-)-α-bisabolol were similarly active against the *An. arabiensis* pupae with LC_{50} values of 0.78 and 0.66 µM, respectively (paired t-test: P = 0.1695; t-value: 3.667; df: 1).

3.4. Adulticidal activity

Baseline susceptibility testing against *An. arabiensis* adult females (n = 100) indicated that they were 100% susceptible to the standard WHO propoxur 100 µg/mL test paper; which was in agreement with previous studies (Buxton et al. 2020; Casimiro et al. 2006; Demissew et al. 2022). In contrast, the controls, acetone and OP/C oil observed no mortality 24 hrs after mosquito exposure. The *Anopheles* mortality at each concentration, as well as the calculated lethality (LC_{50} and LC_{99}) values is shown in Table 4. In terms of LC_{50} values, *cis*-nerolidol (LC_{50} = 222.29 µg/mL) and *trans*-nerolidol (LC_{50} = 228.09 µg/mL) were more active than the three other EOCs, namely (-)-α-bisabolol (LC_{50} = 318.39 µg/mL), farnesol (LC_{50} = 316.45 µg/mL), and methyleugenol (LC_{50} = 323.69 µg/mL) (one-way ANOVA: $P < 0.0001$). All EOCs obtained LC_{50} values between 100 and 500 µg/mL indicating moderate activity. The computed LC_{99} values were doubled for comparison with the positive control, propoxur (WHO 2016). Due to its high potency, propoxur was screened at lower concentrations (0.98 – 62.50 µg/mL) to estimate LC_{50} and LC_{99} values (Table 5). The double LC_{99} value of propoxur was 123.48 µg/mL, in agreement to previous studies (Lissenden et al. 2021), and it was approximately 35 times more potent than the EOCs (Table 4). Generally, none of these EOCs were as active as propoxur when comparing either the LC_{50}, LC_{99} or double LC_{99} concentrations (one-way ANOVA: $P < 0.0001$).

Table 4: Concentration-dependent adulticidal activity of EOCs against *An. arabiensis*.

			Mean mortality (%)							Lethal concentrations		
Compound	**No. of *An. arabiensis* females**	**Negative control (acetone)**	**Concentration (µg/mL)**							**LC_{50} (95% CI)**	**LC_{99} (95% CI)**	**2 x LC_{99}**
			31.25	**62.5**	**125**	**250**	**500**	**1000**	**2000**			
cis-Nerolidol	700	0.0 ± 0.0	1.0 ± 2.0	9.0 ± 2.2	20.0 ± 3.5	60.0 ± 2.0	78.0 ± 3.0	98.0 ± 3.0	100.0 ± 0.0	222.29 (198.65 – 248.77)	1573.68 (1237.42 – 2127.25)	3147.36
trans-Nerolidol	701	0.0 ± 0.0	1.0 ± 2.0	9.0 ± 2.2	20.0 ± 3.5	60.0 ± 2.0	78.0 ± 3.0	95.0 ± 1.2	100.0 ± 0.0	228.09 (203.24 – 255.94)	1768.91 (1381.36 – 2409.72)	3537.82
Farnesol	703	0.0 ± 0.0	2.0 ± 3.5	6.0 ± 2.3	12.0 ± 1.0	31.0 ± 2.0	61.0 ± 4.2	99.0 ± 2.0	100.0 ± 0.0	316.45 (217.87 – 471.61)	2289.48 (1197.84 – 8971.87)	4299.98
(-)-α-Bisabolol	702	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 2.3	14.0 ± 3.0	31.0 ± 2.0	61.0 ± 3.8	99.0 ± 2.0	100.0 ± 0.0	318.39 (232.47 – 443.24)	2083.66 (1184.77 – 6238.65)	4167.32
Methyleugenol	700	0.0 ± 0.0	1.0 ± 2.0	6.0 ± 2.3	11.0 ± 2.0	30.0 ± 2.3	61.0 ± 3.4	99.0 ± 2.0	100.0 ± 0.0	323.69 (230.98 – 463.07)	2156.88 (1190.47 – 7128.95)	4313.76

Table 5: Concentration-dependent adulticidal activity of propoxur against *An. arabiensis*.

			Mean mortality (%)							Lethal concentrations		
Positive control	**No. of *An. arabiensis* females**	**Negative control (OP/C oil)**	**Concentration (µg/mL)**							**LC_{50} (95% CI)**	**LC_{99} (95% CI)**	**2 x LC_{99}**
			0.98	**1.95**	**3.91**	**7.81**	**15.63**	**31.25**	**62.50**			
Propoxur	700	0.0 ± 0.0	14.0 ± 4.0	31.0 ± 3.5	57.0 ± 3.8	74.0 ± 2.3	82.0 ± 2.3	98.0 ± 2.3	100.0 ± 0.0	3.57 (3.07 - 4.13)	61.74 (44.31 – 94.42)	123.48

4. Discussion

This study aimed to address the need for insecticides that possess inhibitory activity against multiple stages of *Anopheles*. For the first time, this study shows the multi-targeted potential of essential oil-based insecticides across various life cycle stages of *An. arabiensis*. Of the five EOCs that were screened against all four stages of the *Anopheles* life cycle, the phenylpropanoid methyleugenol showed potent activity against the egg and larva stages, along with moderate activity against the adult stage. The potential of inhibiting *Anopheles* eggs from hatching is crucial for halting the *Anopheles* life cycle at the very first aquatic stage of its lifecycle (Ateyim et al. 2022).

Quantitative structure-activity relationship (QSAR) studies were undertaken to determine if there was a statistical relationship between the structural properties of a molecule and its biological activity (Verma et al. 2010). QSAR is based on the idea that similar structural molecules will potentially have similar biological activities (Sharma et al. 2021). The QSAR of methyleugenol and propoxur should be further investigated by first identifying the biological target in the inhibition of *Anopheles* egg hatching, followed by investigation of their receptor binding profiles. Proteins such as eggshell organizing factor 1 that stimulate eggshell formation and hardening through melanization and sclerotization, are emerging as promising novel targets (Isoe et al. 2019). The *Anopheles* egg is covered by the eggshell that maintains the embryo development environment and protects it from foreign substances. Alteration of the eggshell has been shown to induce embryonic death (Isoe et al. 2019; Malhotra et al. 2000). Using electron microscopy, various small cavities have been identified on the exochorion surface of the eggshell (Malhotra et al. 2000) and it may be possible that smaller molecules such as methyleugenol may gain entry into the egg through these cavities and affect the embryo development. The identified *Anopheles* egg hatching inhibitor, methyleugenol (178.23 g/mol) was the smallest molecules among the assessed EOCs. Notably, propoxur is also a comparatively small molecule (209.24 g/mol). Alternatively, dopa decarboxylase may be a potential target with its role in inducing eggshell hardening through sclerotization, however, inhibition of this enzyme by benserazide, a standard dopa decarboxylase inhibitor, has been shown to have no effect on the egg viability (Monnerat et al. 1999).

All the assessed EOCs showed highly potent larvicidal activities with LC_{50} values less than 5 µM, indicating that the larvae stage was the most vulnerable stage to the EOCs. In doing so, these EOCs have the potential of being integrated into the vector control programs, to support the WHO recommendation of larval source management as an additional measure to prevent

malaria (WHO 2012, 2013). Several studies have shown the benefit of reducing larvae populations. Mpofu et al. (Mpofu et al. 2016) attained approximately 90% reduction in larvae populations in two Sub-Saharan countries, Zimbabwe and Botswana through larviciding intervention. In Kenya, integrated larviciding with the use of insecticide-treated nets against adult mosquitoes substantially reduced the number of new infections (≥70% reduction in new infections); as well as ≥90% larvae reduction with a subsequent 85.9% reduction in adult mosquito abundance (Fillinger et al. 2009).

In a potential breakthrough, this study has identified two sesquiterpene alcohols, farnesol and (-)-α-bisabolol, with both larvicidal and pupicidal activities indicating great potential for their effectiveness in preventing adult mosquito formation. Examination of their chemical structures revealed that the two molecules have a similar chemical formula, $C_{15}H_{26}O$. In fact, it has been shown that (-)-α-bisabolol is produced by the cyclization of farnesol (Figure 4) (de Meireles et al. 2015). This structural similarity may explain the similar potency of farnesol and (-)-α-bisabolol observed in both the larva and pupa stages of *An. arabiensis*. Using the QSAR hypothesis, farnesol and (-)-α-bisabolol may be expected to have similar biological effects and magnitudes of such activities (Sharma et al. 2021). However, molecular modelling and proteomic studies have also suggested that differences in stereochemistry and structural conformation may generate differences in drug-receptor interactions (Brooks et al. 2011; Rants'o et al. 2022).

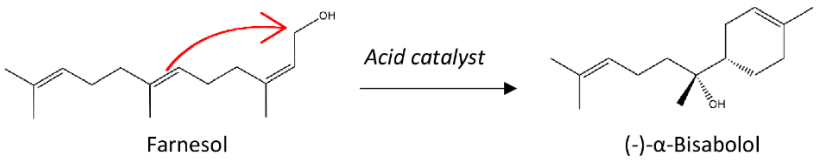


Fig 4: The close structural similarities between farnesol and (-)-α-bisabolol is indicated by the former cyclising to the latter (de Meireles et al. 2015).

Additionally, these two compounds exhibited moderate activity against adult females that are responsible for malaria transmission. These results suggest that farnesol and (-)-α-bisabolol could be able to arrest three stages of the *Anopheles* life cycle, except for the egg stage. (-)-α-Bisabolol has a good safety profile with the United States Food and Drug Administration (FDA) categorizing it under "Generally Regarded as Safe" (GRAS) substances (Kamatou and Viljoen 2010). In general, methyleugenol, (-)-α-bisabolol, and farnesol have shown inhibitory activity against at least two of the three aquatic stages, while the two geometric isomers, *cis*-nerolidol and *trans*-nerolidol were only specific for the larval aquatic stage. Nerolidol is also

classified as a nontoxic substance registered by FDA as a flavouring agent in pharmaceuticals (Chan et al. 2016).

In the adult stage, all EOCs exhibited moderate activity and were all less active than propoxur. The attained double LC_{99} concentrations were comparable to other studies which assessed essential oils for adulticidal effects (Bohounton et al. 2021). This may indicate the potential use of EOCs to enhance the outcome of the interventions against *Anopheles* adult stage. This may include development of nanoparticle formulations to enhance their uptake into the mosquito's systemic circulation (Ibrahim 2019).

This study has shown the potential advantage of using EOCs as *Anopheles* multi-stage targeting bioinsecticides and may have additional benefit if used in combination to overcome resistance as used in many malaria vector programs (WHO 2021a). Identification of insecticides acting at multiple stages of *Anopheles*, especially the early stages, clearly indicates their benefit to be integrated into current malaria vector programs that heavily rely on late stage-acting insecticides (Choi et al. 2019; Koella et al. 2009). Even more beneficial is that these identified larvicides have a natural origin in essential oils, thereby creating an opportunity for cost-effective intervention (Isman 2020a; Isman et al. 2011). To complement the larvicidal properties, the EOCs also possessed moderate adulticidal effects. Therefore, combination of these with current synthetic insecticides may demonstrate inhibitory activities at early stages and enhance activity of late-stage insecticides. The potential to enhance the activity of conventional insecticides, as well as to reverse resistance has been widely reported (Faraone et al. 2015; Gaire et al. 2021; Norris et al. 2019; Waliwitiya et al. 2012). Moreover, the potential of essential oil-based insecticides as alternatives to current insecticides for which global resistance has been reported, has been recommended (Gnankiné and Bassolé 2017; Pavela 2015). Future studies may investigate the potential additive or synergistic effects of the identified EOCs together with conventional insecticides. Moreover, appropriate pharmaceutical formulation of these EOCs for experimental application as bioinsecticides against the various life stages of *An. arabiensis* should be investigated. Nanotechnology is a promising tool for modern formulation, delivery and controlled release of essential oil-based insecticides (Esmaili et al. 2021; Ibrahim 2019; Palermo et al. 2021; Yang et al. 2009). For application in the aquatic stages, formulation of EOCs in nanoemulsions and nanoparticle encapsulation has proven effective, while for adulticidal effects, nanoparticles have also shown compatibility for formulation into sprays, fumigants and granules (Gupta et al. 2022; Mohafrash et al. 2020; Palermo et al. 2021).

5. Conclusion

This study shows the potential of the multi-stage targeted activity of essential oil-based bioinsecticides against *An. arabiensis*. The assessed EOCs were highly active against *Anopheles* aquatic stages. Among the five identified EOCs with larvicidal activity, one of these, methyleugenol, also possessed potent *Anopheles* egg hatching inhibition. In particular, methyleugenol was over ten times more potent in inhibiting egg hatching compared to a commonly used insecticide, propoxur. This study revealed for the first time that the remarkable structural similarity between methyleugenol and propoxur which may explain their observed potent inhibitory effects against *Anopheles* egg hatching. Methyleugenol was also very potent in killing the *Anopheles* larvae. This is an indication that, if applied to the mosquito breeding ponds, methyleugenol may prevent the emergence of *Anopheles* larvae from the egg and in addition, kill larvae that may emerge or in pre-existence. In addition to their potent larvicidal activities, (-)-α-bisabolol and farnesol exhibited potent pupicidal effects. This coordinated activity against *Anopheles* aquatic stages indicates an opportunity to integrate early stage-acting insecticides with current late stage-acting insecticides.

Further, these EOCs also exhibited moderate activity against adult female mosquitoes. Being of natural origin, the identification of these EOCs indicates a potential for cost-effective and eco-friendly insecticides. This study indicates that methyleugenol, (-)-α-bisabolol, farnesol *cis*-nerolidol and *trans*-nerolidol are potent early stage-acting agents against *An. arabiensis.* All five EOCs displayed potent larvicidal activity with moderate adulticidal activity that may offer an auxiliary bioactivity, when integrated with current synthetic insecticides. The findings of this study warrant further investigations into the development of these agents as bioinsecticides against egg, larva and pupa stages of various *Anopheles* vectors.

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CHAPTER 7

The insecticidal activity of essential oil constituents against insecticide resistant *Anopheles* with overexpressed P450 monooxygenases

Following the toxicity analysis of terpenoids against *Anopheles arabiensis*, *An. funestus*, *An. gambiae* and *An. coluzzii* in Chapter 6, the next step was to assess these essential oil constituents against resistant mosquitoes with overexpressed P450 monooxygenases. This chapter presents a manuscript that reports identification of terpenoids with toxicity against pyrethroid resistant *Anopheles* and the impact of overexpressed P450 monooxygenases on the efficiency of select terpenoids. The paper has been published in Parasitology International.

Chapter type: Published ISI paper (Appendix E.1)

Title of manuscript: The insecticidal activity of essential oil constituents against pyrethroid-resistant *Anopheles funestus* (Diptera: Culicidae).

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Conceptualization	✓		✓
Method	✓		
Data analysis	✓		
Writing – original draft	✓		
Writing – review and editing	✓	✓	✓
Supervision		✓	✓
Funding acquisition		✓	✓

Abstract: Malaria vector control relies on the use of insecticides for indoor residual spraying and long-lasting bed nets. However, insecticide resistance to pyrethroids amongst others, has escalated. *Anopheles funestus,* a major African malaria vector, has attained significant levels of resistance to pyrethroids. Overexpressed P450 monooxygenases have been previously identified in pyrethroid resistant *An. funestus.* The escalating resistance against conventional insecticides signals an urgent need for identification of novel insecticides. Essential oils have gained recognition as promising sources of alternative natural insecticides. This study investigated six essential oil constituents, farnesol, (-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, methyleugenol, santalol (α and β isomers) and one essential oil; sandalwood oil, for the adulticidal effects against pyrethroid-resistant *An. funestus*. The susceptibility against these terpenoids were evaluated on both pyrethroid-susceptible and resistant *An. funestus*. Furthermore, the presence of overexpressed monooxygenases in resistant *An. funestus* was confirmed. Results showed that both the pyrethroid-susceptible and resistant *An. funestus* were susceptible to three EOCs; *cis*-nerolidol, *trans*-nerolidol and methyleugenol. On the other hand, the pyrethroid-resistant *An. funestus* survived exposure to both farnesol and (-)-α-bisabolol. This study however does not show any direct association of the overexpressed *Anopheles* monooxygenases and the efficacy of farnesol and (-)-α-bisabolol. The enhanced activity of these terpenoids against resistant *An. funestus* that has been pre-exposed to a synergist, piperonyl butoxide, suggests their potential effectiveness in combination with monooxygenase inhibitors. This study proposes that *cis*-nerolidol, *trans*-nerolidol and methyleugenol are potential agents for further investigation as novel bioinsecticides against pyrethroid-resistant *An. funestus*.

Keywords: Malaria, insecticides, terpenoids, monooxygenases, synergy

1. Introduction

Malaria is a plasmodial disease transmitted through infected female mosquito bites [1]. There were approximately 241 million malaria cases worldwide in 2020. In addition, malaria-related deaths were estimated at 627 000, 80% of which were children under the age of five. Ninety-five (95) percent of these cases and 96% deaths were attributed to the African region [2]. Anopheline mosquitoes are responsible for transmitting the malaria parasites. Among these, *Anopheles funestus* Giles (Diptera: Culicidae) is a major malaria vector, especially in Africa [3, 4].

Insecticides are the core interventions used for malaria vector control. Pyrethroid-based insecticides have a long history of use in the malaria vector control programmes. Historically, pyrethrum extracted from the flowers of *Chrysanthemum cinerariifolium,* was used against indoor *Anopheles* mosquitoes [5]. Currently, pyrethroid insecticides are still utilized in the form of insecticide-treated nets as well as indoor residual sprays [6-8]. Pyrethroids are the only insecticides recommended by World Health Organization (WHO) for use in the insecticide-treated nets [8, 9]. Malaria cases have however risen despite the use of insecticides and is most likely due to the increased level of insecticide resistance [7]. Insecticide resistance have been reported in all the main African malaria vector species, including *An. funestus*. The pyrethroid metabolic resistance mechanisms are well studied in this species since it was first described by Hargreaves et al. [10].

Biochemical and synergist bioassays have suggested the elevated levels of detoxifying monooxygenase as the major mechanism related to the pyrethroid resistance in *An. funestus* populations in Sub-Saharan Africa [7, 11]. Specific P450 genes like *CYP6P9a* and *CYP6P9b* are usually upregulated in pyrethroid-resistant *An. funestus* [12-15]. These overexpressed genes convey the cross-resistance among the type I and type II pyrethroids, invalidating any possible substitutions in these groups [16, 17]. The same mechanism also results in cross resistance to carbamate resistance [7, 18]. The importance of the monooxygenases in detoxifying insecticides was confirmed using piperonyl butoxide (PBO), a monooxygenase (P450) inhibitor. PBO has also been incorporated into bed nets and these are currently being evaluated by the WHO [8, 19, 20]. In addition, biochemical assays are used to identify the elevated enzyme activities responsible for conveying resistance [21, 22]. This is achieved by measuring the levels of heme-containing P450 enzymes in mosquito populations. The peroxidase activity of the inherent heme group is directly proportional to the P450 levels, hence useful in

establishing enzyme activity differences between resistant and susceptible colonies [22]. Brogdon et al. [22] highlighted the importance of evaluating the monooxygenase enzyme levels upon detection of resistance in disease vectors. This assay has previously been used to complement synergist studies in confirming the P450-based resistance mechanism in *An. gambiae* [23].

Insecticide resistance reports have significantly increased and have signalled the urgency for the identification of novel insecticides to combat malaria. Plants often naturally biosynthesize constituents to protect themselves against insect pests, including mosquitoes [24, 25]. Due to this, plant constituents and essential oils serve as potential sources of effective chemical agents against insect species including *Anopheles* mosquitoes. The essential oils, hydrophobic extracts from aromatic plants, along with their constituents, have attracted recognition as potential alternative insecticides [9, 25, 26]. Isman [27] stated that the current trend shows great promise towards manufacturing, approval and commercialization of botanical insecticides.

The essential oil constituents (EOCs) are mainly terpenoids and phenylpropanoids [28]. The sesquiterpene alcohols farnesol and (-)-α-bisabolol are naturally found as major phytoconstituents in the essential oils of various plants including *Rosa x. damascene* Mill., *Polianthes tuberosa* L., *Matricaria chamomilla* L., *Cymbopogon citratus*, *Cymbopogon nardus* [29, 30]. The *C. citratus* (lemongrass) and *C. nardus* (citronella) essential oils have shown adulticidal effects against *An. gambiae [31].* Nerolidol geometric isomers, cis-nerolidol and trans-nerolidol, are *also* sesquiterpene alcohols that have been identified as main constituents in the essential oils of *Piper* species such as *P. claussenianum* (Miq.), *P. umbellatum, P. nigrum* and *P. capense* [32, 33]. Nerolidol has previously shown larvicidal activity against *An. sinensis* at a low LD_{50} value of 20.84 mg/L [34]. Similarly, methyleugenol, a phenylpropanoid, has been identified in the essential oils of more than 20 plant species including *C. nardus, Ocimum basilicum* and *Colocasia antiquorum* [35, 36] and while that of *C. nardus* has been shown to exert the adulticidal activity against *An. gambiae,* that of *O. basilicum* (basil) has exhibited larvicidal effects against *An. subpictus* [31, 36]. Additionally, sandalwood oil is an essential oil of medicinal interest with diverse biological activities including insecticidal effects. It is obtained from *Santalum album* L. [37, 38]. Interestingly, sandalwood oil has shown larvicidal and adulticidal effects against *An. stephensi* and *An. gambiae*, respectively [31, 39]. Major phytoconstituents in sandalwood oil are the sesquiterpene alcohols α- and β-santalol isomers [40, 41]. This study aimed to assess farnesol,

(-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, methyleugenol, sandalwood oil, and santalol (α and β) for the adulticidal effects against pyrethroid-resistant *An. funestus*.

2. Materials and methods

Two laboratory reared colonies of *An. funestus*, namely FANG (insecticide susceptible) and FUMOZ-R (pyrethroid-resistant) were used in this study, and these were maintained under standard insectary conditions as reported by Hunt et al. [4] and Zengenene et al. [42]. All EOCs including santalol (76% α-santalol and 6% β-santalol; GC-MS; Catalog No. 75831) and the EO sandalwood oil, deltamethrin, cytochrome C from equine heart and PBO were purchased from Sigma Aldrich (South Africa) with >90% purity. PBO was further diluted to 4% v/v in olive oil:acetone (50:50) solvent [7].

2.1 P450 biochemical analysis

To confirm that increased P450 activity was still present in FUMOZ-R colony as per previous studies [4, 7], the biochemical analyses were conducted [23]. The unexposed samples from both FANG and FUMOZ-R were anesthetized on ice and homogenized in sodium phosphate buffer (pH 7.2) [22]. The protein content was assessed using the standard Lowry protein assay [43]. The P450/monooxygenase assay was conducted with slight modifications as reported by Brogdon et al. [22] as well as Penilla et al. [21]. The mosquito homogenate (20 µL) was added to 80 µL sodium phosphate buffer (pH 7.2). The freshly prepared substrate, 3,3′,5,5′-tetramethylbenzidine (TMB; 2% (w/v) in absolute methanol), was diluted in 18 mL of 0.25M sodium acetate buffer (pH 5.0) before 200 µL of this was added to the homogenate incubation mixture. The reaction was then initiated by the addition of 25 µL of 3% (v/v) hydrogen peroxide. Standard curves for TMB peroxidation were assessed with equine heart cytochrome C oxidase in place of mosquito homogenate. Cytochrome C oxidase is an electron-carrying protein found in the mitochondria and is bound to two heme groups and carries out cellular oxidation biological processes making it a suitable positive control for P450 peroxidase activity [21, 44, 45]. Absorbances were read at 650 nm using the Multiskan Go^{TM} microplate spectrophotometer (Thermo Fisher Scientific) at 5 min intervals. The obtained absorbance values were then compared with a standard curve of the known concentrations of cytochrome C oxidase and values reported as equivalent units of cytochrome P450/mg protein. The relative P450 activity was calculated using the P450 absorbance/mg protein.

2.2 WHO bioassays

Susceptibility tests were conducted using the WHO standard bioassay [46]. Batches of 25-30 non-blood fed female mosquitoes aged 2-5 days were assessed in WHO exposure tubes. The mosquitoes were exposed to WHO 0.05% deltamethrin test papers for 1 hr after which they were transferred into the holding tubes. The control batch was exposed to the WHO pyrethroid control paper. The knockdown was then recorded, and mosquitoes were provided with 10% (w/v) sugar solution. The final mortality was recorded 24 hours post-exposure. Mortalities on FANG and FUMOZ-R were compared. For each of the experiments, four replicates were conducted. The testing room conditions were maintained at 27 °C and ≥75% relative humidity, as well as a 12-hr light and 12-hr dark photoperiod. Mortality rate was calculated using the formula: % Mortality = (Mean number of dead mosquitoes / Mean number of treated mosquitoes) x 100 [46, 47]. Furthermore, pyrethroid resistance was identified using the WHO criteria [46].

2.2.1 Essential oil constituent susceptibility

Whatman Grade 1 (12 × 15 cm^2) filter paper was impregnated with 2 mL of the EOC diluted in acetone [9]. The mosquitocidal activities were assessed at seven EOC concentrations spanning a range of 2% to 0.0313% with 2-fold dilutions [48, 49]. Deltamethrin (0.05%) was used as a reference insecticide paper and the EOC control included Whatman filter papers impregnated with 2 mL HPLC grade acetone. The standard susceptibility assay was conducted as done for the reference insecticides with four replicates. Both FANG and FUMOZ-R colonies were assessed, and 24-hr mortality rates compared. The resultant data afforded the calculation of the mean lethal concentrations for 50% and 99% mortality (LC_{50} and LC_{99}, respectively) with 95% confidence intervals by probit analysis using the SPSS Statistics 27.0 (IBM, New York, USA). The diagnostic concentration for susceptibility tests was defined as twice the LC_{99} obtained with the susceptible colony, FANG, in line with WHO guidelines [46, 50]. These EOC susceptibility assays were conducted following the same procedure as for deltamethrin susceptibility.

2.3 Synergist bioassay

The synergist assay was carried out to determine the involvement of metabolic P450 resistance against deltamethrin and identified EOCs [23]. For this assay, the mosquitoes were pre-exposed to 4% PBO for an hour before being exposed to 0.05% deltamethrin or EOC at the determined diagnostic dose. Synergized batches were assessed concurrently with nonsynergized cohorts

for direct comparison. The control assays included the exposure to the synergist alone, exposure to the WHO pyrethroid control paper, to acetone and to olive oil:acetone (50:50). Four test replicates were generated, and the results were discarded and repeated should any of the controls induce >20% mortality [48]. The potentiation of the efficacy of deltamethrin or EOC in the presence of PBO was assessed.

Additionally, the synergistic relationship between EOCs and PBO was characterized using the co-toxicity factor calculated with the following equation [51, 52]:

$$Co - toxicity\ factor = \frac{(observed\ \%\ mortality - expected\ \%\ mortality)}{(expected\ \%\ mortality)} \times 100$$

Where expected mortality is the sum of percentage mortalities obtained by PBO alone and EOC alone. The co-toxicity factor values less than −20 indicated an antagonistic relationship, values between −20 and 20 specified an additive interaction, and those greater than 20 proposed synergism [52].

3. Results

3.1 Pyrethroid susceptibility status of Anopheles funestus

Prior to the analysis with EOCs, pyrethroid susceptibility of the two *An. funestus* colonies, FANG and FUMOZ-R, was confirmed. Results confirmed that FANG was fully susceptible to deltamethrin, while FUMOZ-R was resistant with 29.5% mortality 24 hours post exposure (Table 1). This mortality difference was statistically significant [53] between the two colonies (unpaired *t*-test: $P < 0.0001$; t-value: 17.38; df: 6) and in agreement with previous studies [4, 7, 54].

Table 1: WHO susceptibility analysis of *An. funestus* colonies, FANG and FUMOZ-R, to % deltamethrin

An. funestus **colony (n)**	**Insecticide**	**24 hr % Mortality ± SD**
FANG (104)	Deltamethrin	100.0 ± 0.0
FANG (100)	Pyrethroid control	0.0 ± 0.0
FUMOZ-R (100)	Deltamethrin	29.5 ± 3.5
FUMOZ-R (102)	Pyrethroid control	1.0 ± 1.0

3.2 *Anopheles funestus monooxygenase levels*

The FUMOZ-R resistance has been associated with elevated P450 activity [4, 7, 54], this increased activity was assessed using the monooxygenase assay on the current colonies, FANG and FUMOZ-R [21]. Before this, the linearity of substrate peroxidation and reaction time were optimized. With a standard, cytochrome C oxidase, a good linearity ($R^2 = 0.986$) of TMB

peroxidation was obtained (Figure 1). In addition, the linearity was maintained for up to 60 min at concentrations up to 33.3 µM (R^2 = 0.985), above which linearity was lost (Figure 1). As a result, the monooxygenase activity for FANG and FUMOZ-R was assessed for a period of 60 min, at 5 min intervals.

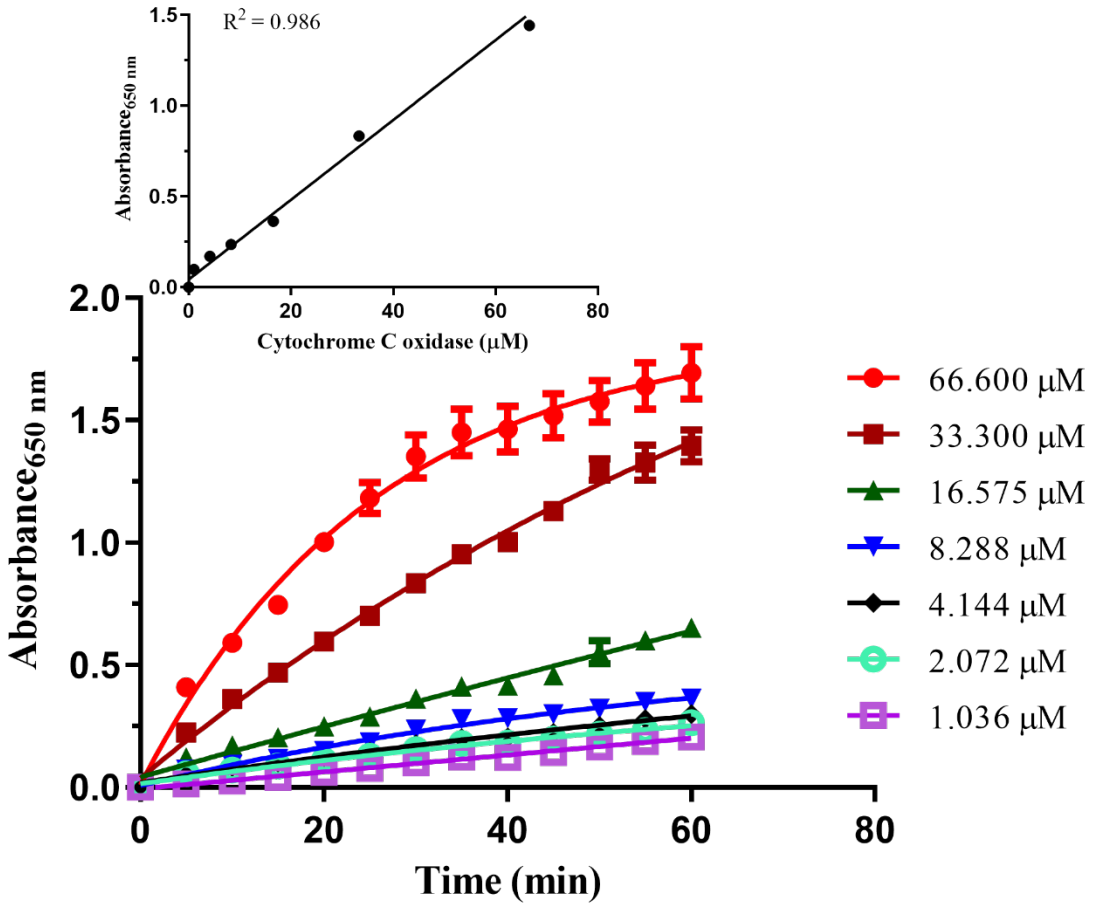


Figure 1: Cytochrome C oxidase standard curve (inset graph) used to determine the linearity of TMB substrate peroxidation (main graph) (cytochrome C concentration: 1.036 to 66.600 µM).

The monooxygenase levels on FANG were significantly lower than that on FUMOZ-R (unpaired *t*-test: $P < 0.0001$; t-value: 95.95; df: 4) (Figure 2). In addition, the P450 activity on FUMOZ-R samples was significantly higher (~ 4-fold), in comparison to FANG (unpaired *t*-test: $P < 0.0001$; t-value: 70.49; df: 4) (Figure 2).

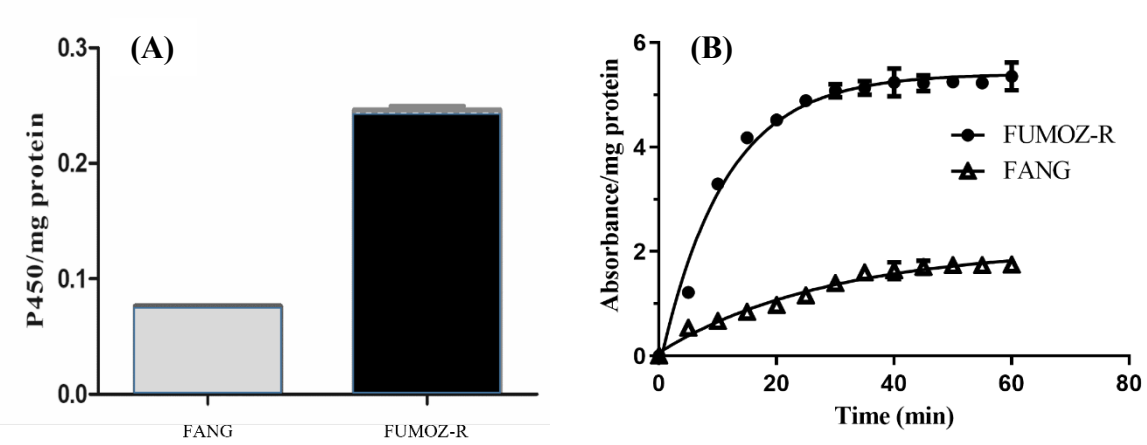


Figure 2: Monooxygenase activity in *An. funestus*. **(A)** Comparison of cytochrome P450/mg protein levels in *An. funestus* FUMOZ-R and *An. funestus* FANG**; (B)** Comparison of the P450 activity in *An. funestus* FUMOZ-R and *An. funestus* FANG.

3.3 Anopheles funestus terpenoid susceptibility

The two colonies, FANG and FUMOZ-R were subsequently used to evaluate *An. funestus* susceptibility to the EOCs. The EOCs were screened at concentrations 0.03, 0.06, 0.13, 0.25, 0.50, 1.00, and 2.00% which span from 30 to 2000 µg/mL. The LC_{99} value for each EOC against the susceptible strain, FANG, was noted [46]. This concentration was then doubled and compared to that of a positive control, deltamethrin. The nerolidol isomers, *cis*-nerolidol and *trans*-nerolidol obtained the lowest LC_{50} values (lethal concentrations at which 50% female mosquitoes died) of 0.14% (140 µg/mL) and 0.17% (170 µg/mL) followed by methyleugenol at 0.24% (240 µg/mL). These along with (-)-α-bisabolol (LC_{50}: 320 µg/mL), santalol (LC_{50}: 350 µg/mL) and sandalwood oil (LC_{50}: 360 µg/mL) showed LC_{50} values in the moderate *Anopheles* toxicity range (100 – 500 µg/mL) while farnesol exhibited low insecticidal activity (LC_{50}: >500 µg/mL) [55, 56]. Considering the concentrations at which 99% female mosquitoes died (LC_{99} values) and the estimated 2x LC_{99} concentrations, three EOCs *cis*-nerolidol, *trans*-nerolidol, and methyleugenol consistently showed relatively lower values, however, higher than that of deltamethrin (Table 2; one-way ANOVA: $P = 0.0001$). In correlation to previous studies, a positive control deltamethrin obtained the LC_{50} and LC_{99} concentrations of 0.007% and 0.05% [57, 58].

Table 2: The lethality concentrations of EOCs against pyrethroid-susceptible *An. funestus.*

	***An. funestus* (FANG)**		
Essential oil constituent	**LC_{50} (%) (95% CI)**	**LC_{99} (%) (95% CI)**	**2x LC_{99} concentration (%)**
cis-Nerolidol	0.14 (0.08-0.23)	1.65 (0.71-12.42)	3.30
trans-Nerolidol	0.17 (0.10-0.29)	1.92 (0.79-18.97)	3.84
Methyleugenol	0.24 (0.15-0.37)	2.47 (1.16-12.32)	4.94
Farnesol	0.74 (0.54-1.11)	30.31 (11.88-146.13)	60.62
(-)-α-Bisabolol	0.32 (0.27-0.37)	8.69 (5.75-14.69)	17.38
Santalol (α and β)	0.35 (0.30-0.41)	9.83 (6.34-17.28)	19.66
Sandalwood oil	0.36 (0.27-0.50)	8.90 (4.35-28.20)	17.80
Deltamethrin	0.007 (0.005-0.008)	0.05 (0.04 – 0.10)	0.10

Following this, the baseline susceptibility of *An. funestus* to the terpenoids was evaluated by screening them at 2x LC_{99} concentrations against both FANG and FUMOZ-R and compared with deltamethrin [59]. However, farnesol, (-)-α-bisabolol, santalol (α and β), and sandalwood oil could not be screened at the double LC_{99} concentrations due to requirements for high concentrations at which solubility was lost. Since the double LC_{50} values have also been used in previous studies [60], these were used to assess the baseline activity of farnesol, (-)-α-bisabolol, santalol (α and β), and sandalwood oil [59]. Interestingly, *cis*-nerolidol, *trans*-nerolidol, and methyleugenol induced 100% mortality in both FANG and FUMOZ-R, where no less than 100 females were assessed against each EOC (Figure 3a). Differently, deltamethrin showed a significantly higher mortality in the susceptible colony (100%) than the resistant colony (29.5%) (unpaired *t*-test: $P = 0.0013$; t-value: 28.20; df: 2) in agreement to the literature [4, 7, 57].

Similar to deltamethrin, there was a significant difference in the susceptibility of FANG and FUMOZ-R to farnesol, (-)-α-bisabolol, santalol (α and β) and sandalwood oil (Figure 3b). However, deltamethrin could reach 100% 24-hr mortality against FANG (n=104) at double LC_{99} concentration, while none of these EOCs reached this mortality rate. On the other hand, this pyrethroid could only attain 21.8% against FUMOZ-R at this concentration (n=106) (unpaired *t*-test: $P < 0.0001$; t-value: 54.59; df: 6). Farnesol induced 65% (n = 103) mortality after 24 hours in the pyrethroid susceptible colony, FANG, and this was significantly different from the 6% (n = 106) mortality recorded for the pyrethroid resistant colony, FUMOZ-R (unpaired *t*-test: $P < 0.0001$; t-value: 22.90; df: 6). Similarly, for (-)-α-bisabolol, 78.3% (n = 100) mortality was recorded for FANG 24 hours after exposure, and this was statistically different from the 39.5% (n = 108) mortality recorded for FUMOZ-R (unpaired *t*-test: $P < 0.0001$; t-value: 9.80; df: 6). Santalol (α and β) showed 76.5% mortality against FANG versus

28% FUMOZ-R mortality (unpaired *t*-test: $P < 0.0001$; t-value: 9.32; df: 6). Likewise, sandalwood oil recorded 79% FANG mortality and only 27% mortality against FUMOZ-R (unpaired *t*-test: $P = 0.0003$; t-value: 7.40; df: 6). Moreover, the activity levels of sandalwood oil and its main constituent, santalol (α and β), were compared. These were similar for FANG (76.5; santalol (α and β) and 79%; sandalwood oil) (unpaired *t*-test: $P = 0.7347$; t-value: 0.36; df: 6) and they showed no statistical difference in their intensity against FUMOZ-R (unpaired *t*-test: $P = 0.8533$; t-value: 0.19; df: 6).

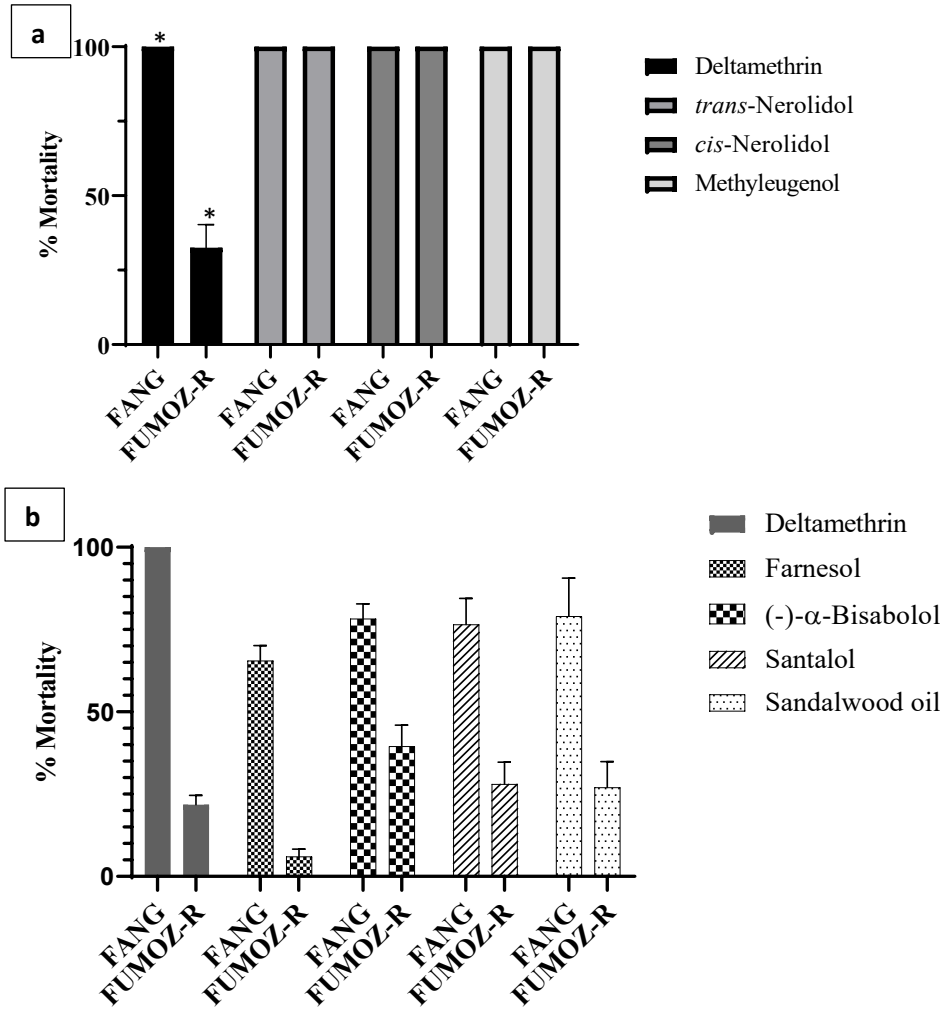


Figure 3: The susceptibility status of FANG and FUMOZ-R against three most active EOCs assessed at their double LC_{99} concentrations (a). The comparison of FANG and FUMOZ-R susceptibility to three less active EOCs and one EO (b). The terpenoid-induced mortalities were compared to deltamethrin. *Only deltamethrin in (a) showed significantly different mortality rates between FANG and FUMOZ-R strains ($p<0.05$) while all test compounds in (b) showed significant difference in the mortality against FANG versus FUMOZ-R ($p<0.05$) [53].

Given the low activity observed for farnesol, (-)-α-bisabolol, santalol (α and β), and sandalwood oil against FUMOZ-R, where they respectively reached 6%, 39.5%, 28%, and 27% mortality at baseline screening (Figure 3b), these compounds could not generate accurate LC_{50} and LC_{99} values. However, the LC_{50} and LC_{99} values of three most active EOCs were determined against FUMOZ-R (Table 2). Similar to their screening against FANG (Table 2), these EOCs still maintained moderate toxicity (LC_{50} range between 100 µg/mL and 500 µg/mL) against FUMOZ-R (Table 3).

Table 3: Activities of select EOCs against *An. funestus* FUMOZ-R

	***An. funestus* (FUMOZ-R)**	
Essential oil constituent	**LC_{50} (%) (95% CI)**	**LC_{99} (%) (95% CI)**
cis-Nerolidol	0.23 (0.16-0.34)	2.09 (1.08-7.64)
trans-Nerolidol	0.26 (0.19-0.37)	2.18 (1.19-6.76)
Methyleugenol	0.27 (0.23-0.31)	5.03 (3.57-7.74)
Deltamethrin	0.14 (0.11-0.18)	1.74 (1.06-3.75)

To confirm similar effects against pyrethroid-susceptible and resistant *An. funestus*, it was worth comparing the LC_{50} and LC_{99} values of these three active terpenoids obtained against FUMOZ-R to those acquired in screening against FANG. *cis*-Nerolidol had similar LC_{50} values against these two colonies (Table 4) (unpaired *t*-test: $P = 0.7646$; t-value: 0.30; df: 198). Comparably, *trans*-nerolidol as well showed similar LC_{50} values against FANG and FUMOZ-R (unpaired *t*-test: $P = 0.7711$; t-value: 0.29; df: 198). Finally, methyleugenol also attained similar LC_{50} values against the susceptible and resistant colonies (unpaired *t*-test: $P = 0.8403$; t-value: 0.20; df: 198). Notably, deltamethrin displayed significantly higher LC_{50} values against FOMOZ-R, in comparison to the susceptible colony (unpaired *t*-test: $P < 0.0001$; t-value: 0.5832; df: 198). In terms of LC99, deltamethrin still had significant decline in potency against FUMOZ-R (LC99=1.74) in comparison to FANG (LC99=0.05) (unpaired *t*-test: $P < 0.0001$; t-value: 7.41; df: 198) (Table 4). These potency differences suggested that its activity has dramatically been reduced in pyrethroid resistant *An. funestus* (FUMOZ-R).

In contrast, *cis*-nerolidol showed similar LC_{99} values between FANG (1.65%) and FUMOZ-R (2.09%) (unpaired *t*-test: $P = 0.1443$; t-value: 1.466; df: 198) (Table 4). In the same way, *trans*-nerolidol obtained LC_{99} values with nonsignificant differences against FANG (1.92%) and FUMOZ-R (2.18%) (unpaired *t*-test: $P = 0.4009$; t-value: 0.8418; df: 198). Nevertheless, methyleugenol had different LC_{99} values between the two strains (LC_{99} (FANG) = 2.47 and LC_{99} (FUMOZ-R) = 5.03) (unpaired *t*-test: $P < 0.0001$; t-value: 17.22; df: 198).

Table 4: Comparison of terpenoid potencies between pyrethroid-susceptible and resistant *An. funestus* strains

	Lethality concentrations					
	LC_{50} (%) (95% CI)			LC_{99} (%) (95% CI)		
Compound	**(FANG)**	**(FUMOZ-R)**	***p*-value**	**(FANG) LC_{99}**	**FUMOZ-R)**	***p*-value**
cis-Nerolidol	0.14 (0.08-0.23)	0.23 (0.16-0.34)	0.7646	1.65 (0.71-12.42)	2.09 (1.08-7.64)	0.1443
trans-Nerolidol	0.17 (0.10-0.29)	0.26 (0.19-0.37)	0.7711	1.92 (0.79-18.97)	2.18 (1.19-6.76)	0.4009
Methyleugenol	0.24 (0.15-0.37)	0.27 (0.23-0.31)	0.8403	2.47 (1.16-12.32)	5.03 (3.57-7.74)	< 0.0001*
Deltamethrin	0.007 (0.005-0.008)	0.14 (0.11-0.18)	< 0.0001*	0.05 (0.04 – 0.10)	1.74 (1.06-3.75)	< 0.0001*

*Statistically significant difference (p<0.05) [53].

3.4 Synergist assays

Since they generally showed similar activity levels in FANG and FUMOZ-R (Figure 3a and Table 4), the three most active EOCs, *cis*-nerolidol, *trans*-nerolidol and methyleugenol were not included in the synergy study. On the other hand, due to the 24-hr mortality profile that showed statistically significant differences between the two colonies when exposed to deltamethrin (Figure 3b), the role of P450 monooxygenases was investigated. The colonies are no longer under pyrethroid selection pressure [4], therefore the role of monooxygenases was confirmed when PBO pre-exposed FUMOZ-R *An. funestus* females restored deltamethrin susceptibility (Table 3) in line with recent studies [11, 61]. In a similar fashion, the impact of synergising monooxygenases prior to farnesol and (-)-α-bisabolol exposure on pyrethroid resistant *An. funestus* was investigated. Their assessment in the synergist assay revealed that, in the presence of a P450 inhibitor, PBO, 100% mortalities were recorded 24 hours after exposure for both farnesol and (-)-α-bisabolol (Table 3). On the other hand, the combination of either santalol (α and β) or sandalwood oil with PBO did not result in a 100% 24-hr mortality in FUMOZ-R, despite having increased mortality rates (Table 3). This mortality increase was not statistically significant for santalol (α and β) (unpaired *t*-test: $P = 0.3480$; t-value: 1.02; df: 6) but significant in the case of sandalwood oil (unpaired *t*-test: $P = 0.0167$; t-value: 3.29; df: 6).

Table 5: Evaluation of *An. funestus* FUMOZ-R susceptibility to deltamethrin and EOCs with and without a synergist

Deltamethrin synergy		
***An. funestus* colony (n)**	**Insecticide**	**24 hr Mortality (% ± SD)**
FUMOZ-R (106)	Deltamethrin	21.8 ± 2.9
FUMOZ-R (101)	PBO	0.0 ± 0.0
FUMOZ-R (101)	Deltamethrin + PBO	100 ± 0.0
FUMOZ-R (104)	Pyrethroid control	1.0 ± 1.0
FUMOZ-R (101)	PBO control	0.0 ± 0.0
EOCs synergy		
FUMOZ-R (106)	Farnesol	6.0 ± 2.3
FUMOZ-R (106)	Farnesol + PBO	100 ± 2.0
FUMOZ-R (100)	(-)-α-Bisabolol	39.5 ± 0.7
FUMOZ-R (106)	(-)-α-Bisabolol + PBO	100 ± 0.0
FUMOZ-R (101)	Santalol (α and β)	28.0 ± 3.4
FUMOZ-R (101)	Santalol (α and β) + PBO	37.3 ± 6.4
FUMOZ-R (100)	Sandalwood oil	27 ± 3.9
FUMOZ-R (100)	Sandalwood oil + PBO	42.5 ± 5.2
FUMOZ-R (101)	PBO	0.0 ± 0.0
FUMOZ-R (102)	Acetone	0.0 ± 0.0
FUMOZ-R (99)	PBO control*	0.0 ± 0.0

All the control tests including PBO alone did not cause any mortality.

*Olive oil:acetone (50:50).

In addition to the results from the synergist assay, the calculated co-toxicity factor [52] confirmed that deltamethrin, farnesol and (-)-α-bisabolol exhibit a synergist character when in combination with PBO (Table 5). Moreover, synergism was still responsible for the 10 – 15-fold increase in FUMOZ-R toxicity when santalol (α and β) or sandalwood oil were combined with PBO (Table 5). The mortality levels of synergized santalol (α and β) (37.3%) and sandalwood oil (42.5%) were still statistically similar (unpaired *t*-test: $P = 0.8358$; t-value: 0.24; df: 2).

Table 6: Co-toxicity effects of four select EOCs in combination with PBO against *An. funestus* FUMOZ-R.

		% Mortality					
Synergist	**EOC**	**Synergist alone**	**EOC alone**	**Expected**	**Observed**	**Co-toxicity factor**	**Co-toxicity type**
PBO	Farnesol	1.0	6.0	7.0	99.0	1314.3	Synergism
PBO	(-)-α-Bisabolol	1.0	39.5	40.5	100.0	146.9	Synergism
PBO	Santalol (α and β)	0.0	28.0	28.0	38.0	35.7	Synergism
PBO	Sandalwood oil	0.0	27.0	27.0	42.5	57.4	Synergism
PBO	Deltamethrin	0.0	21.8	21.8	100.0	358.7	Synergism

4. Discussion

This study reports for the first time the potential insecticidal activity of EOCs on pyrethroid-resistant *An. funestus*. The current and increasing insecticide resistance has rendered many conventional insecticides ineffective and is a threat for vector control programs. Resistance to pyrethroids, the only insecticides used for insecticide-treated nets, poses a major challenge in the future of malaria vector control programs [6]. Due to this high occurrence of resistance, the use of pyrethroids is reported to have decreased by 53% in the recent 10-year WHO insecticide use survey [62]. The *An. funestus*, a major African malaria vector, possesses significant pyrethroid resistance mainly through rapid detoxification of insecticides by P450 enzymes [4, 7]. Identification of novel insecticides from natural products is a relatively cost-effective alternative [26]. Isman [25] suggested that commercialization of essential oil-based insecticides will be highly facilitated by the exemption of some of them from normal lengthy regulatory processes, given their known safety profiles and long history of use. Many studies have proven that essential oils are promising sources of alternative natural insecticides [9, 49, 63] and some have shown their potential in reversing pyrethroid resistance in mosquito species such as *Aedes aegypti* [64]. The identification of EOCs with potent insecticidal activity against pyrethroid-resistant *An. funestus* has potential for greater benefits for malaria vector control.

In this study, six EOCs farnesol, (-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, methyleugenol, and santalol (α- and β) along with one EO, sandalwood oil, were assessed for this potential. The WHO insecticide susceptibility assay has confirmed the pyrethroid susceptibility of the *An. funestus* colony, FANG, and pyrethroid resistance in the second *An. funestus* colony, FUMOZ-R. Further, the synergist assay confirmed that the monooxygenase-based mechanism of resistance in FUMOZ-R is still present, as described in previous studies [4, 7]. This was also confirmed by increased monooxygenase levels in FUMOZ-R were compared to fully susceptible FANG strain using the P450 biochemical assay. Brooke et al. [7] showed that the monooxygenase levels on the resistant *An. funestus* colony could reach more than 100 times higher than the susceptible colony. At a transcriptional level, Riveron et al. [17] reported that the specific genes; *CYP6P9a* and *CYP6P9b*, were significantly overexpressed (~50-fold) in pyrethroid-resistant *An. funestus* from Mozambique and Malawi. Similarly, Wondji et al. [65] and Christian et al. [50] reported that the *CYP6P9* gene was highly overexpressed in FUMOZ-R as opposed to FANG.

Following confirmation of the monooxygenase-based pyrethroid resistance, FUMOZ-R colony as well as a pyrethroid susceptible colony, FANG, were investigated for susceptibility to six

EOCs and one EO. Insecticide susceptible *Anopheles funestus* females were used as a reference for determining the double LC_{99} concentrations for test compounds [59]. The three lowest double LC_{99} concentrations were attained with *cis*-nerolidol (3.30%), *trans*-nerolidol (3.84%) and methyleugenol (4.94%). These three EOCs showed similar LC_{50} values regardless of whether pyrethroid resistant or susceptible *An. funestus* were used. This appears to indicate that the efficacies of these EOCs are not negatively impacted on by the pyrethroid resistant genotype of *An. funestus.*

On the other hand, four other test compounds; farnesol, (-)-α-bisabolol, santalol (α and β), and sandalwood oil showed statistically lower activity against pyrethroid resistant *An. funestus* with less than 50% 24-hr mortality. The association of overexpressed monooxygenases in the pyrethroid *An. funestus* on the activity of these essential oils was assessed through standard synergist assays. When the monooxygenases were inhibited by PBO, both farnesol and (-)-α-bisabolol, showed 100% 24-hr mortality in pyrethroid resistant *An. funestus*. The enhanced activity of farnesol and (-)-α-bisabolol against resistant *An. funestus* when synergized by PBO, indicates their potential effectiveness in combination with P450 monooxygenase inhibitors. As an example, the combination of pyrethroids with PBO has already shown great impact in reversing resistance in the field applications [19, 20].

On the other, sandalwood oil and its major constituent, santalol (α and β), could still not attain 100% 24-hr mortality in pyrethroid resistant *An. funestus,* even when combined with PBO. Notably, the LC_{50} values of santalol (α and β) and sandalwood oil were similar. Furthermore, the statistical similarity of efficacies of santalol (α and β) and sandalwood oil against either insecticide susceptible or pyrethroid resistant *An. funestus*, suggests that santalol (α and β) may serve as the main active component in this essential oil.

In this study, nerolidol geometric isomers obtained the lowest LC_{50} and LC_{99} values against pyrethroid-susceptible and resistant *An. funestus*. The nerolidol-rich essential oil of *Lantana camara* has previously induced adulticidal effects against *An. gambiae* (susceptible 'Kisumu' strain), with the LC_{50} value of 0.24%, [49] comparable to this study. Nerolidol is currently registered by Food and Drug Administration as a food flavouring agent [66, 67]. It has been proven nontoxic to mammals at concentrations higher than those reported here for insecticidal activity [67]. It is commercially used at concentrations as high as 20% in human fragrance products and showed the LD_{50} at about 10g/kg dose in mice [67, 68]. Therefore, nerolidol is considered safe and for that reason, it may be further investigated for field application as a

novel bioinsecticide. Another EOC that showed comparable LC_{50} value to the nerolidol isomers is a phenylpropanoid methyleugenol. This EOC also showed similar potencies between the pyrethroid-susceptible and resistant *An. funestus*.

5. Conclusion

The findings of this study suggest that both the pyrethroid-susceptible and resistant *An. funestus* colonies are susceptible to *cis*-nerolidol, *trans*-nerolidol, and methyleugenol. This study has also identified two essential oil constituents, farnesol and (-)-α-bisabolol, that have a potential to successfully kill the pyrethroid resistant *An. funestus*, when combined with P450 synergists such as PBO. Therefore, this study reports for the first time, *cis*-nerolidol, *trans*-nerolidol and methyleugenol as potential candidates for further investigations as novel entities for development as bioinsecticides against *An. funestus*. These EOCs should further be evaluated against other malaria vectors with known insecticide resistance phenotypes. The nerolidol geometric isomers, *cis*-nerolidol and *trans*-nerolidol were the most active EOCs and given the well-established mammal safety record of nerolidol, these terpenoids are recommended for further investigations as resistance-breaking bioinsecticides. Furthermore, these sesquiterpene alcohols may serve as templates for further structural modification for generating more active insecticides against pyrethroid resistance.

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CHAPTER 8

Integrative discussion

This chapter discusses the implications of the findings of this PhD study, from Chapter 2 – 7. This thesis displayed the potential of tackling the ever-growing insecticide resistance with the natural product resources, EOCs in this case. Through the intensive literature review (Chapter 2), this study started by establishing AChE as a critical, validated target in *Anopheles*. In the same review, this was followed by identifying EOs as potential sources of novel anticholinesterase insecticides. Chapter 3 optimized the *in silico* approach in assessing the *Anopheles* AChE inhibitory potential of these molecules. Using novel donepezil analogues, Chapter 4 included the established *in silico* methods from Chapter 3 coupled with *in vitro* and *in vivo* assays. The Chapter 4 revealed that although it was hypothesized that donepezil analogues with insignificant activity against mammal AChE, would have the potential to target *Anopheles*, this however was not the case with the analogues tested in this study. However, these compounds were used to develop and optimize the *in vitro*, *in vivo* and *in silico* methods used in this thesis. For that reason, the focus of this Thesis was shifted towards the essential oil constituents (terpenoids) as potential *Anopheles* AChE inhibitors hence entitled "*In vitro* and *in silico* characterization of the anticholinesterase activity of select terpenoids against *Anopheles* vectors".

The next three papers interrogated the feasibility of terpenoids to inhibit *Anopheles* AChE, selectivity potential of EOCs to the *Anopheles* AChE over the corresponding mammal (electric eel) target, their potential to kill various stages of *Anopheles* life cycle and lastly their toxicity potential against insecticide-resistant *Anopheles* strains. More detailed and focussed discussions are found in the discussion sections of each paper.

8.1 Establishment of *Anopheles* acetylcholinesterase as a critical target

Anopheles have thin cuticles which allow for the direct absorption of the compounds into the insect and the short length of the nervous system facilitates an immediate inhibitory action (Lewis, 1980). As a result, all the approved insecticides including organochlorines, pyrethroids, organophosphates, and carbamates, act on the nervous system. The organochlorines act on the peripheral nervous system, pyrethroids on both peripheral and CNSs, while the organophosphates and carbamates act mainly on the CNS (Davies et al., 2007; English and

Webster, 2012). The two latter classes exert their actions by altering the cholinergic activity of the *Anopheles* insects (Metcalf, 1971).

The nervous system maintains the general functional integrity of insects (Scharf, 2008). Neurotransmission is an important process in the *Anopheles* nervous system, where acetylcholine is a key neurotransmitter in the cholinergic system of *Anopheles*. However, acetylcholinesterase (AChE) is the essential enzyme that sustains the cholinergic activity by preventing constant nerve firing and thereby maintaining normal neuronal impulse transmission at cholinergic synapses. Without AChE, the ACh could bind continuously on its nicotinic acetylcholine receptors causing prolonged excitation, paralysis and death (Thapa et al., 2017). As such, AChE is a critical target in malaria vector control.

8.2 Hypothesis of essential oils as sources of novel *Anopheles* acetylcholinesterase

The organophosphates and carbamates have greatly lost effectiveness due to resistance (Aïzoun et al., 2013; Zoh et al., 2018) and these are not used for the insecticide treated nets due to safety concerns (Lu et al., 2015; WHO, 2018). This supports the need to identify novel AChE inhibitors with potential to be active against resistant *Anopheles* strains and with high relative safety level to humans. Most EOCs are certified as safe and employed in the food industry, cosmetic sector and pharmaceutical corporation (Hyldgaard et al., 2012; Cimino et al., 202; Angane *et al*., 2022). Therefore, identification of *Anopheles* AChE inhibitors from EO sources may potentially generate safe and cost-effective bioinsecticides.

The extensive review conducted in Chapter 2 identified several EOs and EOCs that presented with low anticholinesterase IC_{50} values and insecticidal LD_{50} ranges against various *Anopheles* colonies. For instance, *Hyptis suaveolens* Poit. EO resulted in an anticholinesterase IC_{50} value of 0.55 µg/mL and LD_{50} value of 61.71 µg/mL against the egg stage, 159.48 µg/mL against the larva and >1000 µg/mL against the adults of *An. gambiae* (Wangrawa et al., 2015; Wangrawa et al., 2018). This indicated that *H. suaveolens* EO was capable of exerting AChE inhibition whilst killing the eggs and larvae at potent and moderate LD_{50} values, respectively. Against *An. arabiensis*, the EO of *Schinus molle* L. displayed potent larvicidal activity (LD_{50}: 21.0 µg/mL) but was not an effective AChE inhibitor (>1000 µg/mL) (Aboalhaija et al., 2019; Massebo et al., 2009). On the other hand, *Ocimum canum* Sims. showed larvicidal activity (LD_{50} value of 91.2 µg/mL) against *An. funestus*, with a potent anticholinesterase IC_{50} range of 0.21 - 26.16 µg/mL (Gnankiné and Bassolé, 2017; Kiendrebeogo et al., 2011; Wangrawa et al.,

2015; Wangrawa et al., 2018). For the actual EOCs, the monoterpene, terpinolene displayed moderate larvicidal activity (LD_{50}: 404.71 µg/mL) against *An. gambiae* with an anticholinesterase IC_{50} value range of 156.4 to 550.0 µg/mL (Bonesi et al., 2010; Gnankiné and Bassolé, 2017; Peng et al., 2021; Spinozzi et al., 2021). Similarly, terpinen-4-ol was reported to inhibit AChE with an IC_{50} value range of 190 to 320 µg/mL, while exhibiting *An. gambiae* larval toxicity (LD_{50} value of 337.7 µg/mL) (Bonesi et al., 2010; Wang et al., 2016). In contrast, isopulegol is a potent *An. gambiae* larvicidal activity with a LD_{50} value of 49.4 µg/mL (Gnankiné and Bassolé, 2017).

Based on this promising published evidence, Chapters 5-7 discussed the first ever assessment of insecticidal and selective *Anopheles* AChE inhibition by select EOCs; sesquiterpene alcohols that included farnesol, (-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, a monoterpene (*R*)-(+)-limonene, and a phenylpropanoid methyleugenol. These were assessed against the sub-Saharan laboratory strains of *An. gambiae* (COGS), *An. coluzzii* (G3), *An. arabiensis* (KWAG), and *An. funestus* (FANG, FUMOZ, and/or FUMOZ-R).

8.3 Optimization of the *in silico* approach for assessment of novel AChE inhibitors

A reliable molecular docking method is critical in reducing discrepancies between the *in silico* and *in vitro*/*in vivo* studies (Jeong et al., 2019). This thesis focused on *Anopheles* AChE as a biological target for novel insecticides. The current anticholinesterase insecticides such as OPs, are covalent inhibitors of the *Anopheles* AChE (Chambers and Oppenheimer, 2004). However, the covalent docking is rarely reported in literature due being relatively complicated than the noncovalent modelling. The complexity is caused by the chemistry that defines the specific covalent bond formation (Manoharan and Sridhar, 2018). At the time of the execution of this research, there was no reported molecular docking data that displayed a covalent bond between OPs and the *Anopheles* AChE catalytic site. Many research papers had reported the molecular docking of OPs through the noncovalent route, generating data that may lack clinical relevance. Nevertheless, the studies recognized this gap and attributed it to the fact that most docking software packages did not comprise the covalent docking modes; whereby few that had this option could not have the chemical reaction for OP hydrolysis.

To ensure that a reliable molecular modelling data with known *Anopheles* AChE inhibitors was available for comparison with the EOCs, manual customization of the covalent docking reactions in Schrödinger modelling software (2018-2) was carried out, in which the OP hydrolysis reaction was added to the repository and optimized (Chapter 3). This exercise

interrogated the suitability of noncovalent docking versus the customized covalent procedure, to assess the AChE inhibitory activity of OPs by generating the molecular binding conformations and interactions reported by the X-ray diffraction studies (Bjerrum, 2016; Maveyraud and Mourey, 2020). On top of establishing the covalent bond and showing the hydrolyzed OP structural portions, the customized method regenerated the data reported from X-ray studies suggesting high accuracy and reliability (Chapter 3). Therefore, a reliable docking methodology to assess the *Anopheles* AChE binding profiles of OPs was first reported from this study (Rants'o et al., 2022). This was used to generate the crystal structures of *Anopheles* AChE complexed with standard AChE inhibitors for comparative purposes with the test EOCs. This served a critical role of assisting in the identification of the novel AChE inhibitors that have a capacity to inhibit *Anopheles* AChE through a different binding profile.

8.4 *In silico*, *in vitro* and *in vivo* analysis of anticholinesterase activity of terpenoids

The terpenoids farnesol, (-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, and methyleugenol, were assessed for selective *Anopheles* AChE inhibition in the *in vitro* study against *An. arabiensis*, *An. funestus, An. gambiae,* and *An. coluzzii*. These were assessed reliably using AChE samples from larval homogenates of these *Anopheles* species. To extrapolate the *in vitro* anticholinesterase activity to the *in vivo* efficacy, these terpenoids were also assessed for potential to kill larvae. Both anticholinesterase and larvicidal activity of terpenoids were generally ranked as farnesol and (-)-α-bisabolol > *cis*-nerolidol and *trans*-nerolidol > methyleugenol > (*R*)-(+)-limonene (Chapter 5; Tables 1 and 2, Chapter 6; Tables 2 and 3). Interestingly, the *An. arabiensis* colony was the most susceptible of all colonies to the assessed terpenoids in the larvicidal (Chapter 5; Table 1 and Chapter 6; Table 2). This was in correlation with literature that, in comparison to other *Anopheles* species such as *An. funestus* and *An. gambiae*, the *An. arabiensis* has lower level of insecticide resistance (Kawada et al., 2011; Glunt et al., 2018).

Similarly, in terms of the AChE inhibition capacity, the terpenoids were more active against *An. arabiensis* and *An. funestus* than the corresponding *An. gambiae* and *An. coluzzii* (Chapter 5; Table 2). Amongst the assessed terpenoids, only farnesol showed considerable activity against mammal (electric eel) AChE that was used as a human bioequivalent control. Similarly, the positive control, propoxur, was non-selective between the *Anopheles* and electric eel AChE in correlation to the literature (Chapter 5; Table 2) (Neale and Escher, 2013; Carlier et al., 2017). In the screening against *An. arabiensis* AChE, all terpenoids showed low IC_{50} values

(0.5 – 0.8 µM) similar to propoxur (IC_{50}: 0.778 µM). However, all terpenoids except for (R)-(+)-limonene, were more active than propoxur against *An. funestus* AChE ($p < 0.05$). In *An. gambiae*, the terpenoids displayed IC_{50} values ranging from 3 – 7.5 µM as opposed to propoxur (IC_{50}: 28.06 µM), i.e. approximately 4 to 9 times more potent (Chapter 5; Table 2). The *An. coluzzii* AChE was the least sensitive in this study where none of the assessed terpenoids achieved an IC_{50} value less than 50 µM though they were more effective than propoxur (IC_{50} > 100 µM). In fact, only farnesol and bisabolol showed IC_{50} values less than 100 µM (range: 60 – 70 µM) (Chapter 5; Table 2). AChE inhibition was therefore ruled out as the main mechanism of action for the terpenoid-induced larvicidal activity against *An. coluzzii*.

In terms of cytotoxicity against *Artemia franciscana*, which determines the selectivity potential among aquatic organisms, (*R*)-(+)-limonene and methyleugenol were the least toxic making them highly selective to the *Anopheles* species in general (Chapter 5; Table 1). Based on LC_{50} values, farnesol and (-)-α-bisabolol were about twenty times more selective towards *An. arabiensis* larvae, while *cis*-nerolidol and *trans*-nerolidol there was a two-fold selectivity towards this colony. The positive control, propoxur, was similarly toxic between *An. arabiensis* (LC_{50}: 8.672 µM) and *Artemia* (LC_{50}: 9.202 µM) in agreement with the literature about its aquatic toxicity (Chapter 5; Table 1) (Barahona and Sánchez-Fortún, 1999; Deidda et al., 2021).

Further studies were undertaken to assess the *Anopheles* AChE inhibition potential of terpenoids at the molecular level. Chapters 2 (Figure 3) and 4 (Figure 7) assessed the differences between mammal (human and electric eel) and insect (*Anopheles*) AChE catalytic sites, which may stimulate ligand selectivity between these targets. Also, as reviewed in Chapter 2, the amino acid sequence of *Anopheles* AChE is only 48-49 % identical to that of human (Carlier et al., 2008; Engdahl et al., 2015). The AChE is characterized by a deep and narrow active-site gorge (Engdahl et al., 2015). However, the current study (Chapter 2; Figure 3, Chapter 4; Figure 7) showed that the entrance to the active site gorge of *Anopheles* AChE possesses a free and smaller cysteine residue (Cys^{447}) (PDB ID: 5YDI) instead of a bulky phenylalanine (Phe^{295}) in human AChE (PDB ID: 7E3H). Additionally, in *Anopheles* AChE, a smaller aspartic acid residue (Asp^{602}) replaces a larger tyrosine residue (Tyr^{449}) at the rim of the active site gorge (Chapter 2; Figure 3, Chapter 4; Figure 7). This is in correlation with the literature whereby these properties have been suggested to create the larger active site gorge of *Anopheles* in comparison to that of the vertebrates (Engdahl et al., 2016; Cheung et al., 2018).

Also, for the first time in this study, our results in Chapter 4 (Figures 7 and 8) showed that the AChEs from human and electric eel are similar in function and structure at a molecular level, in support of previous biochemical studies (Li et al., 2016; Xu et al., 2016). Through homology modelling in Chapter 4 (Figures 6 and 9), the differences between human/electric eel and *Anopheles* were shown to induce ligand binding differences.

After identifying the terpenoid-based *Anopheles* AChE inhibitors in Chapter 5 (Table 2), these were assessed through molecular modelling and molecular mechanics simulations. In correlation to the *in vitro* and *in vivo* data, farnesol was still the best *Anopheles* AChE inhibitor based on the relative binding score and generated target interactions (Chapter 5; Table 3). Additionally, the capability to bind both *Anopheles* and electric eel targets was proven, however with a preference to *Anopheles* based on crystal pose, intermolecular interactions and binding scores (Chapter 5; Figure 2 and Tables 3 and 4). The study showed that farnesol had a capacity to show relative selectivity to *Anopheles* as suggested by the *in vitro* data (Chapter 5; Table 2). This selectivity was further induced by interaction with the *Anopheles* conserved Cys^{447} amino acid residue (Chapter 5; Figure 2). This is important as Cys^{447} is a novel target amino acid in the design of *Anopheles* selective anticholinesterases (Carlier et al., 2017; Pang et al., 2009; Pang et al., 2012). Another highly potent terpenoid from *in vitro* and *in vivo* assessments, (-)-α-bisabolol, formed hydrogen bonds with the catalytic serine (Ser^{360}) which is a validated target site (Chapter 5; Figure 4) (Pang et al., 2012). Generally, the terpenoids interacted with *Anopheles* AChE catalytic site through hydrogen bonds and established the nonpolar interactions with the active site gorge entrance through their hydrophobic chains (Chapter 5; Figure 4 and Table 3). However, in agreement to the *in vivo* data, a nonpolar monoterpene, (*R*)-(+)-limonene was shown as the least active and nonspecific inhibitor due to lack of polar functional groups required for interaction with the active site (Chapter 5; Figure 5).

Therefore, farnesol, (-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, (*R*)-(+)-limonene, and methyleugenol were identified as potent larvicidal agents against major African malaria vectors *An. funestus*, *An. arabiensis*, *An. gambiae*, and *An. coluzzii*. The evaluation of these terpenoids against AChE samples extracted from the four *Anopheles* species revealed them to be strong AChE inhibitors against *An. arabiensis* and *An. funestus* with moderate inhibitory activity against *An. gambiae,* but very weak activity against *An. coluzzii.*

8.5 Terpenoids bioactivity against the *Anopheles* life cycle

The cholinergic system develops early during embryogenesis in the egg stage and sustains the mosquito throughout its lifetime as a key system in the nervous system (Mehrotra, 1960a). Mehrota (1960b) showed that AChE inhibitors could also inhibit the cholinergic activity in insect eggs; however, AChE gene expression is at its minimum during the egg stage. To note, the larva has a higher AChE content than adult, making it a preferred stage for insect AChE inhibition studies (Hemingway and Georghiou, 1983; Lu et al., 2012; Grünewald and Siefert, 2019).

Chapter 5 highlighted that *An. arabiensis* was the most susceptible species to the assessed terpenoids in terms of acetylcholinesterase inhibition and insecticidal activity at a larva stage (Chapter 5; Tables 1 and 2). This is an important finding considering *An. arabiensis* is a major vector in arid African countries, where new insecticides are needed to reduce malaria transmissions (Nyanjom et al., 2003; Dorn et al., 2011; Dandalo et al., 2017). Having confirmed the potent AChE inhibition of select terpenoids, it was then crucial to generate their complete insecticidal profile by assessing them against all developmental stages of *Anopheles*. Given the escalating insecticide resistance against all current insecticides, identification of novel insecticides that simultaneously act at multiple stages of *Anopheles* life cycle presents a cost-effective opportunity. Therefore, in Chapter 6, further analysis was undertaken to assess the capability of the identified terpenoid-based *Anopheles* AChE inhibitors to kill multiple stages of *An. arabiensis*, including three aquatic stages, egg, larva and pupa, and an adult stage (Chapter 1; Figure 1) (WHO, 2022).

Amongst these terpenoids, methyleugenol was the only one that inhibited the egg hatching process (Chapter 6; Table 1), a critical activity to halt the *Anopheles* life cycle at an earliest stage (Chapter 1; Figure 1). Moreover, all terpenoids were potent against the larva stage (Chapter 6; Table 2) in correlation with Chapter 5 (Table 1). In the assessment against pupae, the two sesquiterpene alcohols, farnesol and (-)-α-bisabolol were the only bioactive terpenoids (Chapter 6; Table 3). In the adult stage, all terpenoids showed moderate activity and were less potent than propoxur with diagnostic concentrations about forty times higher than that of propoxur.

In general, this study showed the multi-stage targeted potential of essential-oil based anticholinesterase insecticides across various developmental stages of *Anopheles*. Methyleugenol, farnesol, and (-)-α-bisabolol showed inhibitory activity at more than one of the

aquatic stages of *Anopheles* (Chapter 6; Tables 1-3, Figure 2) while all assessed terpenoids displayed potent larvicidal activity. This shows a potential for integrating these EOCs into larviciding programs (Fillinger et al., 2009; Mpofu et al., 2016). This further indicated an opportunity to integrate early stage-acting insecticides with current late stage-acting insecticides. Additionally, with moderate activity against adults these terpenoids may offer an auxiliary bioactivity when integrated with current synthetic insecticides. Being from natural resources, the identification of these EOCs indicates a potential for cost-effective and eco-friendly insecticides against malaria vectors. Amongst these, (-)-α-bisabolol, nerolidol and limonene are certified as generally safe, by the FDA (Kamatou and Viljoen, 2010; Chan et al., 2016).

8.6 Structure-activity relationships

According to QSAR theory, similar structural molecules may produce similar biological activities (Sharma et al., 2021). Propoxur and methyleugenol were the only molecules showing inhibitory activity at *Anopheles* egg stage (Chapter 6; Table 1). Interestingly, this study showed a remarkable structural similarity between methyleugenol and propoxur where both had the 1,2-dimethoxybenze moiety (Figure 1).

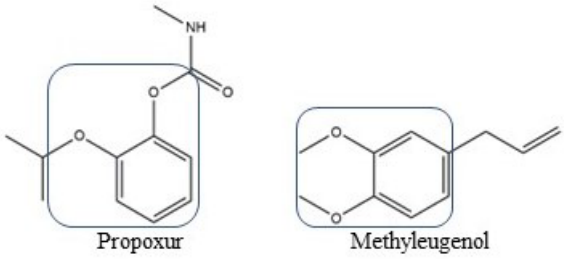


Figure 1. Chemical similarity of propoxur and methyleugenol. The common 1,2-dimethoxybenze group is enclosed in a rectangular box.

Therefore, the binding profiles of propoxur and methyleugenol against *Anopheles* AChE (PDB: 5YDI) were compared (Figure 2). It was noted for the first time that the shared 1,2-dimethoxybenze group plays a critical role in target site binding of these two agents. The benzene ring was stabilized by aromatic phenylamine (Phe^{490}) residues, while the methoxy groups interacted strongly with the catalytic triad amino acids His^{600} and Ser^{360}. This study also suggested that although methyleugenol has a structural portion that is similar to propoxur, it however has a higher chance of becoming effective against AChE-resistant strains of *Anopheles*. This is based on the observation that unlike methyleugenol, propoxur interacts with a mutable Gly^{280} (Figure 2A) that causes AChE resistance (Pang et al., 2009; Essandoh et al.,

2013). This was afforded by the amide substituent attached to one methoxy functional group on propoxur, while the methoxy groups are uncapped in methyleugenol structure. Moreover, unlike propoxur, methyleugenol selectively inhibited *Anopheles* AChE while it did not show the inhibitory activity against mammal AChE, suggesting a potential to be relatively selective to the *Anopheles* target (Chapter 5; Table 2).

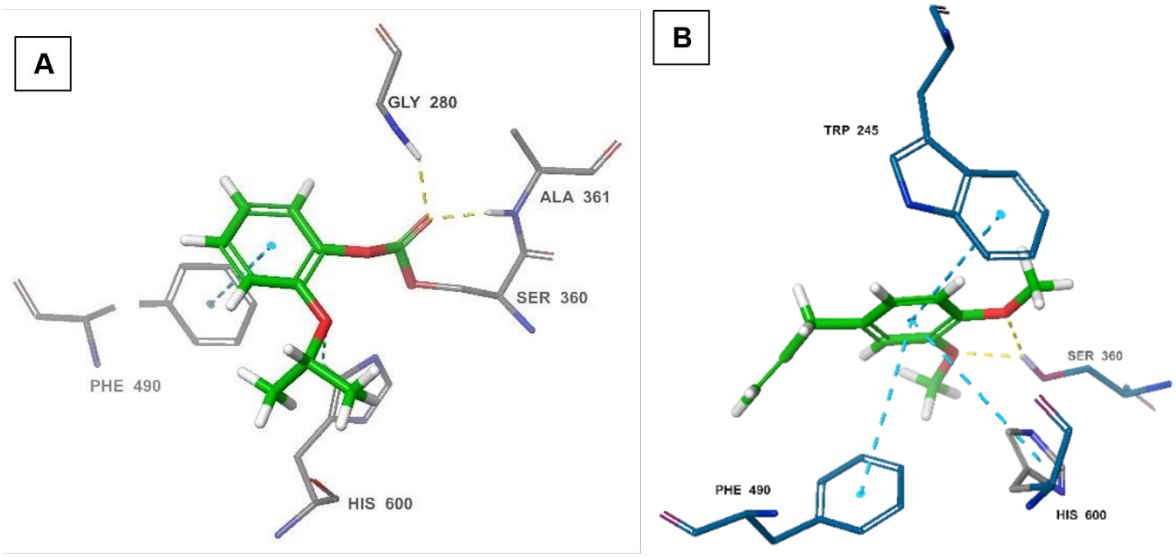


Figure 2. Propoxur (A) and methyleugenol (B) in the *Anopheles* AChE binding site.

The two other molecules that have been showing similar activities across all the assessed *Anopheles* species and performed biological assays, are farnesol and (-)-α-bisabolol (Chapter 5 - 7). This prompted us to do a QSAR study on these two terpenoids. It was noted that these molecules have a similar chemical formula ($C_{15}H_{26}O$). Additionally, studies have shown that the acid-catalyzed cyclization of farnesol generates (-)-α-bisabolol (de Meireles et al., 2015) (Figure 3).

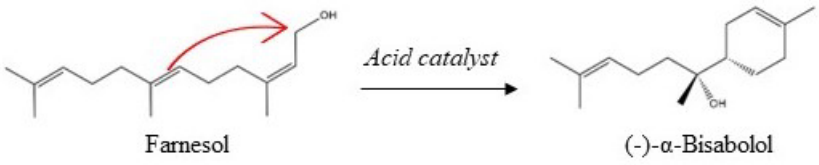


Figure 3. The close similarity of farnesol and (-)-α-bisabolol chemical structures. Farnesol is aliphatic while (-)-α-bisabolol is cyclic (de Meireles et al., 2015).

Interestingly, the molecular modelling work however showed differences in the binding profiles of these sesquiterpene alcohols (Figure 4). Due to the aliphatic form of farnesol, it could extend out to the active site gorge with its hydroxyl group and favourably interact with a free Cys^{447} residue (Figure 4A) that is absent in the mammal targets (Carlier et al., 2017). On

the other hand, the cyclic nature of (-)-α-bisabolol directed its hydroxyl group towards the catalytic site where it also formed an important interaction with catalytic Ser^{360} (Figure 4B). While farnesol established a unique binding profile with the *Anopheles* target, it was shown to also possess activity against mammal target in the *in vitro* study (Chapter 5; Table 2) though it assumed a completely different binding pose at this site, by interaction with the aromatic amino acids Phe^{288} and Phe^{331} (Figure 4C). The flexible nature of farnesol imparted by the bis-allylic group (circled in Figure 4C) allows it to interconvert between *cis* and *trans* conformations (Overton and Roberts, 1974; Wang et al., 2008). This interconversion may have contributed to having farnesol retain the *trans*-isomer form at *Anopheles* target (Figure 4A) while it bent to assume the *cis* conformation at the electric eel target site (Figure 4C). This isomerization of farnesol upon interacting with biological enzymes has previously been reported (Overton and Roberts, 1974; Capellini et al., 1976; Ahmad-Sohdi et al., 2015).

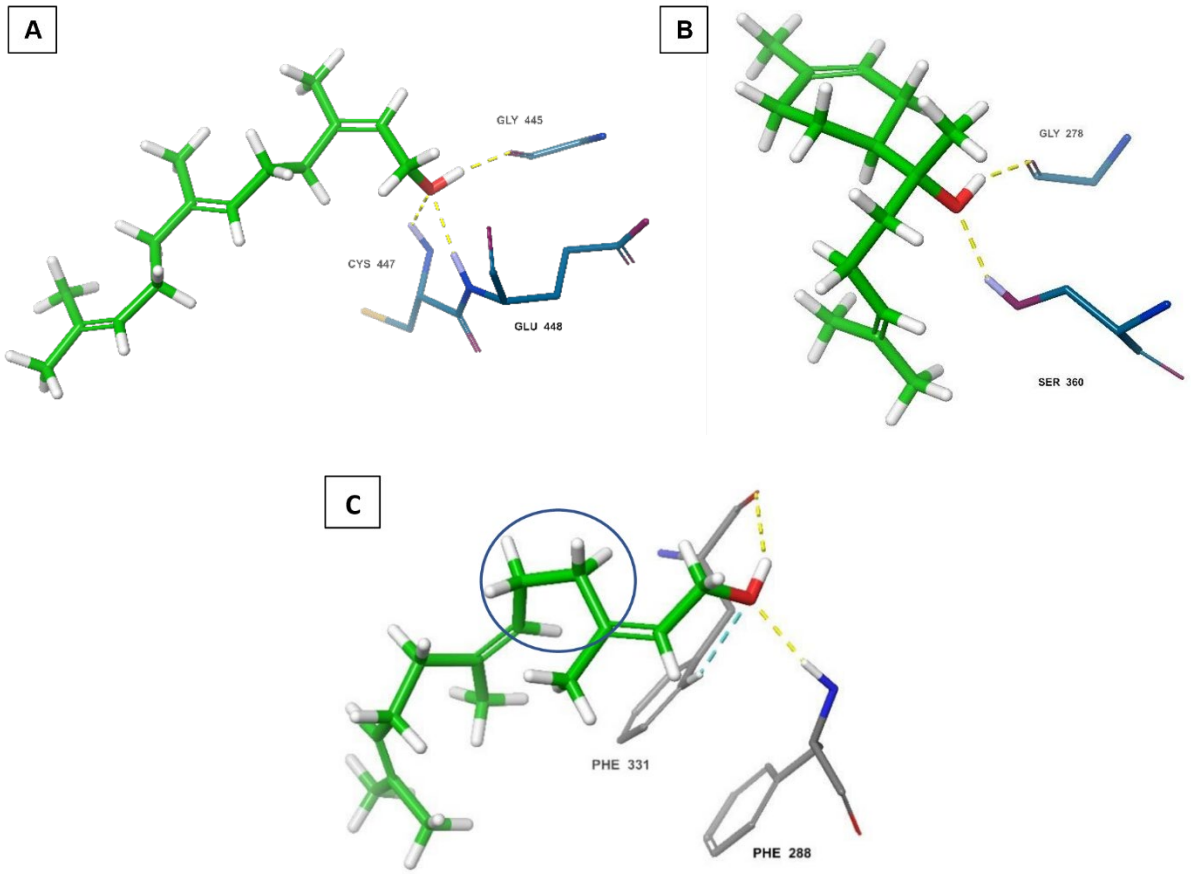


Figure 4. Comparison of the molecular interactions of farnesol (A) and (-)-α-bisabolol (B) with the *Anopheles* AChE binding site. C) Shows the conformation of farnesol at the electric eel AChE binding site with the flexible portion of the bis-allylic group circled in blue.

The nerolidol geometric isomers, *cis*-nerolidol and *trans*-nerolidol, are the third group of compounds that steadily showed similar *in vitro* and *in vivo* potencies (Chapter 5; Tables 1 and 2, Chapter 6; Tables 1-4, Chapter 7; Tables 2 and 3, Figure 3a) as well as the *in silico* binding profiles (Chapter 5; Table 3). Therefore, molecular modelling showed that these two isomers induce similar biological activities because they bind similarly at the *Anopheles* AChE site. This was shown by the generated crystal poses and molecular interactions (Figure 5) as well as the binding scores (-40.41 kcal/mol and -42.74 kcal/mol for *cis*-nerolidol and *trans*-nerolidol, respectively).

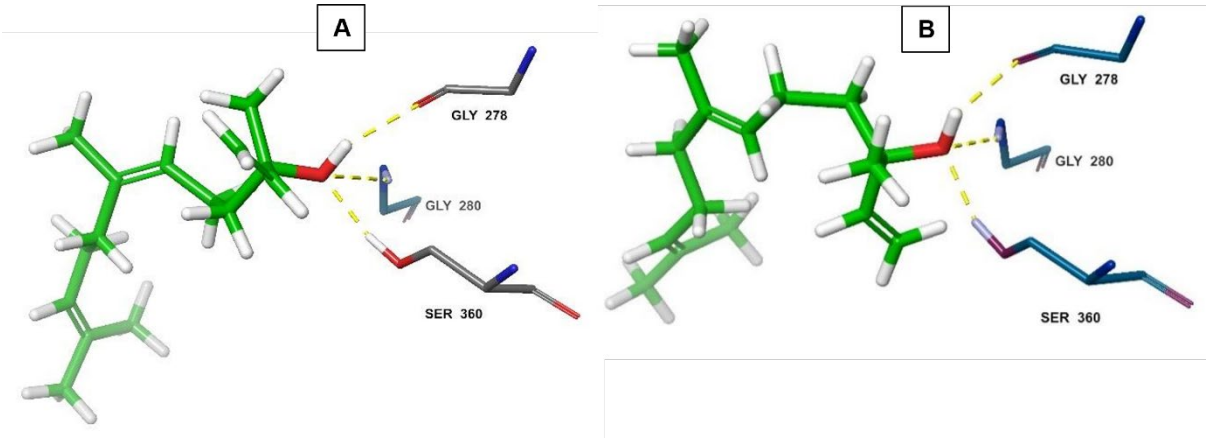


Figure 5. Comparison of the *Anopheles* AChE binding profiles of farnesol *cis*-nerolidol (A) and *trans*-nerolidol (B).

8.7 Terpenoids activity against resistant *Anopheles* strains

Finally (Chapter 7), these identified *Anopheles* anticholinesterase terpenoids with potential to kill various developmental stages of *Anopheles*, were assessed for the insecticidal effects against a pyrethroid- and carbamate-resistant strain of *An. funestus* (FUMOZ-R) (Brooke et al., 2001; Chanda et al., 2020) and compared to the insecticide susceptible strain of *An. funestus* (FANG) (Brooke et al., 2001). The insecticide resistance and susceptibility of these two strains were confirmed by exposure to a pyrethroid, deltamethrin. The resistance to pyrethroids poses a major threat in malaria vector control programs since these are the only insecticide class used for the treatment of bed nets (Chrustek et al., 2018). The involvement of P450 monooxygenases in the resistant strain was confirmed through the synergist assay. Wherein, pre-exposure of the pyrethroid resistant *An. funestus* to the P450 monooxygenase inhibitor, PBO completely reversed the pyrethroid resistance. Moreover, the relative levels and activity of P450 monooxygenases were assessed through the peroxidase biochemical assay and it was observed

as previously reported that the monooxygenase levels and activity in resistant strain were substantially (~ 4-fold) higher than in the susceptible strain (Brooke et al., 2001).

The terpenoid screening results from this study revealed that all the terpenoids sustained the moderate toxicity (LC_{50} range: 100 – 500 µg/mL) against the *An. funestus* FANG adults (Chapter 7: Table 2) as was observed in other species (Chapter 6; Table 4). However, in the resistant *An. funestus* FUMOZ-R, only three terpenoids *cis*-nerolidol, *trans*-nerolidol, and methyleugenol maintained their moderate toxicity, while farnesol and (-)-α-bisabolol drastically lost effectiveness (Chapter 7; Table 2, Figure 3b, and Table 5). Using PBO to synergize their effects, the effectiveness of farnesol and (-)-α-bisabolol was restored (Chapter 7; Table 5). These results suggested that *cis*-nerolidol, *trans*-nerolidol, and methyleugenol were not susceptible to the insecticide resistance in *An. funestus* FUMOZ-R, while the efficacies of farnesol and (-)-α-bisabolol were negatively impacted by this. However, the enhanced activity of farnesol and (-)-α-bisabolol against resistant *An. funestus* when combined with PBO opens another possibility of future interest which will be to assess their potential application in combination with P450 monooxygenase inhibitors. Also, the structural modification of farnesol and (-)-α-bisabolol to conjugate various P450 monooxygenase inhibitors is another recommended area of exploration.

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CHAPTER 9

Conclusions and recommendations for future research

Concluding remarks

Two of the most potent sesquiterpene alcohols, farnesol and (-)-α-bisabolol, were highly active across the various *Anopheles* species and biological assays until they were assessed against the *Anopheles* strain with P450-based metabolic resistance. In the event that these P450 monooxygenases were suppressed by a synergist, these terpenoids regained their efficacies. Therefore, it is concluded that it would be essential to investigate a combination of these EOCs with P450 inhibitors to overcome resistance.

Overall, *cis*-nerolidol, *trans*-nerolidol, and methyleugenol were identified as the best of the evaluated terpenoids with potent *Anopheles* AChE inhibition accompanied with insecticidal activity against both the insecticide susceptible and resistant *Anopheles* strains. These terpenoids displayed insecticidal activities at various developmental stages of *Anopheles* including egg, larva, and adult. The AChE inhibition was alluded to as their mechanism of action by both the *in vitro* and *in silico* studies. These EOCs present as potential candidates for further investigations as novel entities for development as bioinsecticides.

Limitations of the current study

Although this study showed that the presence of increased activity on resistant *Anopheles funestus* as opposed to the susceptible strain, it was beyond the scope of the current study to confirm the P450 monooxygenase overexpression at a genetic level nor identify the upregulated P450 enzymes. As a result, though there is an indication of possible interaction between the *Anopheles* P450 monooxygenases with the assessed terpenoids, this cannot be ascertained as the actual metabolic study whereby the terpenoids are incubated with the specific *Anopheles* P450 enzymes was not conducted.

Recommendations for future research

For future, it will be worthwhile to assess the possible detoxification on farnesol and (-)-α-bisabolol by *Anopheles* amplified P450 monooxygenases at a biochemical level, and preferably using a liquid chromatography coupled to mass spectrometry detector (LC/MS). This recommended study will show if these terpenoids are substrates of overexpressed *Anopheles*

P450 enzymes, determine the corresponding metabolic pathway and identify the specific metabolites. Such a study will then inform next steps which may include structural modification of these two terpenoids to alter their susceptibility to the P450 enzymes.

From the results of the current study, it is strongly recommended that three other terpenoids that were not affected by overexpressed P450 enzymes, *cis*-nerolidol, *trans*-nerolidol, and methyleugenol, be developed and assessed for field application in the experimental hut conditions. Moreover, the assessment of a suitable formulation of these EOCs to reduce their volatile nature to lengthen their duration of action and clinical effectiveness is highly imperative.

CHAPTER 10

Appendices

Appendix A.1: Potential of essential oil-based anticholinesterase insecticides against *Anopheles* vectors: A review (Chapter 2; published paper)

molecules

MDPI

Review

Potential of Essential Oil-Based Anticholinesterase Insecticides against *Anopheles* Vectors: A Review

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Abstract: The insect nervous system is critical for its functional integrity. The cholinergic system, of which acetylcholinesterase (AChE) is a key enzyme, is essential to the *Anopheles* (consisting of major malaria vector species) nervous system. Furthermore, the nervous system is also the primary target site for insecticides used in malaria vector control programs. Insecticides, incorporated in insecticide-treated nets and used for indoor residual spraying, are a core intervention employed in malaria vector control. However, *Anopheles* resistance against these insecticides has grown rapidly. Due to this major setback, novel agents with potential activity against resistant *Anopheles* and/or capacity to overcome resistance against current WHO-approved insecticides are urgently needed. The essential oils have the potential to be natural sources of novel insecticides with potential to inhibit the *Anopheles* AChE target. In the current review, the scientific evidence highlights the ability of essential oils and specific essential oil constituents to serve as anticholinesterase insecticides. For this reason, the published data from scientific databases on the essential oils and essential oil constituents on anticholinesterase, ovicidal, larvicidal, pupicidal and adulticidal activities were analyzed. The identification of major constituents in active essential oils and their possible influence on the biological activity have also been critically evaluated. Furthermore, the toxicity to mammals as well as potential activity against the mammalian AChE target has also been reviewed. The importance of identifying novel potent insecticides from essential oils has been discussed, in relation to human safety and cost-effectiveness. Finally, the critical insights from this review can be used to inform future researchers towards potent and safe anticholinesterase insecticides for the management of *Anopheles* malaria vectors.

Keywords: malaria; insecticides; terpenoids; acetylcholinesterase

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1. Introduction

Malaria is a devastating disease caused by a protozoan parasite, namely *Plasmodium falciparum* which is the major causative agent in the pathogenesis of this infectious disease [1–3]. *Anopheles* vectors are infected with malaria after they ingest blood from an infected human host. The female *Anopheles* vectors effectively bite the human hosts between dusk and dawn [3] and it is during this time that she ingests gametocytes. The *Plasmodium* gametocytes develop into an oocyst in the mosquito midgut, which then matures into sporozoites. The sporozoites are released into the hemolymph and migrate to the salivary glands [1,4]. This parasite developmental process within the vector takes approximately

11–16 days before the female mosquito is able to transmit the parasite to the next human host during a blood feeding. This means that a long lifespan of the *Anopheles* vector is required for the successful completion of the parasite development and reinfection of the human host. Vertebrate blood is needed every 2–3 days by the female mosquito for nutrition, as well as egg development. The eggs are oviposited into water and fertile eggs hatch into larvae a few days later. Larvae will develop into pupae and finally adults will emerge after a few days [3]. There are more than 400 *Anopheles* species of which about 30 are major malaria vectors. The African *Anopheles* vectors have both long lifespans and a higher preference for human feeding and, collectively, these account for the high malaria cases and mortality that is recorded in Africa [3,5]. Other factors, such as climate conditions and political and economic stability, also affect the intensity of transmission and enhance the problem [3].

The malaria vectors have long been controlled by using insecticides. Insecticide classes include organophosphates, carbamates, pyrethroids, organochlorines and neonicotinoids [6]. Larvicides including insect growth inhibitors as well as bacterial larvicides, such as *Bacillus thuringiensis* subspecies *israelensis, Bacillus sphaericus* and spinosyns from *Saccharopolyspora* species, have also gained popularity in mosquito control activities [6–8]. The implementation of large-scale larviciding is however challenging in Sub-Saharan Africa and these may be used as a complementary intervention [6,9–11]. The *Anopheles* vectors have developed substantial resistance against almost all current insecticides [12–15]. To compound the issue, the commercial development of insecticides through various and often complicated synthetic mechanisms is expensive and time-consuming [16,17]. We propose that the identification of potential insecticides from natural product resources, such as essential oils (EOs), is a relatively cost-effective and faster alternative. Target identification and the corresponding mechanism of action are critical components of the drug discovery process [18]. Acetylcholinesterase (AChE) is a validated target in the insect nervous system and inhibitors of this critical enzyme have been useful in the control of malaria vectors for over eighty years [6,19,20].

To facilitate future research in EOs and potential insecticidal activity through AChE inhibition, this review will first discuss malaria vector control systems and current challenges, and we will explore the insect nervous system with relevance to a specific neurotransmitter, acetylcholine (ACh), and provide an in-depth discussion on AChE function and inhibition. Finally, we identify the potential of various EOs and essential oil constituents (EOCs) as anticholinesterase agents against *Anopheles* vectors. This was achieved by collecting scientific data using keywords such as anticholinesterase/acetylcholinesterase inhibition, EOs, terpenoids/terpenes, *Anopheles*, larvicidal, pupicidal, adulticidal and insecticidal in the search engines of PubMed, Google Scholar, ScienceDirect, SciFinder, SCOPUS and Web of Science.

2. Malaria Vector Control

The early vector control strategies adopted vast activities to reduce larval populations, which included, amongst others, the drainage of breeding sites such as swamps or the application of copper (II) acetoarsenite (Paris green), a highly toxic inorganic compound to the breeding sites [21,22]. In addition, the screening of windows and doors to prevent vectors entering houses and the use of mosquito nets have been at the forefront in protecting people against mosquito bites [6]. The plant-based insecticides were the first preparations used historically. Pyrethrins extracted from the flowers of *Chrysanthemum cinerariifolium* and *Chrysanthemum roseum* were used against indoor *Anopheles* mosquitoes in the 19th century [23,24]. However, the structural modifications of the natural pyrethrins and the generation of first synthetic pyrethroids were first reported in the period 1924 to 1970 [25]. The discovery of an organochloride, namely, dichlorodiphenyltrichloroethane (DDT), was reported in 1939 [25,26]. DDT has been highly effective against malaria vectors. However, in recent times increasing safety concerns have seen it being replaced in many countries by newer insecticides with reduced toxicity profiles [27–29].

Currently, malaria vector control adopts an integrated vector management program through the use of insecticides targeting both the larval and adult stages [6,27]. This is achieved through two main interventions, namely, insecticide-treated mosquito nets and indoor residual spraying, with additional interventions including larviciding [6]. The insecticide-treated nets provide both a physical barrier and insecticidal activity against *Anopheles* vectors. Indoor residual spraying (IRS) on the other hand, provides host protection through the *Anopheles* insecticidal effect [3]. Pyrethroids, pyrethroid-PBO combinations and pyrroles are the only insecticide classes used for the insecticide-treated nets, as the latter insecticides pose a low toxicity risk to humans [30,31]. The pyrethroids used in IRS include deltamethrin, alpha-cypermethrin, etofenprox, lambda-cyhalothrin, bifenthrin and cyfluthrin, while the organochlorines include DDT. On the other hand, the organophosphates approved by WHO for IRS include malathion, fenitrothion, pirimiphos-methyl and the carbamates such as propoxur and bendiocarb [6,32–34].

2.1. Insecticide Resistance in Main African Malaria Vectors

Insecticide resistance has been reported in all of the main African malaria vectors and this resistance against WHO approved insecticidal agents is rapidly increasing in intensity and geographical distribution [5,35,36]. An overview of mosquito resistance has been highlighted below. To keep this brief, only a few examples will be provided to explain the extent of the problem mainly on the African continent. Insecticide resistance in the main vector species has been reported for pyrethroids [35,37–41], organochlorides [38,41–43], organophosphates [12,44] and carbamates [20,38,43,45].

Common insecticide resistance markers associated with pyrethroid and organochloride resistance include the L1014F and L1014S mutation of the voltage-gated sodium channel gene, known as knockdown resistance (*kdr*). These mutations shift activation voltage dependence of sodium channels stabilizing them in the closed state. This antagonizes the action of pyrethroids and organochlorines since these compounds bind to open sodium channels [25,41,46,47]. Apart from the *kdr* mutations, elevated metabolic enzymes, including P450 monooxygenases, glutathione-S-transferases and non-specific esterases, also convey high resistance to pyrethroids and organochlorides [13,39,40,44,48,49]. On the other hand, the resistance mechanism commonly conferring organophosphate and carbamate resistance is a single point polymorphism resulting from glycine conversion to a serine residue at position 119 (G119S; *Torpedo californica* AChE numbering) or more precisely, position 280 (G280S; *Anopheles gambiae* AChE numbering) in the AChE target [41,47,50,51]. Resistance mechanisms often prevent the intended biological activity of a specific insecticide; therefore, it is important to discuss modes of action of the major insecticide classes.

2.2. Modes of Action of Main Insecticides Used in Malaria Vector Control

Regardless of their small size, insects have a high surface area for the penetration and subsequent systemic distribution of an insecticide from contact exposure. Furthermore, the small size generates short pathways to the insect's nervous system and as a result, most insecticides act on the insect's nervous system [52]. Organochlorines act specifically on the peripheral nervous system, where they bind and stabilize the open voltage-gated sodium channels [25]. The stabilized open state of the sodium channels allows for continuous sodium influx and prolonged action potentials leading to spontaneous neuronal firings succeeded by muscle twitches and sustained body tremors [46]. In contrast to the organochlorines, pyrethroids act on both the peripheral and central nervous systems; however, they act in a similar manner to prevent the closing of the voltage-gated sodium channels, resulting in continuous neuronal discharges followed by paralysis [46]. Similarly, the organophosphates and carbamates also exert their effects on the central nervous system. However, these insecticide classes inhibit acetylcholinesterase, a principal enzyme in the insect nervous system, which leads to an increase in the neurotransmitter ACh levels in the synapse. This leads to enhanced ACh effects on the cholinergic receptors

resulting in constant neurotransmission and neuronal hyperexcitation [53]. On the other hand, the neonicotinoids enhance cholinergic activity by acting as agonists on nicotinic acetylcholine receptors (nAChRs). Similarly, this disrupts neuronal transmission in the insect nervous system, causing paralysis and subsequent insect death [54]. Since the insect nervous system is the target site for all of the major insecticide classes, it is worth discussing it in more detail.

2.3. Insect Nervous System

The insect nervous system is composed of central, visceral and peripheral nervous systems [55]. The insect central nervous system (CNS) is composed of the ventral nerve cord and brain connected to various ganglia including supra- and sub-esophageal ganglia, thoracic ganglia and abdominal ganglia (Figure 1). The sub-esophageal ganglion transmits impulses to the mouthparts and salivary glands. The insect brain is composed of three cephalic neuromeres, including the protocerebrum, deutocerebrum and tritocerebrum. The deutocerebrum carries out olfactory and sensory functions through the antennae; where the olfactory signal transduction is important in host identification and interaction by the insect. The tritocerebrum nerves innervate the ventral nerve cord and internal organs including the anterior digestive canal [55–57]. The insect's peripheral nervous system, commonly referred to as a stomatogastric nervous system is composed of the peripheral ganglia complex and nerves that innervate visceral organs. This system mainly controls food intake and digestion. Generally, the insect CNS ganglia receive sensory impulses from the appendages and body cuticle after which the efferent signals are sent to the body muscles, internal organs and genitalia [58,59]. The protocerebrum controls the insect's vision through compound eyes and ocelli. Most importantly, neurosecretory cells are located in the protocerebrum [59,60]. Most insecticides including the organophosphates and carbamates affect neurotransmitter secretion and action [61,62]. As a result, the insect neurotransmitters, more specifically ACh and its transmission cascade, will be discussed in more detail.

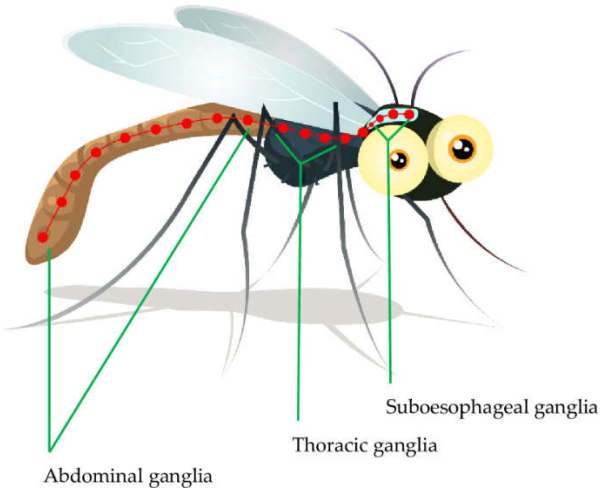


Figure 1. The nervous system ganglia of *Anopheles* [55].

2.3.1. Insect Neurotransmitters

The protocerebrum of the insect brain has nerves that innervate the corpora cardiaca, an organ located posterior to the brain that is composed of the neurohemal and endocrine sections. This enables the corpora cardiaca to perform the important role of neurotransmitter storage and release [55]. Biogenic amines play an important role in the insect nervous system as neuromodulators, neurohormones and neurotransmitters. Moreover, biogenic amines are also a key role player in associative learning and memory for insects. Common neurotransmitters found in the insect CNS include biogenic amines such as

dopamine, 5-hydroxy-tryptamine, octopamine, noradrenaline and ACh [58,63,64]. In addition, other neurotransmitters important in the insect nervous system are histamine, glutamate and gamma-aminobutyric acid (GABA). GABA is a main CNS inhibitory neurotransmitter, while glutamate plays an excitatory role both centrally and peripherally [55,65]. For example, the insect heart rate and contraction are regulated by glutamate and other neurotransmitters including ACh, norepinephrine, dopamine, serotonin and octopamine [66]. ACh is a key excitatory neurotransmitter in the insect nervous system [67].

2.3.2. Acetylcholine and Its Function in Insects

ACh is synthesized from acetyl-coenzyme A and choline in insect cholinergic neurons and stored in nerve terminals inside presynaptic vesicles from which they are released upon detecting the nerve impulse. This impulse activates voltage-gated calcium channels leading to the influx of calcium ions that then induce ACh release from vesicles via exocytosis [68]. After being released in the synapse, ACh exerts its actions by binding to the postsynaptic nAChRs [69]. ACh is the main excitatory neurotransmitter in insects and its binding to nAChRs and generation of excitatory action potential controls the rapid synaptic neurotransmission process [70]. The nAChRs are ligand-gated channels controlled by binding of ACh and its subsequent removal from the binding site. As long as ACh is bound on the postsynaptic nAChRs, the nerve impulses are transmitted. However, this is short-lived as ACh is rapidly hydrolyzed from the binding site by a specialized enzyme, namely AChE. This ensures that the action potentials are initiated at precise and accurate intervals for efficient neurotransmission [71,72].

2.3.3. Acetylcholinesterase and Its Function in Insects

AChE belongs to the general family of cholinesterases; these are the specialized hydrolase enzymes that hydrolyze the choline ester bonds [73]. Therefore, AChE rapidly hydrolyzes the neurotransmitter ACh, to the resultant products, choline and acetate (Figure 2). Through this, it prevents constant nerve firings and maintains normal neuronal impulse transmission at cholinergic synapses and neuromuscular junctions [74,75].

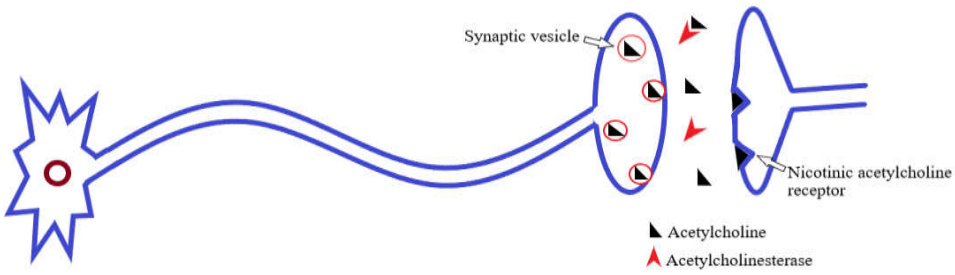


Figure 2. Acetylcholine release, postsynaptic receptor binding, and hydrolysis by acetylcholinesterase [74].

Exclusively in insects, the cholinergic system is localized centrally and is absent at the neuromuscular junctions [76]. The cholinergic system is essential for the functioning of the insect nervous system and AChE is a key enzyme in this system [77]. The functional integrity of insects is maintained by its nervous system, and it is for this reason that most insecticides act on the insects' nervous system [72,78].

2.4. Molecular Characterization of Acetylcholinesterase

There are apparent AChE structural differences between insects and mammals. These span from their distinct genomics, amino acid sequences to their active and peripheral anionic site conformations [76]. Recent biochemical studies have revealed critical differences between the *Anopheles* AChE and human AChE that could serve as potential drug targets for directed insecticide design.

The amino acid sequence of *Anopheles* AChE is reported to be 48–49% identical to that of the human AChE [79,80]. Unlike humans where there is a single *ace* gene coding for AChE, mosquitoes have two *ace* genes, *ace*-1 and *ace*-2, coding for AChE1 and AChE2 enzymes, respectively [81,82]. These genes are crucial in all life stages of the mosquito, ranging from egg through to adult stages [83]. AChE1 is the main catalytic enzyme, while AChE2 is involved in non-catalytic activities such as reproduction. As a result, target site insensitivity on insect AChEs, such as G280S genotype, is linked to mutations in *ace*-1 but not *ace*-2 [51,62,81]. AChE is characterized by a deep and narrow active-site gorge (Figure 3). There are differences in these gorge structures between *Anopheles* and human AChEs and this may affect ligand binding and specificity [51,79,84]. Notably, a free cysteine residue (Cys^{447}) is available at the entrance to the active site gorge of *Anopheles* AChE (Figure 3A,B), but not in human AChE. Instead, a human AChE has a bulky phenylalanine (Phe^{295}) at the active site entrance (Figure 3C). Additionally, in *Anopheles* AChE, a smaller aspartic acid residue (Asp^{602}; Figure 3A,B) replaces a larger tyrosine residue (Tyr^{449}) at the base of the active site gorge [51]. Moreover, a conserved arginine residue (Arg^{339}; not shown in order to maintain the catalytic side resolution) has also been identified in *Anopheles* AChE [85]. In addition, the displayed *An. gambiae* AChE catalytic site in Figure 3B shows a G280S mutated site (pointed).

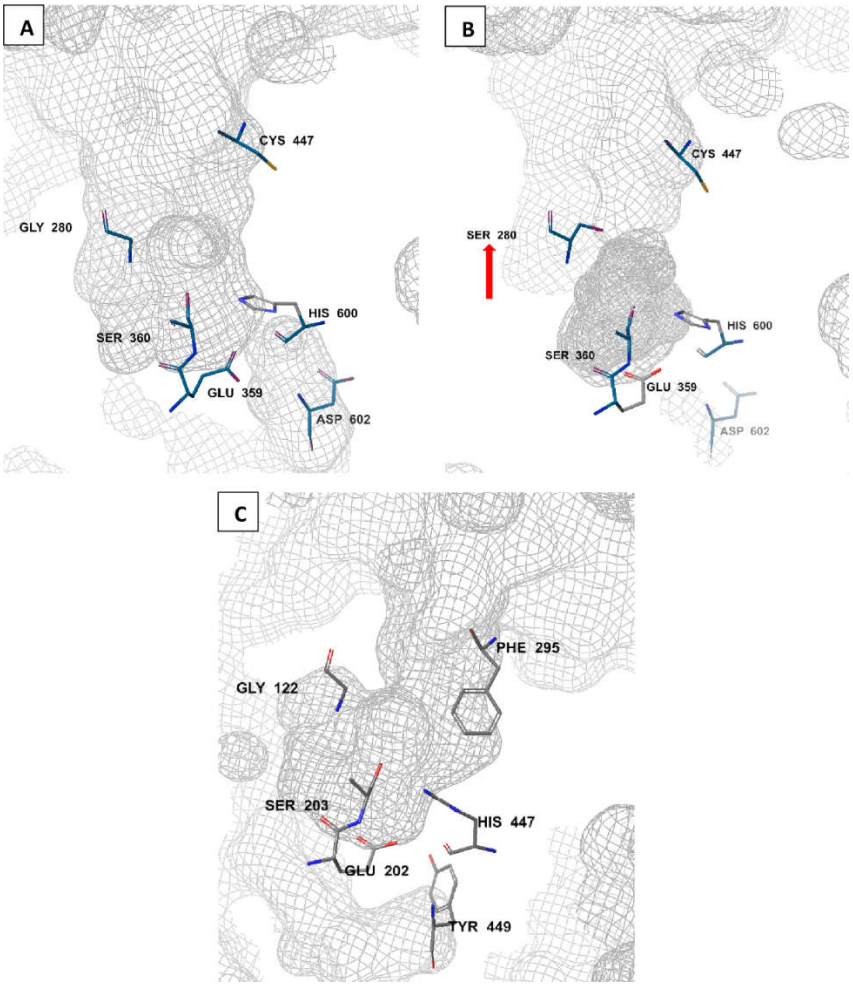


Figure 3. Molecular comparison of *An. gambiae* wild-type (**A**) and resistant (**B**) AChE catalytic sites (PDB IDs: 5YDI and 6ARY, respectively) to the human AChE (PDB ID: 7E3H) (**C**). generated by Schrodinger's Maestro 2018-2 software (New York, NY, USA). The G280S mutation is shown (red arrow) in the resistant *Anopheles* AChE phenotype (**B**) [51,75].

2.4.1. Acetylcholinesterase Inhibition in Anopheles

The catalytic site in *Anopheles* is characterized with a catalytic triad made of His-Ser-Glu (His^{600}-Ser^{360}-Glu^{359}; Figure 3A) amino acid combination. The catalytic serine (Ser^{360}; Figure 3A) is the target for covalent insecticides, including organophosphates and carbamates [61,62]. These insecticides establish a covalent bond with AChE through phosphorylation and carbamoylation, respectively [67]. The inhibition of AChE leads to ACh accumulation that eventually results in over-stimulation of postsynaptic cholinergic receptors [76]. This neuroexcitation causes rapid insect paralysis and death [72,76]. The *Anopheles* resistance to the anticholinesterase insecticide classes is usually caused by *ace*-1 G280S mutation (Figure 3B) and metabolic resistance resulting from the elevated levels of monooxygenases, glutathione-S-transferases and general esterases [62,86–88]. Given the widespread resistance that has largely rendered organophosphates and carbamates non-effective, there is an urgent need to identify novel anticholinesterase insecticides. AChE has proven to be a valid target in *Anopheles* vectors [79] and EOs have also shown to be the promising sources of novel insecticides [89–91]. Interestingly, the EOs have shown activity against resistant *Anopheles* species and a capability to synergize conventional insecticides including the pyrethroids [92,93]. Additionally, the EOs and their constituents inhibit the P450 monooxygenase and glutathione-S-transferase detoxification enzymes involved in multiple insecticide resistance including the pyrethroids, organophosphates and carbamates. The insect toxicity of pyrethroids, organophosphates and carbamates has been greatly enhanced by synergy with EOs including cedarwood oil, geranium oil, clove oil, patchouli oil, cinnamon oil, basil oil, oregano oil, purple nutsedge oil, thyme, coriander and galangal oil [94–96]. The identified individual EOCs capable of synergizing conventional insecticides, especially pyrethroids, include thymol, eugenol, carvacrol, geraniol and linalool [96,97].

Essential Oils and Constituents as Potential Anopheles AChE Inhibitors

EOs are hydrophobic secondary metabolites extracted from different aromatic plant parts by steam distillation, hydro-distillation, head-space analysis, solvent extraction or liquid carbon dioxide extraction [93,98]. Various terpenoids including sesquiterpenes, monoterpenes, diterpenes and phenylpropanoids are major phytoconstituents in EOs [99,100]. Many EOs and certain EOCs have exhibited significant anticholinesterase activity in in vitro studies. In insects, many EOs have been reported to exhibit neurotoxic effects characterized by rapid paralysis and death [98], which links them to AChE inhibition. Interestingly, most EOCs can only inhibit mammalian AChE very weakly. In addition, most EOs and EOCs are relatively non-toxic to mammals with very low potency for acute oral toxicity, whereby lethal doses for pure compounds range from 800 to 3000 mg/kg and more than 5000 mg/kg for EOs or EOCs incorporated in pharmaceutical formulations [93,101,102]. The EOs are natural resources and therefore, the identification of novel insecticides from such sources is relatively cost-effective. Production of insecticides from EOs can be relatively less expensive translating into low costs for the malaria endemic countries [103,104]. In addition, the discovery of insecticides is relatively rapid since the lead compounds are screened on the actual insect as opposed to pharmaceuticals intended for human use that undergo a lengthy translational science process from the in vitro to the in vivo studies including animal models and human clinical trials [105].

In vitro anticholinesterase activity is usually assessed with the Ellman assay, a globally accepted method to assess the AChE activity and potential inhibition thereof [106]. For such studies, the insect homogenate may be used as a crude enzyme source [107–109]. However, some studies use AChE from *Electrophorus electricus* (electric eel) to estimate

insect AChE activity [110,111]. The electric eel and *Anopheles* AChEs are reported to have nearly the same backbone conformation [85]. For the purpose of this review, EOs or EOCs inhibiting AChE in the in vitro studies are only considered to have potential *Anopheles* anticholinesterase activity if they have shown *Anopheles* insecticidal activity in addition to the observed AChE inhibition. Furthermore, where available, the selectivity between human and *Anopheles* AChE targets, as well as human toxicity potential for the EOs and EOCs, are extensively discussed.

The EOs as Potential Anticholinesterase Insecticides

Several EOs have shown insecticidal activity against *Anopheles* species [93]. Given their high volatility, the EOs can reach their target through inhalation by the insect species, ingestion or contact [112]. The absorption of EOs is facilitated by the lipophilic character and low molecular weights of their active constituents [100,112]. This behavior is also important for the partitioning of EOs into the insect midgut plasma membrane [112,113].

Some of the EOs have not only shown the insecticidal effects, but also exhibited anticholinesterase activity indicating a potential to be anticholinesterase insecticides [102]. With increasing resistance against current insecticides, the potential use of EOs as alternative insecticides has been suggested [93]. Various EOs have shown potential to be active against resistant colonies including pyrethroid resistant *Anopheles* species, such as *An. gambiae* and *An. stephensi* [93,114,115]. Furthermore, the EOs and EOCs have shown potential to synergize with conventional insecticides and to inhibit those enzymes responsible for insecticide detoxification, which shows promise in overcoming resistance [88,92,94]. This study identified essential oils from 16 plant species with anticholinesterase potential and corresponding insecticidal capabilities (Table 1). Seven of these exhibited both anticholinesterase and insecticidal activities at IC_{50} and LC_{50} values less than 100 μg/mL, indicating high potencies [116]. These highly potent EOs with potential of being anticholinesterase insecticides include *Hyptis suaveolens*, *Hyptis spicigera*, *Ocimum canum*, *Lantana camara*, *Ferulago carduchorum, Ferulago trifida*, *Salvia officinalis* and *Curcuma longa* (Table 1); the latter EOs belong to the general plant families of Lamiaceae, Verbenaceae, Apiaceae and Zingiberaceae [93,117,118].

Table 1. Anticholinesterase and insecticidal activities of EOs.

Essential Oil	Anticholinesterase IC_{50}	Insecticidal Activity	Insecticidal LD_{50}	*Anopheles* Species (Laboratory Strain)	Reference(s)
Carum carvi	0.82 ± 0.05 mg/mL	Larvicidal	72.00 μg/mL	*An. dirus*	[93,119]
Citrus limon	0.85 ± 0.01 mg/mL	Larvicidal	13.75 μg/mL	*An. gambiae (Kisumu)*	[120,121]
			32.28 μg/mL	*An. gambiae Logbessou*	
Curcuma longa	34.70 ± 3.10 μg/mL	Larvicidal	33.61 μg/mL	*An. cracens*	[122–124]
		Larvicidal	1.8–3.7 μg/mL	*An. quadrimaculatus*	
Eucalyptus globulus Labill.	0.13 ± 0.01 mg/mL	Larvicidal	0.09 mg/mL	*An. arabiensis*	[121,125]
Ferulago carduchorum	23.6 μg/mL	Larvicidal	12.00–12.78 μg/mL	*An. stephensi*	[93,126]
Ferulago trifida	21.5 ± 2.2 mg/mL	Larvicidal	10.46 μg/mL	*An. stephensi*	[127]
Foeniculum vulgare Mill.	1.19 ± 0.01 mg/mL	Larvicidal	35.30 μg/mL	*An. dirus*	[93,121,128]
		Larvicidal	20.10 μg/mL	*An. stephensi*	
Hyptis spicigera Lam.	6.3 ± 0.43 μg/mL	Ovicidal Larvicidal Adulticidal	69.61 μg/mL 45.18 μg/mL 1.45% *w/v*	* *An. gambiae*	[110,129]
		Adulticidal	1.04% *w/v*	*An. gambiae* (Kisumu)	
Hyptis suaveolens Poit.	0.55 ± 0.12 μg/mL	Ovicidal Larvicidal Adulticidal	61.71 μg/mL 159.5 μg/mL 1.86% *w/v*	* *An. gambiae*	[110,129]
		Adulticidal	0.85% *w/v*	*An. gambiae* (Kisumu)	
Lantana camara L.	1.75 ± 0.12 μg/mL	Ovicidal Larvicidal Adulticidal	53.59 μg/mL 61.69 μg/mL 0.84% *w/v*	* *An. gambiae*	[110,129]
		Adulticidal	0.24% *w/v*	*An. gambiae* (Kisumu)	
Mentha pulegium L.	108.75 μg/mL	Larvicidal	40.13–113.6 μg/mL	*An. stephensi*	[130–132]

		Larvicidal	118.0 μg/mL	*An. gambiae*	
		Larvicidal	58.9 μg/mL	*An. atroparvus*	
Ocimum canum Sims.	0.21–26.16 μg/mL	Ovicidal	71.56 μg/mL	* *An. gambiae*	[93,110,129,133]
		Larvicidal	138.7 μg/mL		
		Adulticidal	1.84% *w/v*		
		Adulticidal	1.22% *w/v*	*An. gambiae* (Kisumu)	
		Larvicidal	74.12 μg/mL	*An. funestus*	
Salvia leucantha	>250 μg/mL	Larvicidal	10.9 μg/mL	*An. quadrimaculatus*	[93,134]
Salvia officinalis	47.68–77.51 μg/mL	Larvicidal	14.1 μg/mL	*An. quadrimaculatus*	[93,135]
Schinus molle L.	6.54 ± 0.15 mg/mL	Larvicidal	21.0 μg/mL	*An. arabiensis*	[136,137]
Thymus vulgaris	0.22–0.54 mg/mL	Larvicidal	351.63 μg/mL	*An. labranchiae*	[121,138,139]

* Wild population.

Notably, one EO could exhibit considerable differences in anticholinesterase and/or insecticidal activity (Table 1). This is probably due to variations in the identity or quantity of its specific phytoconstituents. Three *Salvia officinalis* EOs from different locations in Italy exhibited anticholinesterase activity at IC_{50} values of 47.68, 58.35 and 77.51 μg/mL [135]. While these EOs had a similar content of camphor (16.84%, 16.16% and 18.92%, respectively) as the main constituent, it was notable that the borneol content in the third EO (IC50: 77.51 μg/mL) was approximately half (2.34%) of that yielded from the former EOs (4.48% and 4.68%, respectively) [135]. Borneol has been previously shown to inhibit AChE [121]. Using similar experimental conditions and AChE enzyme concentrations, the *Ocimum canum* Sims. EOs from Burkina Faso inhibited AChE at the IC_{50} value of 0.21 μg/mL [110] and a relatively higher value of 36.16 μg/mL [133]. The latter EO had 59.9% content of 1,8-cineole as the main constituent, while the composition of the former and more active EO was not mentioned [110,133]. *Mentha pulegium L.* EO from Iran exhibited the larvicidal activity against *An. stephensi* with the LC_{50} value of 40.13 μg/mL [130], while that from Portugal attained the LC_{50} of 113.6 μg/mL against the same *Anopheles* species [132]. *Mentha pulegium* L. EO from Portugal had 61.4% pulegone and 20% menthone as the main constituents, while the phytochemical analysis was not reported for the *Mentha* species from Iran [130,132].

Major constituents in EOs are often responsible for the observed biological activity associated with such a sample [100,140,141]. For this reason, this review collected phytochemical data on the major components in the identified bioactive EOs (Table 2). Some of these major constituents possess anticholinesterase and insecticidal activity as displayed in Table 3. Common constituents in most EOs include α-pinene, β-pinene, p-cymene, γ-terpinene and β-caryophyllene. Certain EOCs such as terpinen-4-ol, 1,8-cineole, menthone, menthol, fenchone, γ-terpinene, (-)-bornyl acetate, linalool, citral and pulegone have been reported as competitive AChE inhibitors [98,142–144]. Additionally, common constituents in seven of the most active EOs, especially *Hyptis suaveolens*, *Hyptis spicigera*, *Ocimum canum*, *Lantana camara*, *Ferulago carduchorum, Ferulago trifida* and *Salvia officinalis* are α-pinene, β-pinene, β-caryophyllene and γ-terpinene (Table 2). The *Curcuma longa* EO has a unique composition profile of ar-turmerone, tumerone, curlone, α-curcumene and β-sesquiphellandrene [123]. The main constituent ar-turmerone has only attained anticholinesterase activity against human AChE with an IC_{50} value of >100 μg/mL (IC_{50}: 191.1 ± 0.3 μg/mL), indicating a low potency [116]. *Curcuma longa* EO has been reported as possessing an anticholinesterase with an IC_{50} value of 34.70 ± 3.10 μg/mL. The *Curcuma longa* EO has also been reported to possess strong larvicidal activity with the LC_{50} range of 1.5 to 34 μg/mL [122,123], indicating that it may be selectively targeting the insect.

Table 2. Major constituents of EOs with anticholinesterase insecticidal activity.

Essential Oil	Major Constituents	References
Carum carvi	γ-Terpinene, β-pinene, bornyl acetate, carvone, *p*-cymene	[119]
Citrus limon	Limonene	[121]
Curcuma longa	ar-Turmerone, tumerone, curlone, α-curcumene, β-sesquiphellandrene	[123]
Eucalyptus globulus Labill.	Eucalyptol, α- pinene, *p*-cymene, β-cymene, 1,8-cineole, limonene	[121,145,146]
Ferulago carduchorum	(*Z*)-β-ocimene, α-pinene, bornyl acetate	[126]
Ferulago trifida	Isobornyl acetate, trans-verbenol, (*E*)- β-caryophyllene	[127]
Foeniculum vulgare Mill.	Thymol, estragole, α-phellandrene, limonene, (*E*)-anethole, fenchone,	[121,123,147]
Hyptis spicigera Lam.	β-caryophyllene, α-pinene, β-pinene, α-phellandrene, α-thujene, sabinene	[129,148,149]
Hyptis suaveolens Poit.	1,8-cineole, sabinene, terpinolene α-thujene, α- pinene, β-pinene	[129,150,151]
Lantana camara L.	β-caryophyllene, α-humulene, γ-curcumene, germacrene D	[152,153]
Mentha pulegium L.	Pulegone, neomenthol, menthone	[131,132]
Ocimum canum Sims.	Thymol, *p*-cymene, γ-terpinene, estragole, linalool	[154,155]
Salvia leucantha	Bornyl acetate, 6,9-guaiadiene, (*E*)-β-caryophyllene, bicyclogermacrene, camphene, α- pinene, β-pinene	[117,134]
Salvia officinalis	Camphor, camphene, α-thujone, 1,8-cineole, α- pinene, β-pinene	[135]
Schinus molle L.	α-Phellandrene, β-phellandrene, α- pinene, β-pinene, γ-terpinene, *p*-cymene	[136]
Thymus vulgaris	*p*-Cymene, borneol, thymol, carvacrol	[121]

The EOCs as Potential Anticholinesterase Insecticides

Some studies have isolated the active principles from EOs and demonstrated the potential of these to exert *Anopheles* anticholinesterase activity [98,156]. Moreover, some of these have also exerted *Anopheles* mortality in insecticide susceptibility assays [93,131,157]. Gnankiné and Bassolé (2017) reported that several constituents in EOs possess ovicidal, larvicidal and adulticidal effects against *Anopheles* species. In this study, 22 EOCs from various terpenoid classes such as sesquiterpene alcohols, sesquiterpene oxides, monoterpenoids and monoterpene alcohols, as well as phenylpropanoids, have been identified as possessing both anticholinesterase and insecticidal activity against *Anopheles* species (Table 3). Some of these EOCs, such as 1,8-cineole, α-pinene, camphor, linalool, borneol, (+)-3-δ-carene, γ-terpinene, caryophyllene oxide, *p*-cymene, (*E*)-anethole, terpinen-4-ol, pulegone and limonene, have shown low potency towards human AChE [98,102,158–160].

The α-pinene, estragole, carvacrol, (+)-δ-3-carene, eugenol and camphor were the most active in terms of both AChE inhibition and insecticidal activity (Table 3); thus, indicating that these EOCs show potential to serve as anticholinesterase insecticides. Interestingly, previous studies have shown that these EOCs have a lower activity against human AChE. For example, camphor and α-pinene could only inhibit AChE from human erythrocytes with IC_{50} values of >10 mM and 0.4–0.7 mM, respectively [159,161]. Similarly, an IC_{50} range of 0.2 to 0.3 mM of (+)-3-δ-carene was needed to inhibit bovine and human erythrocytes AChE [159,161]. These EOCs may therefore produce insecticides with high selectivity towards *Anopheles* AChE inhibition over the human target.

Monoterpenoids: The monoterpenoid α-pinene is the main constituent in many essential oils [93]. Orhan et al. (2008) reported that α-pinene, but not β-pinene, has anticholinesterase properties [162]. α-Pinene has shown anticholinesterase activity at an IC_{50} value as low as 22 μg/mL [163]; however, this was higher than its parent EOs from *Hyptis suaveolens* and *Hyptis spicigera* that obtained IC_{50} values between 0.5 and 6.5 μg/mL [110]. Apart from the fact that different *Anopheles* species were used, this suggests the possibility of synergism among the EOCs in such EOs. Due to their smaller molecular weights, more than one monoterpenoid can bind to the AChE catalytic and/or peripheral site and, usually, binding of one monoterpenoid facilitates binding of the other [102]. On the other hand, α-pinene exhibited larvicidal activity (LC_{50} of 32.1 μg/mL) against *An. subpictus* that was comparable to its anticholinesterase activity [93,163]. In another study, Wojtunik-Kulesza et al. (2017) reported a higher anticholinesterase IC_{50} value of 102.0 mM for α-pinene along with declaration that insolubility of the EOCs affected the accuracy of

spectrophotometric measurements [164]. Generally, the observed variations were caused by the use of different AChE types and protein contents. For example, using 0.25 U/mL AChE, Farag et al. (2016) determined an IC_{50} of 0.337 μM for estragole; meanwhile, Lopez et al. (2015) doubled the AChE concentration and obtained an IC_{50} value of 12.6 mM [102,165]. The purity and stability of reagents and EOs or EOCs used in AChE activity assays may also affect the outcome [166,167]. Additionally, AChE activity is a pH-dependent reaction [168] and variations in pH across studies may cause potential differences in inhibition kinetics.

A major constituent in the EO of *Echinophora lamondiana*, (+)-δ-3-carene, resulted in comparable anticholinesterase (IC_{50} value: 36 μg/mL) and larvicidal (LC_{50} value of 42.9 μg/mL) activity against *An. quadrimaculatus* [169,170]. The latter larvicidal LC_{50} value of the EOC was observed to be similar to that obtained for the EO of the flowers (LC_{50}: 46.9 μg/mL) with 61.9% (+)-δ-3-carene content, but higher than the EOC of the leaves (LC_{50}: 26.2 μg/mL) with comparable (+)-δ-3-carene content (75.0%) [169]. Though less predominant than (+)-δ-3-carene in the *Echinophora lamondiana* EO, both terpinolene (2.7 to 3.3%) and α-phellandrene (12.8 to 20.3%) were reported to be even more potent larvicidal agents than (+)-δ-3-carene, with LC_{50} values of 20.9 and 15.6 μg/mL, respectively [169].

Camphor, a monoterpenoid ketone and the major component in the EOs of *Ocimum africanum* and *Ocimum americanum*, was also shown to exhibit low anticholinesterase activity (IC_{50} value of 21.43 μM) [165]. Moreover, camphor is known to non-competitively inhibit nAChRs [98,102,171]. Camphor is also active as a repellent against *An. culicifacies*, *An. gambiae* and *An. funestus* [172–174]. However, poor larvicidal activity by camphor was obtained with a LC_{50} value >100 μg/mL [175].

Aazza et al. (2011) reported that the EO of *Thymus vulgaris* consists of 16% carvacrol. Interestingly, carvacrol is more than three times as active (IC_{50} value of 63.0 μg/mL) as its parent EO (IC_{50} value of 216.9 μg/mL) in AChE inhibition studies [121,139]. This indicates the potential effect of antagonistic interactions within the complex mixture of the *Thymus vulgaris* EO resulting in decreased activity. However, the *Anopheles* anticholinesterase potential of carvacrol (IC_{50} value of 63.0 μg/mL) is non-specific as its activity is comparable to that against AChE from bovine serum (IC_{50} value of 70.3 μg/mL) [139,162]. Stereochemistry in AChE inhibition also plays a role, whereby carvone (a monoterpenoid ketone) attained weak AChE inhibition (IC_{50} value of 830 μg/mL), in comparison to the structurally related phenolic monoterpenoid, carvacrol (IC_{50} value of 63.0 μg/mL) [93,139,164]. Carvone has previously been reported to be a potent non-competitive AChE inhibitor of mammal AChE [102,162].

Another phenolic monoterpene, thymol, is ineffective in inhibiting AChE, but is a positive allosteric modulator of the insect's GABA-A receptors [118,164]. Thymol has also been reported to act at the octopamine receptors; this is where octopamine is an analogue of norepinephrine, functioning as a neurotransmitter, neuromodulator and neurohormone in insects [176]. Most studies have shown thymol to be as ineffective as its parent EO *Thymus vulgaris* against the AChE target [121,139].

Phenylpropanoids: Estragole is a phenylpropanoid isolated from the EOs of the *Ocimum* species. Along with strong anticholinesterase activity (IC_{50} value of 0.337 μM) [165], it has shown similarly potent larvicidal activity against *An. stephensi* (LC_{50} value of 11.01 μg/mL) and *An. atroparvus* (LC_{50} value of 15.7 μg/mL) [102,118]. Estragole was reported to be potentially selective for *Anopheles*, as it has shown no inhibition of mammal AChE [162].

Eugenol, an allyl chain-substituted guaiacol (phenol), is a major constituent (31.12%) in the *Plectranthus barbatus* EO, an EO with the LC_{50} value of 84.2 μg/mL, against *An. subpictus* larvae [177]. However, eugenol is three times more potent (LC_{50} value of 25.45 μg/mL) than this latter parent EO as a larvicide against *An. subpictus* [93]. Again, this suggests possible antagonism with other constituents. Eugenol and (*E*)-anethole (a phenylpropanoid) reportedly interacted in an antagonistic manner when tested for larvicidal activity [178]. Although eugenol had an IC_{50} value of 40.32 μg/mL for AChE inhibition, this inhibitory activity was however comparable to that attained against bovine

erythrocytes AChE (42.44 ± 1.21 μg/mL) [165,179]. In contrast, Dohi et al. (2009) reported a much higher IC_{50} value for eugenol (480 μg/mL) against electric eel AChE [163]. Based on these two findings, eugenol has a potential nonselective AChE inhibition, and to be an antagonist at insect octopamine receptors [93].

Table 3. Anticholinesterase and insecticidal activities of EOCs.

Essential Oil Constituent	Anticholinesterase IC_{50}	Insecticidal Activity	Insecticidal LD_{50}	*Anopheles* Species (Laboratory Strain)	Reference(s)
(E)-Anethole	1.324 ± 0.011 mg/mL	Larvicidal	25.11 μg/mL	* *An. sinensis*	[121,178]
Borneol	0.132 ± 0.012 mg/mL	Larvicidal	35.89 μg/mL	*An. anthropophagus*	[121,180]
Camphor	0.003–1.7 mg/mL	Larvicidal	129.17 μg/mL	*An. anthropophagus*	[102,165,175]
(+)-δ-3-Carene	0.036–0.64 mg/mL	Larvicidal	42.9 μg/mL	*An. quadrimaculatus*	[169,170]
Carvacrol	0.063–0.092 mg/mL	Larvicidal	21.1–24.06 μg/mL	*An. subpictus*	[121,139,181]
		Larvicidal	21.15 μg/mL	*An. stephensi*	
Carvone	0.437–0.83 mg/mL	Larvicidal	19.3 μg/mL	*An. stephensi*	[102,157,164]
Caryophyllene oxide	>200 μg/mL	Larvicidal	49.46 μg/mL	*An. anthropophagus*	[93,175,182]
Estragole	0.337 μM–12.6 mM	Larvicidal	15.7 μg/mL	*An. atroparvus*	[102,118,165]
			11.01 μg/mL	*An. stephensi*	
Eucalyptol	266.0 ± 11.87 mM	Larvicidal	>200 μg/mL	*An. anthropophagus*	[93,102,164]
Eugenol	40–480 mg/mL	Larvicidal	93.14 μg/mL	*An. stephensi*	[163,165,178,181,183]
			25.45 μg/mL	*An. subpictus*	
			31.09 μg/mL	* *An. sinensis*	
Isopulegol	233.0 ± 10.08 mM	Larvicidal	49.4 μg/mL	*An. gambiae s.s*	[93,102]
Limonene	220–586 μg/mL	Larvicidal	8.8 μg/mL	*An. stephensi*	[121,184–186]
		Larvicidal	18.91 μg/mL	*An. sinensis*	
Linalool	1.69–2.4 mg/mL	Larvicidal	35.87 μg/mL	*An. anthropophagus*	[102,164,180]
(Z)-*β*-Ocimene	4.7 ± 0.20 mM	Larvicidal	25.84 μg/mL	*An. stephensi*	[118,164]
			30.86 μg/mL	*An. subpictus*	
α-Phellandrene	0.12–3.68 mg/mL	Larvicidal	15.6 μg/mL	*An. quadrimaculatus*	[102,169]
α-Pinene	0.022–1.43 mg/mL	Larvicidal	32.1 μg/mL	*An. subpictus*	[163–165,187]
Pulegone	9.0 ± 0.41 mM	Larvicidal	48.9 μg/mL	*An. stephensi*	[141,164]
Sabinene	176.5 ± 2.8 μg/mL	Larvicidal	19.67 μg/mL	*An. stephensi*	[118]
Terpinen-4-ol	0.19–3.2 mg/mL	Larvicidal	47.73 μg/mL	*An. subpictus*	[143,156,163,186,188]
		Larvicidal	43.27 μg/mL	*An. stephensi*	
		Larvicidal	62.09 μg/mL	*An. sinensis*	
		Larvicidal	337.7 μg/mL	*An. gambiae s.s*	
γ-Terpinene	5.8 mM	Larvicidal	44.61 μg/mL	*An. anthropophagus*	[102,180,186]
		Larvicidal	36.42 μg/mL	*An. sinensis*	
α-Terpineol	1.3 ± 0.06 mg/mL	Larvicidal	39.98 μg/mL	*An. sinensis*	[163,186]
Terpinolene	156.4–550.0 μg/mL	Larvicidal	404.71 μg/mL	*An. gambiae s.s*	[93,118,156,169,170]
		Larvicidal	20.9–25.7 μg/mL	*An. quadrimaculatus*	
Thymol	0.05–0.74 mg/mL	Larvicidal	10.3–22.06 μg/mL	*An. subpictus*	[118,139,141,189,190]
		Larvicidal	48.88 μg/mL	*An. stephensi*	

* Wild population.

While showing potent insecticidal activity, some EOs and EOCs did not show any evidence that their mode of action involved targeting AChE. Pulegone has a potent insecticidal activity against *An. stephensi*, however, it could not efficiently inhibit AChE [141,164]. In contrast, the epoxide form of pulegone, the pulegone-1,2-epoxide isolated from the *Lippia steochadifolia* EO, has be reported to be an insect neurotoxin, acting as an irreversible inhibitor of AChE [191]. In addition, the *Citrus limon* EO and it major EOC, limonene (99%) both possess weak AChE inhibition properties; whilst both are potent larvicides against *An. gambiae* and *An. stephensi* [120,121,184]. This suggests that a different mode of action may be responsible for their insecticidal activity. In *Cimex cimicidae* (bedbugs), limonene has been proposed as acting by destroying the wax layer of the insect respiratory system [192].

This review identified EOs and specific EOCs with anticholinesterase activity and indeed the capacity to cause *Anopheles* mortality. The EOs and EOCs spectrum of activity

against *Anopheles* included ovicidal, larvicidal and adulticidal activities, where they were regarded as active if they obtained LC_{50} values less than 100 μg/mL [193]. The common *Anopheles* species involved in global malaria transmission that have been assessed include *An. stephensi*, *An. gambiae*, *An. arabiensis*, *An. subpictus*, *An. anthropophagus*, *An. quadrimaculatus*, *An. dirus*, *An. cracens*, *An.labranchiae*, *An. sinensis* and *An. atroparvus* [194,195]. Interestingly, almost all of these have already developed clinically significant resistance against current insecticides [194,196–198]. The commonly assessed *An. Stephensi*, that was susceptible to many of the EOs and EOCs, is predominant in Asia and recently invaded Africa [199,200]. This species is resistant to conventional AChE insecticides, including organophosphates and carbamates, and has also shown resistance to other insecticide classes such as pyrethroids and organochlorines [201,202]. The EO of *Ferulago carduchorum* has shown both larvicidal and anticholinesterase activity at LC_{50} and IC_{50} values around 12 μg/mL, which are extremely promising [126].

Interestingly, the EOs of *Hyptis suaveolens*, *Hyptis spicigera*, *Ocimum canum* and *Lantana camara* have shown activity against all three developmental stages of *An. gambiae*, a main African vector. Meanwhile, the EO of *Mentha pulegium* and *Ocimum canum* have been shown to target the larval stage of *An. gambiae* [93,131] and *An. funestus* (LC_{50} value of 91.2 μg/mL), respectively [93,195,203]. Of all the species, *An. arabiensis* is the main vector in Sub-Saharan Africa [48] against which the EOs of *Schinus mole* and *Eucalyptus globulus* have displayed larvicidal activity with LC_{50} values less than 100 μg/mL. However, the anticholinesterase activity of these latter EOs, as well as their major EOCs, α-phellandrene and eucalyptol, respectively, were in the millimolar range indicating that their larvicidal activity is not primarily through AChE inhibition. Both *Schinus mole* and *Eucalyptus globulus* EOs are non-toxic towards mammals and the *Eucalyptus globulus* EO is recommended for skin application as a repellent [93,204].

It is crucial to identify novel insecticides from EOs as most are relatively safe to mammals [93,118]. An EO of *Foeniculum vulgare* could only exhibit acute oral toxicity at the LD_{50} of 3120 mg/kg in the rat model [118]. This EO is active against *An. stephensi* and *An. dirus* with LD_{50} values between 20 and 35 μg/mL, thus indicating a high safety index for human use [93]. The EOCs, (*E*)-anethole, eugenol, limonene, thymol and γ-terpinene, displayed acute oral toxicity at the LD_{50} values ranging from 980 to 4600 mg/kg [118] where all attained insecticidal LC_{50} values less than 100 μg/mL (Table 3); thus, indicating their potential as bioinsecticides.

3. Conclusions and Future Perspective

The *Anopheles* vectors are responsible for malaria transmission across the world; additionally, most of these vectors have acquired resistance against current insecticide classes. *Anopheles* AChE is a valid target by conventional AChE inhibitors in the market. Due to the current resistance status towards anticholinesterase insecticides, organophosphates and carbamates, the identification of novel insecticides is critical. The EOs and EOCs have shown potential to serve as anticholinesterase insecticides against *Anopheles* vectors. This is afforded by the capability of some EOs and EOCs to exhibit both anticholinesterase and insecticidal activity. In this review, seven EOs from *Hyptis suaveolens*, *Hyptis spicigera*, *Ocimum canum*, *Lantana camara*, *Ferulago carduchorum*, *Ferulago trifida*, *Salvia officinalis* and *Curcuma longa* plant species which showed potent anticholinesterase and insecticidal activities against various *Anopheles* species were summarized. Along with these, six EOCs, namely, α-pinene, estragole, carvacrol, (+)-3-δ-carene, eugenol and camphor, were identified as being the most active against the *Anopheles* vector and AChE target. All of these, except for eugenol and carvacrol, have the potential to be selective towards *Anopheles* AChE. This scientific data review is important for informing future research towards novel anticholinesterase insecticides for malaria vector control. Future studies in this area should focus on the EOs and EOCs identified in this review for possible development into insecticidal agents. Active constituents should be identified from the EO complex mixtures and possible synergistic interaction taken into consideration. The EOs and the

identified EOCs in this review require further assessment against various stages of the *Anopheles* life cycles and potential activity against insecticide resistant *Anopheles* vectors. Moreover, these promising EOs and EOCs should be assessed for possible activity against human AChE, toxicity against other aquatic lives, as well as for mammal toxicity.

In general, this review supports the future development of EOs as a potential source for novel insecticides with AChE inhibitory potential; including the specific EOs with potential anticholinesterase and insecticidal activities outlined in this review, belonging to the families of Lamiaceae, Verbenaceae, Apiaceae and Zingiberaceae. Meanwhile, the promising EOCs for development into novel anticholinesterase insecticides belong to broader classes of sesquiterpene alcohols, monoterpenoids, monoterpene alcohols and phenylpropanoids.

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Appendix B.1: Optimization of covalent docking for organophosphates interaction with *Anopheles* acetylcholinesterase (Chapter 3; published paper)

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Optimization of covalent docking for organophosphates interaction with *Anopheles* acetylcholinesterase

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ABSTRACT

Organophosphates (OPs) used as potent insecticides for malaria vector control, covalently phosphorylate the catalytic serine residue of *Anopheles gambiae* AChE (*Ag*AChE) in a reaction that liberates their leaving groups. In the recent 10-year insecticide use assessment, OPs were the most frequently used World Health Organization prequalified insecticides. Molecular modelling programs are best suited to display molecular interactions between ligands and the target proteins. The docking modes that generate ligand poses closer to the binding site show high accuracy in predicting the ligand binding mode. The implicit solvation approach such as molecular mechanics-generalized born surface area (MM-GBSA) is a more reliable method to predict ligand onformations and binding affinities. Apart from covalent docking studies being scarce, current molecular docking programs do not adequately possess the covalent docking reaction algorithm to display the molecular mechanism of OPs at the *Ag*AChE catalytic site. This results into OP docking studies commonly being conducted through noncovalent pannels. The aim of this study was to establish the optimim covalent docking system for OPs through manual customization of Schrödinger's Glide covalent docking reaction algorithm. To achieve this, a newly customized covalent reaction algorithm was assessed on a set of ligands covering aromatic, non-aromatic and hydrophobic OPs and compared to the noncovalent docking results in terms of reliability based on the reported X-ray diffraction molecular interactions and crystal poses. The study established that by virtue of omitting the well-known OP hydrolysis, noncovalent mode suggested molecular interactions that were further from the catalytic triad and could not otherwise occur when the molecule is hydrolyzed as in the customized covalent docking mode. Moreover, the MM-GBSA concurred with the optimized covalent docking in eliminating such inaccurate molecular interactions. Additionally, the covalent docking mode confined the interactions and ligand poses to the catalytic site indicating relatively high accuracy and reliability. This study reports the optimized covalent docking panel that effectively confirmed the molecular mechanisms of OPs, as well as indentifying the corresponding amino acid residues required to stabilize the aromatic, non-aromatic and hydrophobic OPs at the *Ag*AChE catalytic site in line with the reported X-ray diffraction studies. As such, the proposed manual customization of the Schrödinger's Glide covalent docking platform can be used to reliably predict molecular interactions between OPs and *Ag*AChE target.

1. Introduction

Acetylcholinesterase (AChE), a serine hydrolase located at the cholinergic brain synapses and neuromuscular junctions, attains normal neuronal impulse transmission by hydrolyzing the neurotransmitter, acetylcholine, forming acetate and choline [1]. This enzyme has been an effective target in malaria vector control using organophosphates (OPs) and carbamates, the largest group of insecticides. The OPs were the most frequently used World Health Organization (WHO) prequalified insecticides in a recent 10-year survey [2]. The OPs are considered more potent than carbamates due to the "aging" process that forms a true covalent bond resulting in the permanent deactivation of AChE enzyme [3,4]. These phosphate ester compounds get hydrolyzed by AChE to a covalently bonded dialkylphosphate and release a leaving group, one of

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the singly bonded substituents with the weakest bond (Fig. 1) [5]. The insecticidal activity of OPs is achieved by this irreversible inhibition of AChE leading to continuous cholinergic impulse transmission and subsequent death from neuronal hyperexcitation [6]. This mecahnism of action is evident by the efficacy of malathion, an OP insecticide used in malaria vector control, that is known to exert 100% mortality to susceptible *Anopheles* populations at 5% (v/v) concentration [7].

Common malaria vectors are of the *Anopheles gambiae* complex [8]. The genes encoding the AChE of *An. gambiae* (*Ag*AChE) and corresponding amino acid sequences have been previously characterized [9]. The amino acid residues involved in the OP hydrolysis is a catalytic serine, Ser^{203} (human AChE numbering) that utilizes the adjacent His^{447} and Glu^{334} to enhance its nucleophilicity in the reaction [10]. This catalytic triad corresponds to Ser^{360}, His^{600} and Glu^{359} in *Ag*AChE [11]. This irreversible binding mechanism has been confirmed by X-ray crystallography studies [12–14]. Furthermore, the phosphyl adducts of aged AChE catalytic site serine have been detected by mass-spectrometry [15].

Molecular modelling is an efficient strategy that has been successfully used to study the interaction of a ligand and a target protein at a molecular level [16]. The molecular docking algorithms are generally important in predicting crystal poses and binding affinities of small molecules at the target site [17,18]. The molecular modelling scoring functions are used to estimate and rank the binding affinities of ligand poses based on the strengths of the ligand-protein interaction [19,20]. The molecular mechanics-based approach of the binding free energy determination, such as molecular mechanics-generalized born surface area (MM-GBSA), is regarded a more reliable method to predict binding affinities and ligand poses [21,22]. While general docking assumes a protein is rigid and gives flexibility to only a ligand, MM-GBSA provides for some protein flexibility in noncovalent complexes [23,24]. The reversible docking has been widely explored yet covalent docking is still scarce even with well-known irreversible inhibitors [25]. The known covalent reaction of OPs with AChE has not been displayed before in the available covalent docking panels of molecular modelling [25]. Most studies attribute this loop hole to either the lack of covalent docking systems [26], unavailable chemical reactions displaying OP hydrolysis in covalent docking panels [25] or limitations of the molecular modelling software [27]. As a result, OPs have been consistently assessed in noncovalent docking in molecular modelling studies [22,25,28]. While still performing the noncovalent docking, some studies opted for predicting a potential for covalent binding of OPs by assessing the binding pose and a distance between a phosphorus group of an OP and the catalytic serine residue [29–31]. The manual development of a covalent bonding feature by defining events during the reaction process has been a suggested solution to the limited exploration of covalent docking studies [27,32,33].

Moreover, the molecular docking can be performed when a crystal structure of the target protein is available [34]. Alternatively, homology modeling can be used to generate a three-dimensional structure of the target protein should its amino acid sequence be obtained [35]. The crystal structure for *Ag*AChE catalytic subunits has only recently been made available in Protein Data Bank (PDB), the main source of proteins for docking studies [19,36]. As a result, there is no available data on OPs docking study on *Ag*AChE, but on other comparable targets including human AChE, though docked in a noncovalent manner [22,37]. Schrödinger's Glide docking suite with added extra components accounting for solvation and repulsion contributions on top of various chemical forces in ligand-protein complex formation, is well-recognized for better binding affinity prediction, higher binding accuracy and consistency together with more reliable discrimination between active and inactive compounds [19,26,38–40]. The Glide program has the noncovalent docking functions standard precision (SP) and extra precision (XP), as well as a covalent docking panel [22,41,42]. Notable is the Glide's covalent docking virtual screening workflow that has been proven highly accurate and fast in covalent docking studies [43].

This study reports the manual customization of the Glide covalent docking panel to define the reactive amino acid residue of the receptor, ligand reactive group, reaction sites on both a ligand and a receptor, formal charge and bonds created and/or broken during a reaction course. To validate the algorithm, a series of common OPs was docked in wild-type *Ag*AChE. Moreover, two OP metabolites, malaoxon (malathion's oxidative desulfuration metabolite by insect monooxygenases [7]) and methamidophos (acephate's hydrolysis metabolite by insect carboxyamidases [44]), known to be more potent than their parent drugs [45,46], were also docked and their binding scores compared to that of their parent forms. The study goes further to explore the differences in binding crystal poses, molecular interactions and binding affinity predictions of OPs in the covalent docking versus in noncovalent docking. The two docking functions were also assessed for reliability by comparing their molecular interactions with those reported in X-ray diffraction studies. This was based on the reports that a best docking is one which regenerates the X-ray pose and interactions [18]. Carleso et al. (2019) stated that the comparison of Glide noncovalent and covalent modes can overcome docking challenges in the difficult-to-explore systems [47]. This research demonstrates for the first time, the covalently phosphorylated catalytic serine residue while releasing the leaving groups of OPs. Further, this is for the first time *Ag*AChE-OP complexes, in both noncovalent and covalent interactions, are reported and compared.

2. Material and methods

2.1. Target proteins preparation

Schrödinger Release 2018-2 docking suite, Maestro version 11.6, (Schrödinger LLC, New York) was used for all the docking studies. The *Ag*AChE protein (PDB: 5YDI) was retrieved from PDB [11,36]. The 5YDI crystal structure (resolution: 3.45 Å) was in a ligand-free state. The protein was prepared with a protein preparation wizard tool for the docking studies [48]. The preparation comprised of removal of water molecules beyond 5 Å from the binding site and those forming less than 3 H-bonds to a protein or ligand. Removing these water molecules unable to form all possible H-bonds has been reported to increase reliability in binding affinity prediction [18]. The non-protein hetero groups were also removed and completion of any missing hydrogens, side chains and loops was done. The protein was optimized to sample hydrogen orientations at pH 7.0 using PROPKA [22,49,50]. To satisfy the conditions of catalytic serine phosphorylation displayed in equation (1), the protonation states of His^{600} and Glu^{359} were reassessed after the

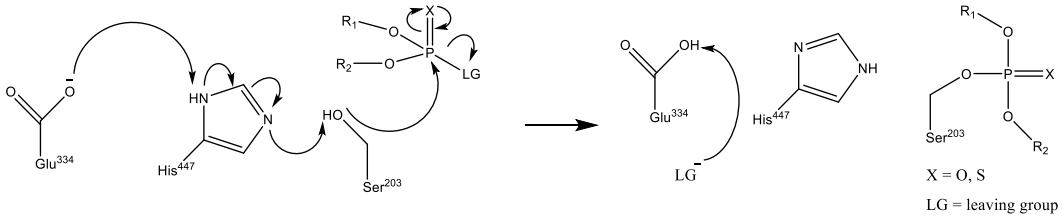


Fig. 1. General OP hydrolysis by catalytic serine in the AChE catalytic site [5].

optimization at pH 7.0 to ensure that they remain neutral and charged, respectively. Should their required protonation states be found to have been changed by optimization at pH 7.0, the new residue states were mutated back to their required original states and conformations [19,50,51]. The protein minimization by OPLS3e force field to fix all molecular overlaps and strains was then performed [49]. The restrained minimization was terminated when the average root mean square deviation (RMSD) of the non-hydrogen atoms was converged to 0.3 Å [22,52].

2.2. Receptor grid generation

The prepared 5YDI had 2 chains (A and B). Unlike humans, mosquitoes are known to have two *ace* genes (*ace*-1 and *ace*-2) coding for AChE1 and AChE2 proteins, respectively [9,53]. Since it is reported that the site of action is AChE1 [54,55], the chains were split before generating the grid and only chain A protein was used for subsequent docking studies thereafter. The grid was generated using OPLS3e force field [49]. The amino acid residues selected to define the active site that enclosed a catalytic Ser^{360}, while incorporating a catalytic triad were Ala^{242}, Trp^{245}, Phe^{277}, Phe^{281}-Tyr- Ser-Gly^{284}, Ala^{361}-Gly^{362}, Tyr^{489}-Phe^{490}, Trp^{441}-Gly-Tyr-Leu-Gly-Ile-Cys-Glu^{448}, Pro^{450}, Tyr^{494}, Phe^{560}, Trp^{592}, Met^{599} and Gly^{601}.

2.3. Ligand preparation

The chemical structures of common OP insecticides [28,56,57] were obtained from PubChem database. The selected OPs were malathion, malaoxon, acephate, methamidophos, dimethoate, thiometon, phosmet, terbufos, ethion and azinphos-methyl (Fig. 2) and all these are known to be effective AChE-inhibitor OP insecticides [58,59]. The selected OPs structurally fall into three subgroups being aromatic, non-aromatic and hydrophobic OPs. Ligands were drawn using Maestro's 2D Sketcher and

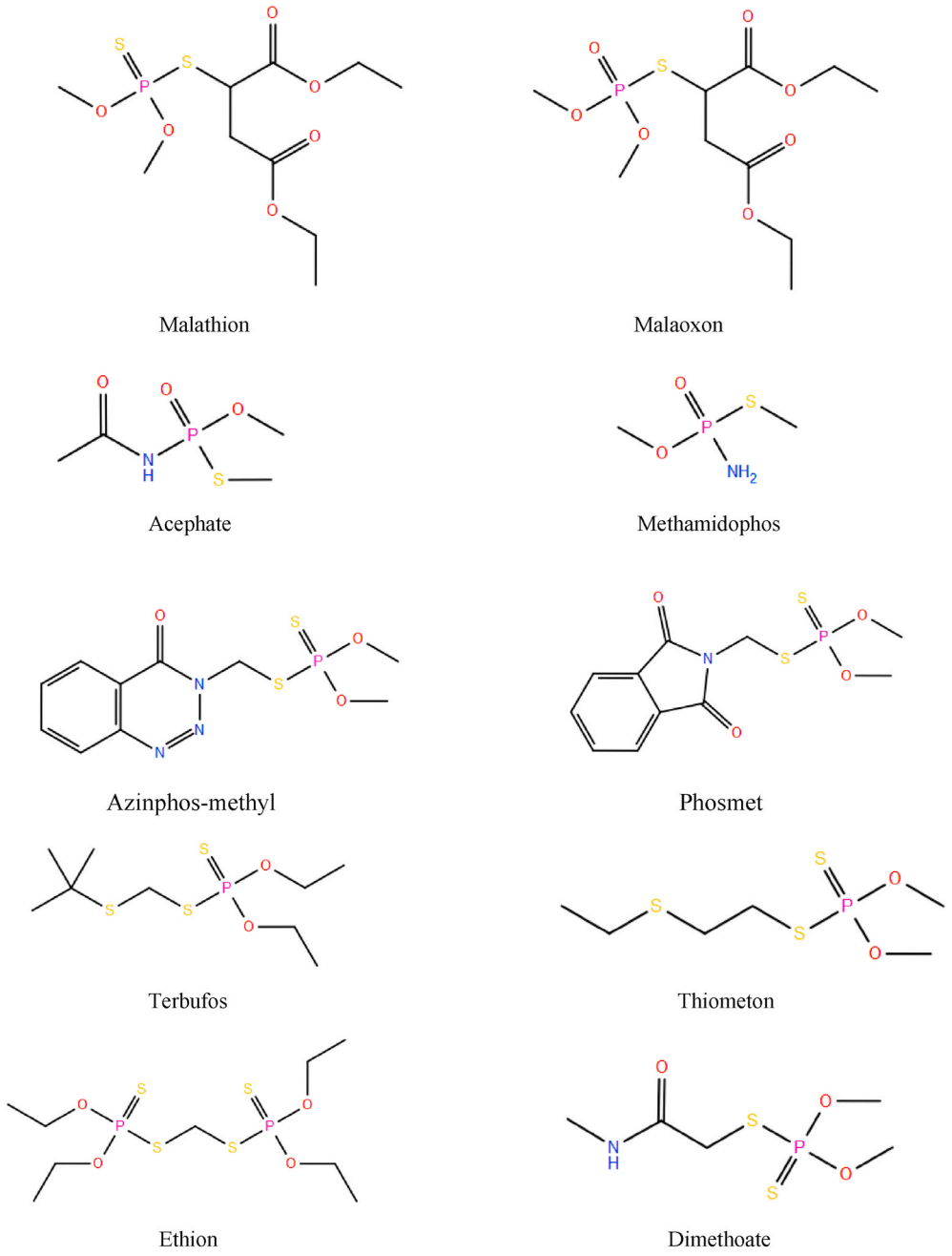


Fig. 2. Chemical structures of ligand OPs.

subsequently subjected to LigPrep (ligand preparation) module of Schrödinger suite [60]. A robust p*Ka* prediction tool, Epik, was used to generate possible ionization and tautomeric states at pH 7.0 (±2.0) [61–63] and each ligand could generate maximum 32 stereoisomers. The ligands were then minimized by the OPLS3e force field [49].

2.4. Noncovalent docking of OPs against AgAChE

Determination of noncovalent interactions of OPs with *Ag*AChE was done through standard precision (SP) and extra precision (XP) modules. The ligand docking panel was displayed on the workspace and the previously generated 5YDI receptor grid retrieved, while the ligands were selected from the project table. The ligands could be flexible and their nitrogen inversions (pyramidal nitrogen atoms) and ring conformations were sampled during the docking process. Only amides were penalized for nonplanar conformations as a default setting. Lastly, Epik state penalties were applied to the docking scores. This is for adopting higher energy states for ligands that were ionized or tautomerized in the preparation stage [62,63]. The SP docking format followed by XP, was then selected. The output poses per ligand was set to 1 best pose, while post-docking minimization was also performed. The SP and XP poses were compared to assess consistency in noncovalent docking, while prediction for covalent bond formation was also done. It is known that a pose that puts an electrophilic ligand atom close enough to the nucleophilic amino acid residue of the receptor resembles a potential for covalent bonding [27,43]. In this study, this was defined as a pose at the catalytic triad placing a phosphorus atom of the OP closer to the catalytic serine residue [25]. Since XP is reported to be more rigorous and accurate than SP [18,22], only XP complexes were kept and further evaluated for binding affinity profiles in the MM-GBSA panel.

2.5. MM-GBSA-based re-docking of OPs against AgAChE

The binding affinities of OP ligands to *Ag*AChE were assessed through MM-GBSA based re-docking. The previously docked noncovalent complexes were retrieved and the solvation model VSGB together with OPLS3e force field were used in the re-docking. Distance from ligand was changed from 0 Å to 5 Å to allow enough flexibility for the target protein [23,24]. The binding affinities for OPs were compared.

2.6. Optimization of covalent docking of OPs against AgAChE

Firstly, the covalent docking reaction indicating the catalytic serine – OP covalent interaction was sought in the Glide's predefined (built-in) and custom covalent reactions available online at Schrödinger's covalent reactions repository (https://www.schrodinger.com/products/covdock). Following the negative results from a reaction's search, the customized Glide covalent docking reaction algorithm was generated to define an electrophilic phosphorus center of OPs, nucleophilic hydroxyl group of serine residue (Ser-OH) on the target protein, ligand attachment atom on a receptor (S), receptor attachment atom on a ligand (P), formal charge (0) and bonds created (1 bond between an oxygen atom of Ser-OH and phosphorus atom of phosphate group on OP) and/or broken (1 bond on a ligand (P–S or P–N (in the case of acephate)) to liberate a leaving group) during a reaction process. The final customized Glide covalent docking algorithm was as follows:

RECEPTOR_SMARTS_PATTERN	2,[C,c]-[S,O;H1,-1]
LIGAND_SMARTS_PATTERN	1,[P] = [O,S]
CUSTOM_CHEMISTRY	('<1>', ('charge', 0, 1))
CUSTOM_CHEMISTRY	('<1>|<2>', ('bond', 1, (1, 2)))
CUSTOM_CHEMISTRY	('<2>[P,S]', ('bond', −1, (1, 2)))[1]
[1] *For acephate*:	
CUSTOM_CHEMISTRY	('<2>[P,N]', ('bond', −1, (1, 2))) based on its distinct chemical structure.

The algorithm was stored in a cdock format and retrieved during the covalent docking study of OPs.

2.7. Covalent docking of OPs against AgAChE

The covalent docking studies were performed using the covalent docking module of the Schrödinger Release 2018-2 suite [42]. The covalent docking panel (CovDock) was used. The CovDock workflow has been reported to be highly accurate in pose prediction of covalent inhibitors [42]. The prepared OP ligands were selected from a project table, while a reactive receptor residue (Ser^{360}) was selected from a 5YDI protein on the workspace. The amino acid residues Phe^{560}, Trp^{245}, Tyr^{489} and Glu^{359} were selected to define the active site that enclosed a catalytic Ser^{360} while incorporating a catalytic triad to generate the binding site displayed in Fig. 3.

The box size enabled docking of ligands of ≤20 Å length. The customized covalent docking algorithm was then selected as the reaction type. No constraints were imposed on the ligand for docking and virtual screening mode was selected. The total energy 2.5 kcal/mol was set as the cutoff to retain poses for further refinement by default, while the maximum number of poses to retain for further refinement, was 200. The output poses per ligand reaction site was set to 1 best pose.

3. Results and discussion

3.1. Noncovalent docking of OPs against AgAChE

The noncovalent docking produced poses at the catalytic triad with the conformations enabling phosphate groups to be closer to the catalytic Ser^{360} residue. The OPs generated H-bonds between Ser^{360} and oxygen or sulfur atoms that were doubly bonded to the phosphorus atom (Figs. 4–5 and Figure S1.0-S1.1 (SI)). Alternatively, this H-bond was formed using one of the two alkoxy groups attached to the phosphorus atom of the OPs as in the case of phosmet, terbufos, thiometon and ethion (Figure S1.2-S1.5 (SI)).

While malathion formed only a Ser^{360} H-bond, its metabolite malaoxon, known to be relatively more potent [45], formed extra H-bonds with Tyr^{282} and Tyr^{489} residues. Moreover, methamidophos displayed a favorable and unique profile of interaction by forming H-bonds with the catalytic triad, Ser^{360}, Glu^{359} and His^{600} residues as opposed to its parent molecule, acephate, that interacted with only Ser^{360} and Gly^{278}. Additional bonds were either an aromatic π-stacking with Tyr^{282} residue or aromatic H-bond with Ser^{283} for the aromatic OPs

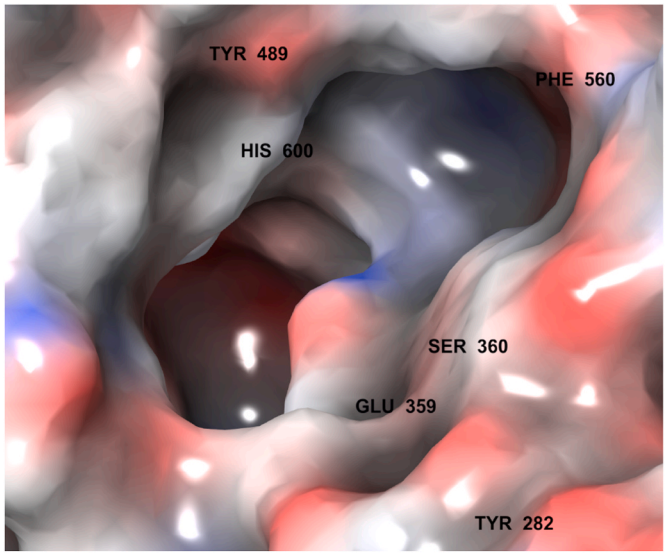


Fig. 3. Generated binding site on the *Ag*AChE.

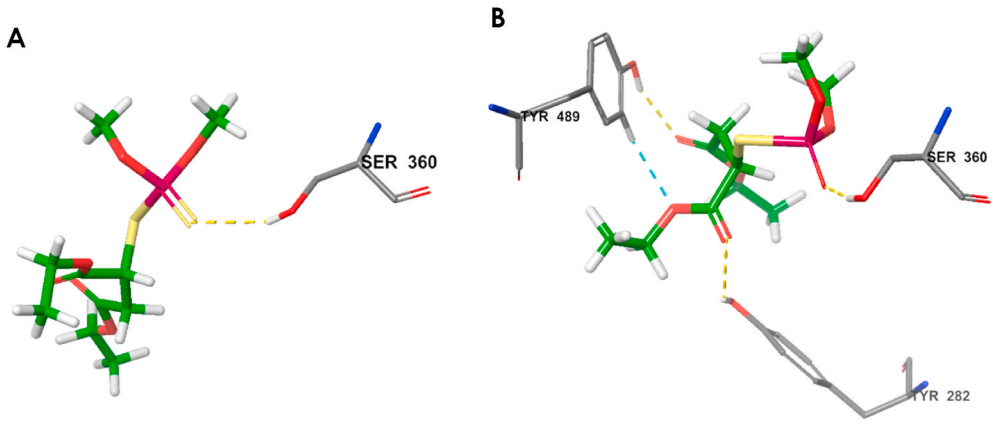


Fig. 4. Malathion (A) and malaoxon (B) docked at *Ag*AChE with a noncovalent function.

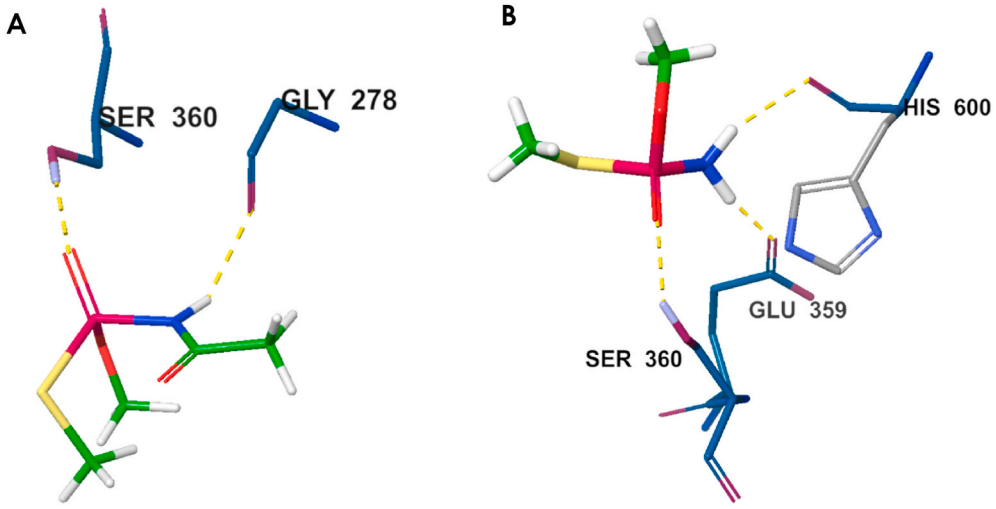


Fig. 5. Acephate (A) and methamidophos (B) docked at *Ag*AChE with a noncovalent function.

such as phosmet and azinphos-methyl, respectively. However, azinphos-methyl formed extra H-bond with Tyr^{489} through its benzotriazine group. Dimethoate also formed the extra H-bond with Ser^{283} through its amide group. The relatively hydrophobic OPs, terbufos, thiometon and ethion could only form the Ser^{360} H-bond (Figure S1.3-S1.5 (SI)) suggesting a potential for none other than a covalent bond in the polar binding site. The orientation of the leaving groups opposite to the catalytic serine observed in noncovalent docking has previously been reported as an indication of the possibility for elimination of the leaving group [13,14]. However, the noncovalent binding scores generally could not show significant difference between the metabolites, malaoxon and methamidophos and their parent drugs, malathion and acephate, respectively (Table 1). Haider et al. (2020) suggested that MM-GBSA scoring and pose generation functions offer more accurate results than the noncovalent mode [64]. The supplementation of noncovalent docking with MM-GBSA scoring has shown efficiency in many studies [64–66]. Conversely, the setback with CovDock scoring system is that of not considering the covalent bond formation. This results in lower scores and may show only a little difference from the noncovalent docking scores [42,47]. As a result, the OPs are ranked according to their MM-GBSA binding scores in Table 1.

Table 1
Glide docking scores for OPs bonded to 5YDI target *Ag*AChE protein.

Glide docking scores (kcal/mol) Wild-type *Ag*AChE (5YDI)			
Organophosphate	Glide-dock XP	MM-GBSA	Optimized CovDock
azinphos-methyl	−5.391	−67.55	−6.546
malaoxon	−5.856	60.01	−7.035
dimethoate	−3.924	−49.14	−5.217
malathion	−5.42	−47.92	−5.184
ethion	−4.541	−44.87	−4.923
phosmet	−5.558	−43.29	−6.827
methamidophos	−4.027	−32.89	−6.12
thiometon	−3.704	−31.93	−4.393
terbufos	−5.459	−26.11	−4.802
acephate	−4.081	−23.45	−5.355

3.2. *MM-GBSA-based re-docking of OPs against AgAChE*

The MM-GBSA module was more rigorous in identifying the amino acid residues involved in the binding of these OPs. For malaoxon, while noncovalent docking suggested two tyrosine amino acid residues Tyr^{282} and Tyr^{489} apart from the catalytic Ser^{360}, MM-GBSA rather indicated the catalytic site amino acid residues His^{600} and Tyr^{282} (Fig. 6). This was also the case with acephate and azinphos-methyl where His^{600} and Tyr^{282}, respectively, were identified by MM-GBSA and not noncovalent mode, as some of the critical amino acid residues for their binding and correct ligand pose generation (Fig. 7 and Figure S2.0). With His^{600} being a catalytic triad amino acid, this shows that MM-GBSA generated poses that were closer to the AChE binding site as previously reported [67]. Interestingly, this favorable interaction with OPs has been reported in the X-ray diffraction studies [12–14,68]. The MM-GBSA study

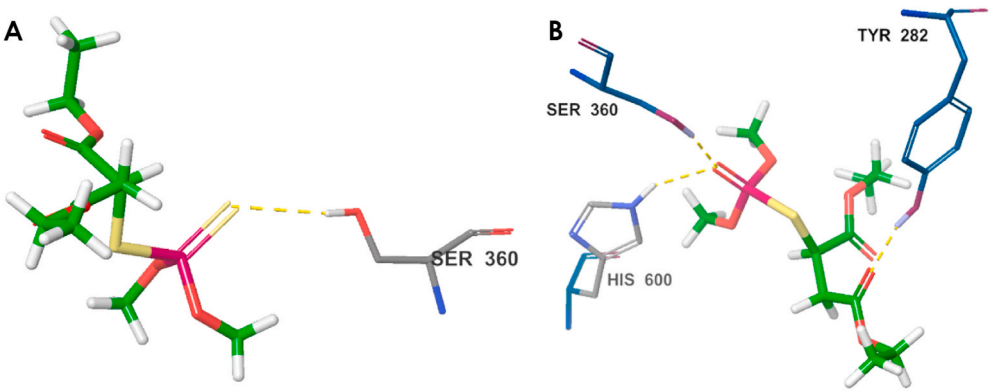


Fig. 6. Malathion (A) and malaoxon (B) re-docked at *Ag*AChE with MM-GBSA method.

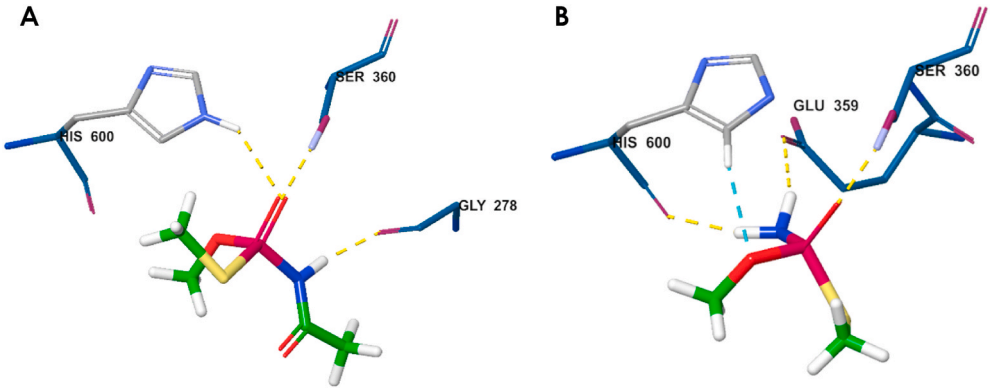


Fig. 7. Acephate (A) and methamidophos (B) re-docked at *Ag*AChE with MM-GBSA method.

showed an increased binding affinity for methamidophos over acephate (Table 1) in agreement with the reported clinical data [46]. In a similar manner, malaoxon had a higher affinity score than malathion in correlation to the literature [45]. This complies with the literature that MM-GBSA is able to differentiate between closely related ligand structures and thereby generate accurate poses and affinity ranking [65,66].

3.3. Optimization of covalent docking of OPs against AgAChE

The customized covalent docking reaction algorithm was successfully generated and utilized, where it produced the covalently phosphorylated catalytic serine residues and displayed the OP leaving groups in agreement with the literature and X-ray diffraction studies [5,12–14]. The concept proved true for both sulfur and oxygen-based OPs (those bearing a P═S or P═O bond) having either sulfur- or nitrogen-based leaving groups.

3.4. Covalent docking of OPs against AgAChE

Using the newly customized covalent docking reaction algorithm, the OPs in this study were successfully covalently docked in *Ag*AChE catalytic site. The CovDock docking scores are displayed in Table 1. The covalently bonded OPs displaying phosphorylated Ser^{360} residues and released leaving groups are displayed in Figs. 8–9 below and in Figure S3.0-S3.5 (SI). While malathion formed the Ala^{361} and Gly^{280} H-bonds with the leaving group, these H-bonds were formed with a

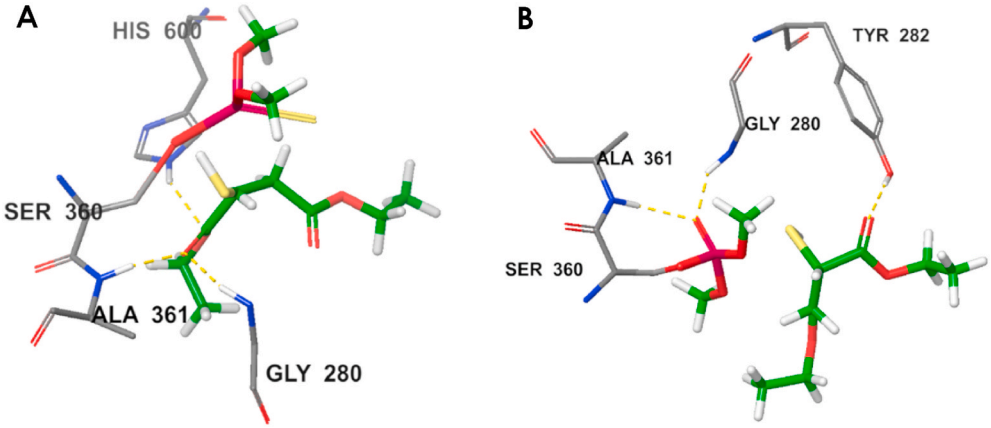


Fig. 8. Malathion (A) and malaoxon (B) docked at *Ag*AChE with new covalent function.

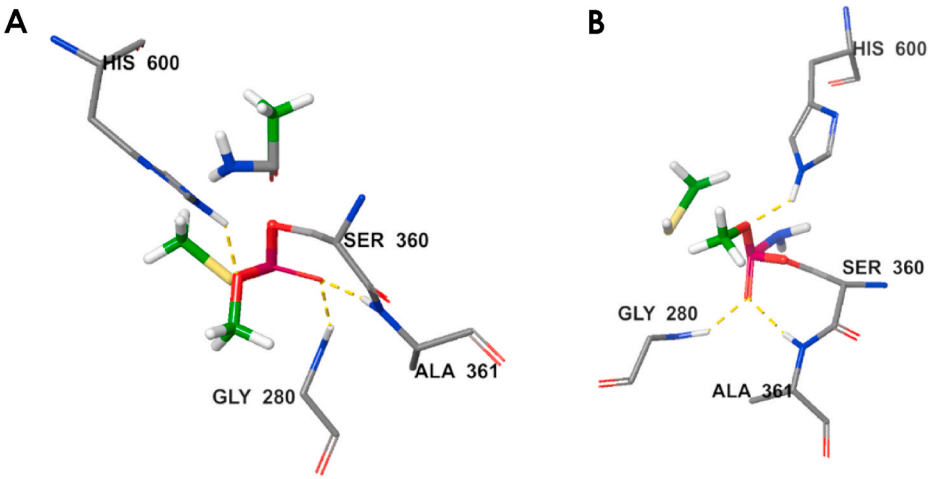


Fig. 9. Acephate (A) and methamidophos (B) docked at *Ag*AChE with new covalent function.

covalently bonded portion in malaoxon, acephate and methamidophos (Figs. 8–9), as well as with dimethoate (Figure S3.1 (SI)). Additionally, malathion, acephate, methamidophos and azinphos-methyl formed extra H-bonds with the His^{600} residue. This type of bonding profile that involves Gly^{280}, Ala^{361} and His^{600}, while covalently bonded to Ser^{360} residue has previously been reported in X-ray diffraction with two of the most toxic chemical warfare OP nerve agents, namely sarin and soman. Similarly, this binding mode has been reported with di*iso*propyl phosphorofluoridate, a potent OP insecticide that was also developed as a warfare agent and is also used for its anticholinergic effects in chronic glaucoma [12,69–72].

Unlike the nonocovalent docking, the customized covalent docking generated poses that were more closer to the catalytic site and interacted mainly with the catalytic site amino acid residues. This was evident with malation, malaoxon, acephate, methamidophos, dimethoate, and azinphos-methyl that interacted with His^{600}, Ala^{361}, Gly^{280}, and Tyr^{282} residues in addition to the Ser^{360} covalent bond. While malathion interacted with only Ser^{360} in the noncovalent mode, it effectively interacted with Ser^{360}, His^{600}, Ala^{361} and Gly^{280} in the covalent docking platform (Table 2). Similarly, malaoxon was predicted to interact with Tyr^{489} and Tyr^{282} in noncovalent docking. However, Tyr^{489} was excluded in both MM-GBSA and CovDock modes that rather indicated increased interaction with catalytic site amino acid residues (Table 2). This was a similar case with azinphos-methyl where this tyrosine residue (Tyr^{489}) located far from the catalytic triad was excluded at MM-GBSA and CovDock stages. Sasmal et al. (2020) stated that high accuracy and reliable affinity prediction is obtainted with poses generated closest to the binding site [67]. On the other hand, it was observed that due to their small size, acephate and methamidophos could effectively reach the catalytic triad and interact with the catalytic site amino acid residues throughout all the docking modes (Table 2). It is agreed that ligands with a similar scaffold display a similar binding conformation [73]. Nevertheless, it is evident from the molecular interactions (Table 2) and displayed conformations (Fig. 4) that the noncovalent mode did not establish a similar pose even with molecules that differ by one atom such as malathion and malaoxon. Kaus et al. (2015) found out that the Glide noncovalent mode could only correctly predict the ligand poses for four of the twenty ligands with a similar scaffold [65]. Interestingly, the optimized covalent docking mode established similar binding modes for similar OPs such as malathion and malaoxon, acephate and methamidophos as well as phosmet and azinphos-methyl (Table 2, Fig. 7, S2.0, and S2.2).

Table 2
Comparison of amino acid residues involved in the binding of OPs to 5YDI target *Ag*AChE protein in different docking methods.

Organophosphate	Amino acid residues		
	Glide-dock XP	MM-GBSA	Optimized CovDock
azinphos-methyl	Ser^{360}, Ser^{283}, Tyr^{489}	Ser^{360}, Tyr^{282}	Ser^{360}, Tyr^{282}, His^{600}
malaoxon	Ser^{360}, Tyr^{282}, Tyr^{489}	Ser^{360}, Tyr^{282}, His^{600}	Ser^{360}, Ala^{361}, Gly^{280}, Tyr^{282}
dimethoate	Ser^{360}, Ser^{283}	Ser^{360}, Ser^{283}	Ser^{360}, Ala^{361}, Gly^{280}
malathion	Ser^{360}	Ser^{360}	Ser^{360}, Ala^{361}, His^{600}, Gly^{280}
ethion	Ser^{360}	Ser^{360}	Ser^{360}
phosmet	Ser^{360}, Tyr^{282}	Ser^{360}, Tyr^{282}	Ser^{360}, Tyr^{282}
methamidophos	Ser^{360}, Glu^{359}, His^{600}	Ser^{360}, Glu^{359}, His^{600}	Ser^{360}, Ala^{361}, His^{600}, Gly^{280}
thiometon	Ser^{360}	Ser^{360}	Ser^{360}
terbufos	Ser^{360}	Ser^{360}	Ser^{360}
acephate	Ser^{360}, Gly^{278}	Ser^{360}, Gly^{278}, His^{600}	Ser^{360}, Ala^{361}, His^{600}, Gly^{280}

Moreover, critical analysis of the generated conformations also idenfitied some differences between noncovalent and covalent modes. By virtue of not considering the OP hydrolysis, noncovalent mode may suggest intermolecular interactions that may not otherwise occur when the molecule is hydrolyzed. This was observed, for instance, in the case of azinphos-methyl, malaoxon and dimethoate where amino acid residues located further from the catalytic triad, namely Ser^{283} and Tyr^{489}, could form H-bonds with such OPs only in the noncovalent mode. In its original structure as retained in the noncovalent docking, dimethoate extended to Ser^{283} while it was however confined closer to the catalytic triad in the covalent mode (Fig. 10).

Similarly, in the covalent mode, malathion was held tightly to the catalytic triad and the leaving moiety was released closer to the catalytic serine hence interactions with Ala^{361} and Gly^{280} residues. Also, malaoxon was bound tightly to the catalytic serine allowing the orientation of its doubly-bonded phosphoryl oxygen (P═O-) to face towards Ala^{361} and Gly^{280} residues. However, both malathion and malaoxon were relatively weakly bound by the catalytic serine in the noncovalent docking with their alkoxy carbonyl groups assuming the orientation facing away from the catalytic triad. This enabled the interaction with Tyr^{489} residue located far from the catalytic triad, in the case of malaoxon (Fig. 11).

3.5. Catalytic site distortion observation

Several amino acid rearrangements occur upon aging [74]; where the catalytic histidine is reported to be mobile during the aging process [12,13,74]. In this study, histidine did not show evidence of rearrangement; however, the catalytic serine showed reorientation with larger ligands, such as malathion and malaoxon (Fig. 12). This was not a

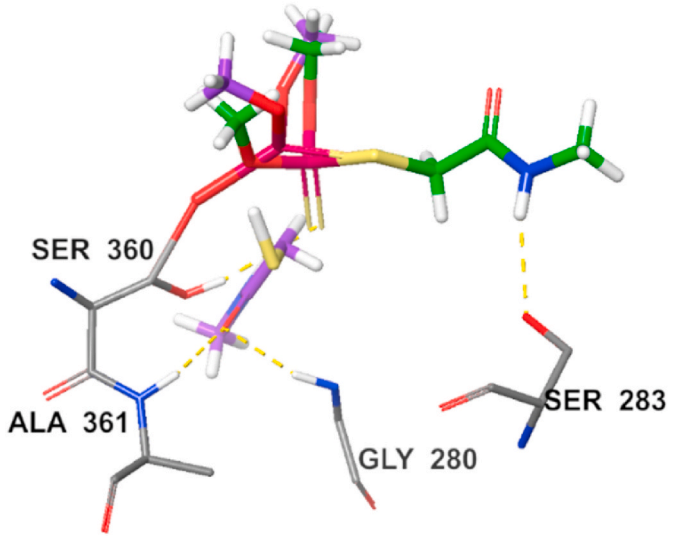


Fig. 10. Superimposed dimethoate in noncovalent (green) and covalent (violet) binding states. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

case with smaller ligands, such as acephate and methamidophos (Fig. 12), which is in agreement with the literature that smaller OPs do not cause distortion at the catalytic site upon dealkylation [13]. It was notable that with malathion and malaoxon, the distance between the catalytic serine and histidine had increased substantially (3.42 Å) from the non-aged form (3.19 Å) or that reported in the original protein (3.10 Å) [36].

While all the subgroups could form a covalent bond with Ser^{360}, there was a noticeable difference in the bonding interactions between aromatic OPs, non-aromatic OPs and hydrophobic OPs with the target sites. The aromatic OPs (azinphos-methyl and phosmet) were stabilized by the aromatic interactions (π - π stacking) mainly from Tyr^{282}, the non-aromatic OPs (malaoxon, acephate, methamidophos and dimethoate) utilized Gly^{280}, Ala^{361} and His^{600} residues, while the hydrophobic OPs only formed a covalent bond. Due to smaller sizes, the elimination of the leaving groups in methamidophos and acephate, has been reported to induce no distortion of the catalytic site [13]. This study has shown catalytic serine reorientation with malathion and maloxon (Fig. 8); which has previously been reported with the high molecular weight ligands such as di*iso*propyl phosphorofluoridate [13]. The increase in distance between the catalytic serine and histidine at the malathion or malaoxon phosphorylated sites indicated a potential for steric effects with these bigger molecules. Additionally, the covalently bonded portions maintained an interaction with the His^{600} residue through their singly-bonded phosphoryl oxygen (P–*O*-) in these two latter OPs, which is a common characteristic seen in X-ray crystallographic complexes involving OPs and AChE targets [12–14,68]. However, the X-ray crystallographic studies focus on the generated catalytic serine adducts rather than the dealkylated or deaminated leaving groups [12]; since aged OP-AChE complexes do not allow direct viewing of the leaving group. Nevertheless, its general topography is inferred from the aged active site [12,75]. As a result, the reliability of the covalent docking mode utilized in this study could be better assessed based on the covalently bonded portions.

Furthermore, good correlation was obtained whereby noncovalent docking modes, MM-GBSA and CovDock, all suggested that malaoxon was among the three OPs with highest binding scores for the *Ag*AChE and was suggested as the best binding OP in the covalent docking panel

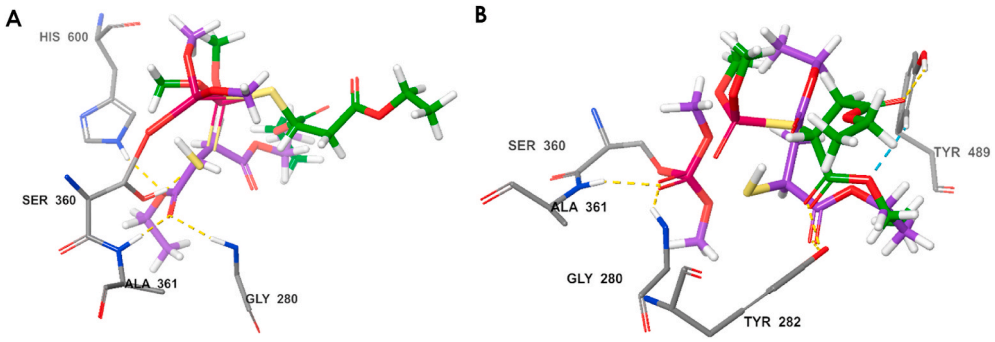


Fig. 11. Superimposed malathion (A) and malaoxon (B) in noncovalent (green) and covalent (violet) binding states. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

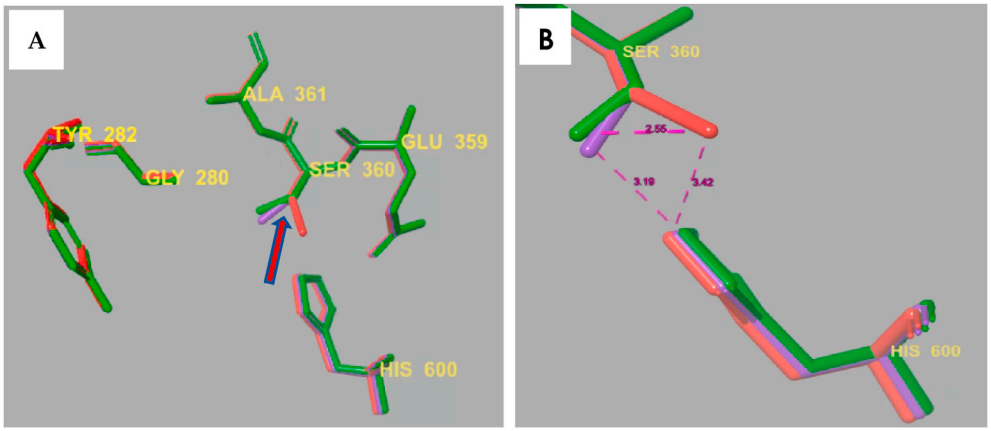


Fig. 12. Superimposed *Ag*AChE active site amino acid residues non-aged (green) and aged by malathion or malaoxon (red) versus acephate or methamidophos (violet). **(A)** Shows the catalytic serine reorientation (arrow) in malathion or malaoxon phosphorylation (red) and **(B)** shows the reorientation distance from the non-aged catalytic serine (2.55 Å) and increased distance from histidine (3.42 Å). Ligands have been undisplayed to enhance visibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Table 1). Generally, the scores obtained in this study correlate to those previously obtained when docking related OPs to the human AChE with Glide XP and MM-GBSA [22].

Comparing the molecular interactions displayed by the malaoxon as opposed to malathion, malaoxon possesses an advantage with oxygen that has replaced sulfur in malathion. This oxygen is on the covalently bonded phosphate group and forms two favorable H-bonds with Gly^{280} and Ala^{361}. This is possibly due to oxygen being more electronegative than sulfur, hence a better H-bond acceptor [76]. This correlates to the literature that reports that the thiophosphoryl OPs are less potent AChE inhibitors compared to other phosphoryl derivatives [77]. The leaving group of malathion formed the other H-bond between its ester group and His^{600}, while malaoxon utilized Tyr^{282}. Together, formation of the Gly^{280} and Ala^{361} H-bonds with a covalently bonded group and Tyr^{282} H-bond with a leaving group, yielded a higher binding score for malaoxon as opposed to malathion. Similar to malathion, dimethoate has a sulfur-based phosphate group and accessible carbonyl group in the leaving moiety that displayed a similar binding profile with malathion and closer binding scores (Table 1).

On the other hand, oxygen-based phosphate groups (P═O), as in acephate and methamidophos, formed H-bonds with Gly^{280} and Ala^{361} with a covalently bonded phosphate group in a similar fashion to malaoxon. Methamidophos may be showing a higher score than acephate due to its lack of steric effects around the electrophilic phosphorus atom as the two insecticides display similar interactions within the *Ag*AChE catalytic site. All other assessed OPs could not form H-bonds with Gly^{280} and Ala^{361} presumably due to the lack of a P═O bond or an accessible carbonyl group in their leaving moieties. Azinphos-methyl and phosmet have carbonyl groups in their amides that are connected to large aromatic rings, facilitating aromatic π-interactions with the Tyr^{282} residue. The lack of a P═O bond and an accessible carbonyl in the leaving group resulted in OPs such as terbufos, thiometon and ethion, with generally hydrophobic leaving moieties, forming only a covalent bond with the catalytic serine residue. The importance of Tyr^{282} in the mechanism of action of the insecticides was evident by its ability to either form a H-bond with non-aromatic OPs that have H-bond acceptors of high electronegativity (malaoxon) or to establish aromatic interactions with aromatic OPs (azinphos-methyl and phosmet). This role of Tyr^{282} was also observed in the noncovalent docking module. The critical role of the aromatic amino acids in stabilizing OPs at the catalytic site has previously been supported by studies on human AChE [28]. This binding profile obtained with the aromatic OPs that utilizes Ser^{360}, Tyr^{282} and His^{600} has been previously reported for aromatic OP analogs in the AChE protein of *Musca domestica* (housefly insect) [78,79].

Therefore, in this study noncovalent docking was coupled to covalent docking for the first time. The noncovalent docking generated poses at the catalytic triad with orientations that suggested a potential for covalent bond formations. However, the noncovalent mode generated poses that were relatively far from the catalytic triad and suggested the intermolecular interactions further from the binding site. This indicated low accuracy and less reliability of OP complexes generated through noncovalent options. On the other hand, in agreement to the literature [66], the MM-GBSA method produced ligand poses closer to the catalytic triad accompanied by increased interactions with the catalytic site amino acid residues. This method was rigorous in excluding amino acid residues that are distant from the catalytic triad such as Ser^{283} and Tyr^{489} that were otherwise suggested by the noncovalent panel. The MM-GBSA procedure showed a greater potential for covalent bond formation and interaction with the catalytic site amino acid residues. This was subsequently confirmed by the establishment of the predicted covalent bond in the covalent docking and favorable interactions with catalytic triad and immediate amino acid residues. In addition, the hydrophobic OPs showed potential for only a covalent bond and indeed, in the covalent docking study, this was the case. The customized covalent model was in agreement with the MM-GBSA for exclusion of amino acid residues located further from the catalytic triad. However, this covalent docking mode generally established more catalytic site interactions than both MM-GBSA and noncovalent docking modes. Notably, with the covalent docking panel ending in two apparent molecules (covalently bonded dialkylphosphate and a leaving group), the intermolecular interactions generated with noncovalent docking that maintained the original OP structure were altered substantially in the covalent docking panel. A docking panel that generates ligand poses closer to the binding site is more reliable [67]. While noncovalent docking created poses and intermolecular interactions far from the catalytic triad, the customized covalent docking method generally confined poses and interactions closest to the catalytic triad. This docking system eliminated distant amino acid residues such as Ser^{283} and Tyr^{489} and maintained interactions with mainly the catalytic site residues such as Gly^{280}, Ala^{361} and His^{600} residues in correlation with the X-ray diffraction studies [12,69,70].

4. Conclusions

The reliable covalent docking for OPs in *Ag*AChE has been optimized through manual customization of the Schrödinger's Glide covalent docking reaction algorithm. The noncovalent docking module generated crystal poses and molecular interactions that were relatively distant from the catalytic site. Additionally, the noncovalent docking could not establish similar binding orientations for the structurally similar OPs showing non-reliability in the investigations of OPs binding mode. The MM-GBSA corrected the binding poses by eliminating interactions with amino acid residues distant from the catalytic triad and established intermolecular interactions that stabilized OPs in the orientations that are more appropriate for a covalent bond formation. Upon submission of the OPs to the newly customized covalent docking reaction algorithm, the required covalently phosphorylated catalytic serine residues and the hydrolyzed leaving groups were effectively generated. Furthermore, the covalent docking mode restricted the interactions and ligand poses to the catalytic site as well as establishing similar binding modes for OPs with similar scaffolds indicating relatively high accuracy and reliability. The binding crystal poses and molecular interactions displayed in the covalent docking studies were similar to those previously reported in X-ray diffraction studies. This study also established that high molecular weight OPs such as malathion and malaoxon may have capacity to induce catalytic site distortion. Moreover, the amino acid residues playing important roles in the binding affinities of OPs and corresponding structural features have been identified. Generally, the aromatic OPs formed H-bonds with Ser^{360} and His^{600} whereby their aromatic rings were stabilized by the Tyr^{282} residue. The non-aromatic OPs on the other hand, utilized Ser^{360}, Gly^{280}, Ala^{361} and His^{600}, while the hydrophobic OPs only formed a covalent bond with the Ser^{360} residue. Therefore, in this study, a covalent docking reaction algorithm that effectively displays the molecular mechanism of OPs is proposed for further evaluation in covalent docking studies involving a larger library of OPs or OP analogs and a wide variety of targets such as G119S mutated *Anopheles* AChE (PDB: 6ARY) and human AChE (PDB: 5HF5).

Supplementary Materials: The following are available as supplementary data: Figure S1.0-S1.5: azinphos-methyl, dimethoate, phosmet, terbufos, thiometon and ethion, respectively, docked in noncovalent function, Figure S2.0-S2.5: azinphos-methyl, dimethoate, phosmet, terbufos, thiometon and ethion, respectively, re-docked with MM-GBSA method, Figure S3.0-S3.5: azinphos-methyl, dimethoate, phosmet, terbufos, thiometon and ethion, respectively, docked in a new covalent function.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this work.

Acknowledgments

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Appendix A. Supplementary data

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Appendix B.2: Supplementary information for Chapter 3 (published paper)

Optimization of covalent docking for organophosphates interaction with *Anopheles* acetylcholinesterase

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Supplementary Information

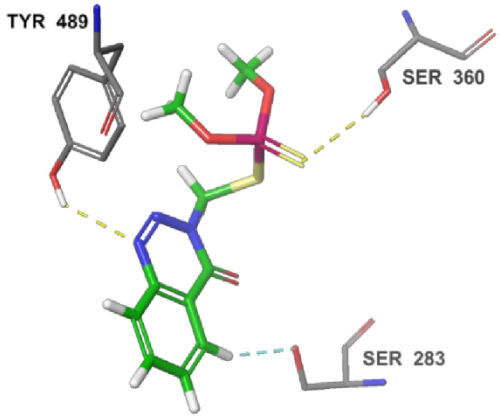


Figure S1.0. Azinphos-methyl docked at *Ag*AChE with a noncovalent function.

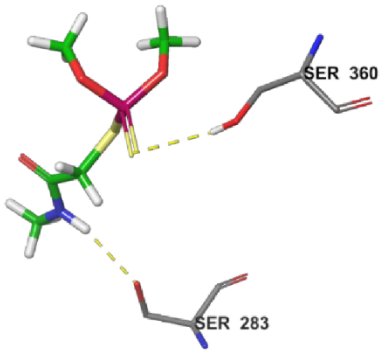


Figure S1.1. Dimethoate docked at *Ag*AChE with a noncovalent function.

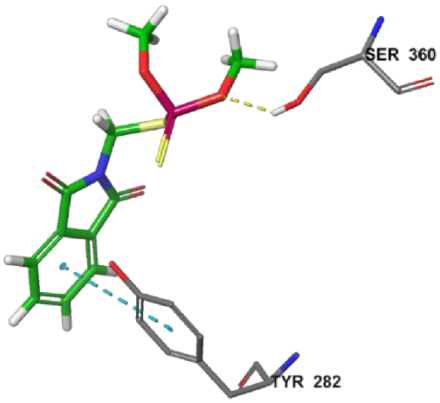


Figure S1.2. Phosmet docked at *Ag*AChE with a noncovalent function.

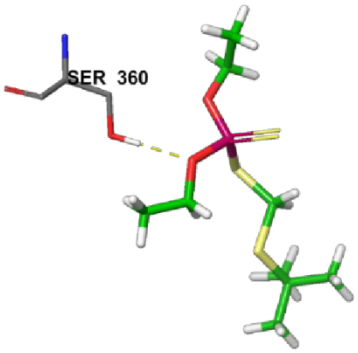


Figure S1.3. Terbufos docked at *Ag*AChE with a noncovalent function.

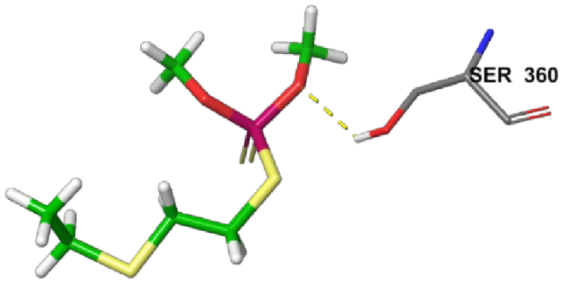


Figure S1.4. Thiometon docked at *Ag*AChE with a noncovalent function.

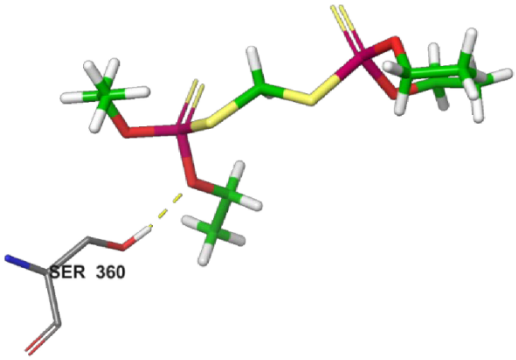


Figure S1.5. Ethion docked at *Ag*AChE with a noncovalent function.

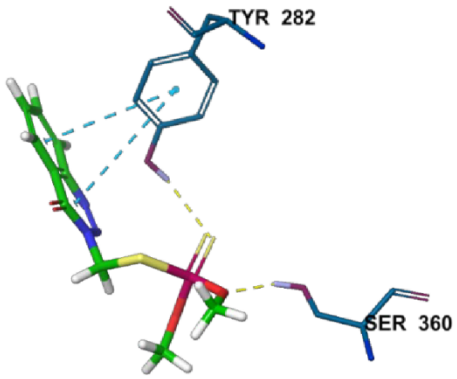


Figure S2.0. Azinphos-methyl re-docked at *Ag*AChE with MM-GBSA method.

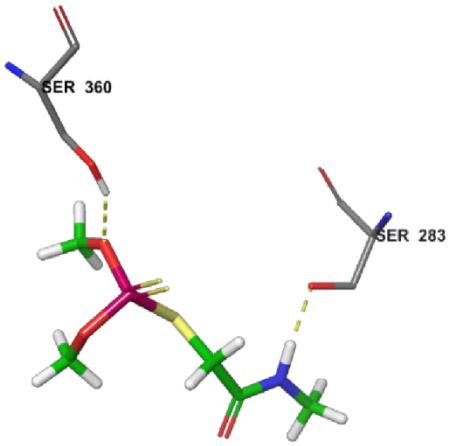


Figure S2.1. Dimethoate re-docked at *Ag*AChE with MM-GBSA method.

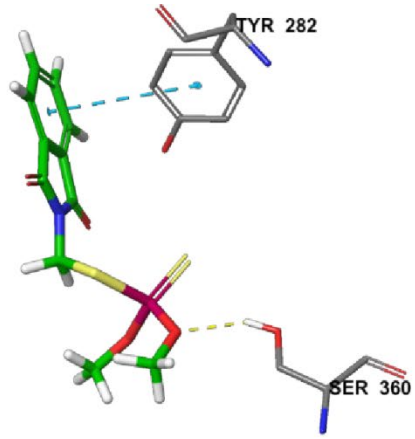


Figure S2.2. Phosmet re-docked at *Ag*AChE with MM-GBSA method.

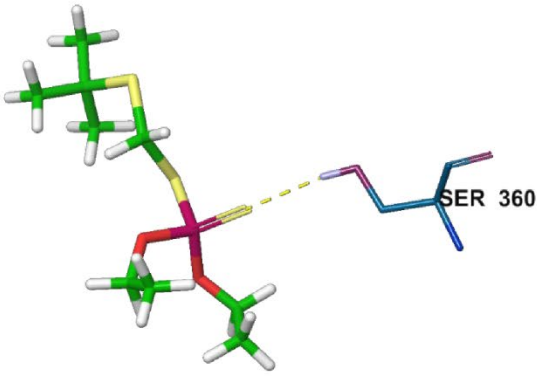


Figure S2.3. Terbufos re-docked at *Ag*AChE with MM-GBSA method.

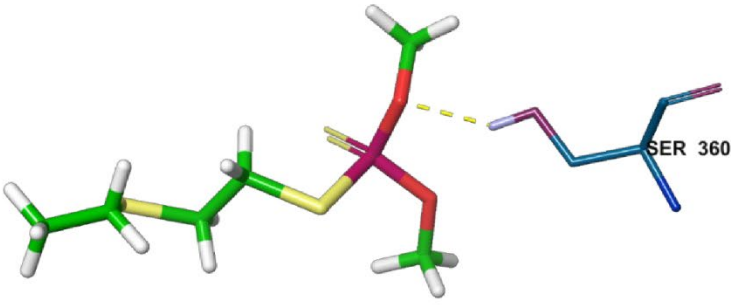


Figure S2.4. Thiometon re-docked at *Ag*AChE with MM-GBSA method.

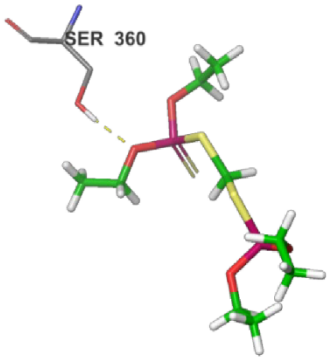


Figure S2.5. Ethion re-docked at *Ag*AChE with MM-GBSA method.

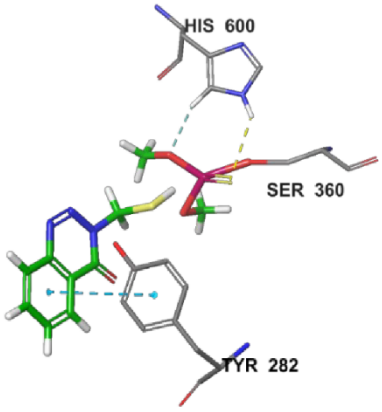


Figure S3.0. Azinphos-methyl docked at *Ag*AChE with new covalent function.

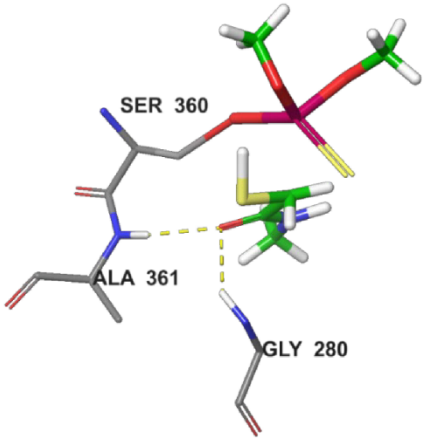


Figure S3. 1. Dimethoate docked at *Ag*AChE with new covalent function.

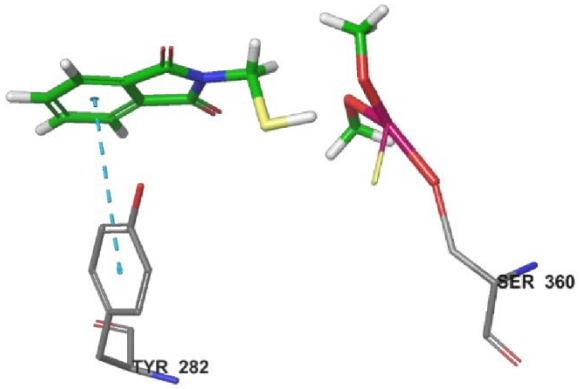


Figure S3. 2. Phosmet docked at *Ag*AChE with new covalent function.

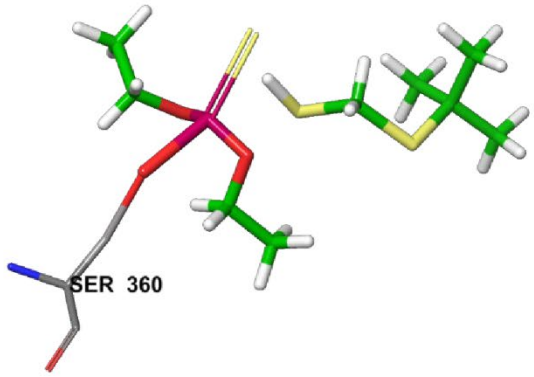


Figure S3. 3. Terbufos docked at *Ag*AChE with new covalent function.

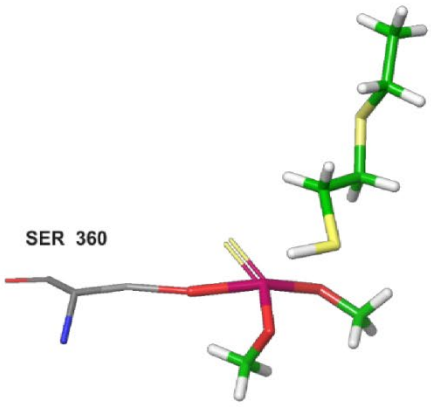


Figure S3. 4. Thiometon docked at *Ag*AChE with new covalent function.

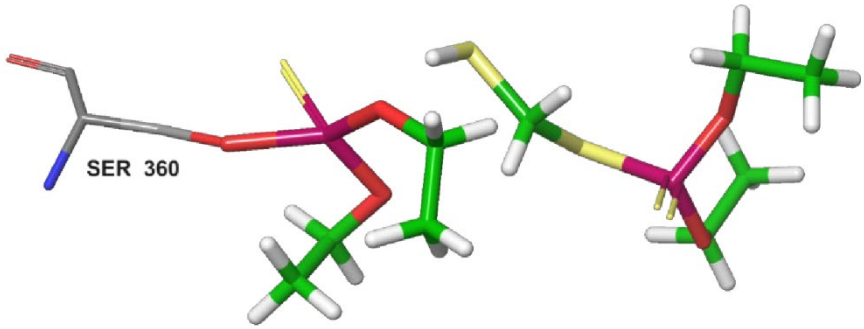


Figure S3. 5 Ethion docked at *Ag*AChE with new covalent function.

Appendix C.1: The *in silico* and *in vitro* analysis of donepezil derivatives for *Anopheles* acetylcholinesterase inhibition (Chapter 4; published paper)

PLOS ONE

RESEARCH ARTICLE

The *in silico* and *in vitro* analysis of donepezil derivatives for *Anopheles* acetylcholinesterase inhibition

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Data Availability Statement: Amino acid sequence data are available UniProt Knowledge Base (Accession numbers: A0A182HKN4, A0A6E8V9T9, and A0A182RZ85). The 3D molecular structure data are available on Protein Data Bank (https://www.rcsb.org/) with the ID numbers: 5YDI, 5YDH, 1EVE, and 4EY7.

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Abstract

Current studies on *Anopheles* anticholinesterase insecticides are focusing on identifying agents with high selectivity towards *Anopheles* over mammalian targets. Acetylcholinesterase (AChE) from electric eel is often used as the bioequivalent enzyme to study ligands designed for activity and inhibition in human. In this study, previously identified derivatives of a potent AChE, donepezil, that have exhibited low activity on electric eel AChE were assessed for potential AChE-based larvicidal effects on four African malaria vectors; *An. funestus*, *An. arabiensis*, *An. gambiae* and *An. coluzzii*. This led to the identification of four larvicidal agents with a lead molecule, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2** showing selectivity for *An. arabiensis* as a larvicidal AChE agent. Differential activities of this molecule on *An. arabiensis* and electric eel AChE targets were studied through molecular modelling. Homology modelling was used to generate a three-dimensional structure of the *An. arabiensis* AChE for this binding assay. The conformation of this molecule and corresponding interactions with the AChE catalytic site was markedly different between the two targets. Assessment of the differences between the AChE binding sites from electric eel, human and *Anopheles* revealed that the electric eel and human AChE proteins were very similar. In contrast, *Anopheles* AChE had a smaller cysteine residue in place of bulky phenylalanine group at the entrance to the catalytic site, and a smaller aspartic acid residue at the base of the active site gorge, in place of the bulky tyrosine residues. Results from this study suggest that this difference affects the ligand orientation and corresponding interactions at the catalytic site. The lead molecule **2** also formed more favourable interactions with *An. arabiensis* AChE model than other *Anopheles* AChE targets, possibly explaining the observed selectivity among other assessed *Anopheles* species. This study suggests that 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2** may be a lead compound for designing novel insecticides against *Anopheles* vectors with reduced toxic potential on humans.

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Competing interests: The authors have declared no competing interests exist.

1. Introduction

Donepezil (1-benzyl-4-((5,6-dimethoxy-1-indanon)-2-yl)methylpiperidine) shown in Fig 1 is a known potent human acetylcholinesterase (AChE) inhibitor used clinically in the management of symptoms associated with mild to severe Alzheimer's disease [1, 2]. The derivatisation of donepezil has been pursued to produce more active AChE agents against the human target with several studies have shown evidence of derivatives with high potency [3–7]. Particularly, van Greunen *et al.* [4] reported a potent derivative by converting the methyl linker (part A; Fig 1) between the piperidine ring and indanone group to an ester linker. This lead compound displayed a 50% inhibitory concentration (IC_{50}) value (0.03 ± 0.07 μM) similar to that of donepezil (0.05 ± 0.06 μM) when screened *in vitro* against AChE from *Electrophorus electricus* (electric eel) [4]. The AChEs from the electric eel and human have been shown to display similar activities, kinetics and inhibition profiles, as a result, electric eel AChE is used as a less expensive alternative to human AChE in bioassays [8, 9]. In a follow-up study, van Greunen and colleagues [3] synthesized and assessed various analogues of this lead compound for improved AChE activity. These new analogues featured the substitution of the ester linker found between the indanone and piperidine ring systems in their previous hit, by an amide that is more stable against rapid metabolism. In addition, the indanone (part B; Fig 1) was replaced with various aryl and aromatic groups [10]. Though at least two analogues were considerably active (IC_{50} <10 μM), none were as active as donepezil against electric eel AChE, and some were biologically inactive (IC_{50} >100 μM) [3].

Current studies on *Anopheles* AChEs are aimed at achieving high selectivity towards *Anopheles* over mammalian targets to reduce human toxicity [11–14]. Utilizing the molecular differences between human and insect AChE binding sites, these studies target conserved amino acid residues in *Anopheles* and compounds with selectivity index more than 100-fold towards *Anopheles* AChE have thus far been reported [11, 12]. The current study assessed the donepezil derivatives prepared by van Greunen *et al.* [3] for potential *Anopheles* AChE inhibition and rationalised their binding profiles through molecular docking. Interestingly, the parent drug, donepezil, has been proven to be active against insect AChE [15, 16], but is known to be approximately 40 times more selective to human AChE than the corresponding *Anopheles* target [16].

2. Materials and methods

This study received the animal research ethics waiver (Waiver Number: 07-11-2017-O) from Wits Animal Research Ethics Committee. Nine donepezil derivatives (Fig 2) with low activity (LC_{50} values >50 μM) against electric eel AChE from a previous study [3] were used for this research. Acetylthiocholine iodide, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dimethylsulfoxide (DMSO), Triton X-100, potassium dichromate, propoxur and sodium phosphate buffer (dibasic) (Na_2HPO_4) were bought from Sigma Aldrich (South Africa) with a purity >90%.

2.1. *Anopheles spp.* rearing

The laboratory-reared colonies of *An. funestus* (FUMOZ), *An. arabiensis* (KWAG), *An. gambiae* (COGS) and *An. coluzzii* (G3) were used for larvicidal and AChE screening assays. These are common *Anopheles* species responsible for malaria transmission in Africa [17–20]. These colonies were maintained under standard insectary conditions as reported by Hunt *et al.* [21] and Zengenene *et al.* [22]. FUMOZ was first collected from Mozambique in 2000 and has a low-level intensity of pyrethroid and carbamate resistance [23–25]. KWAG was collected from

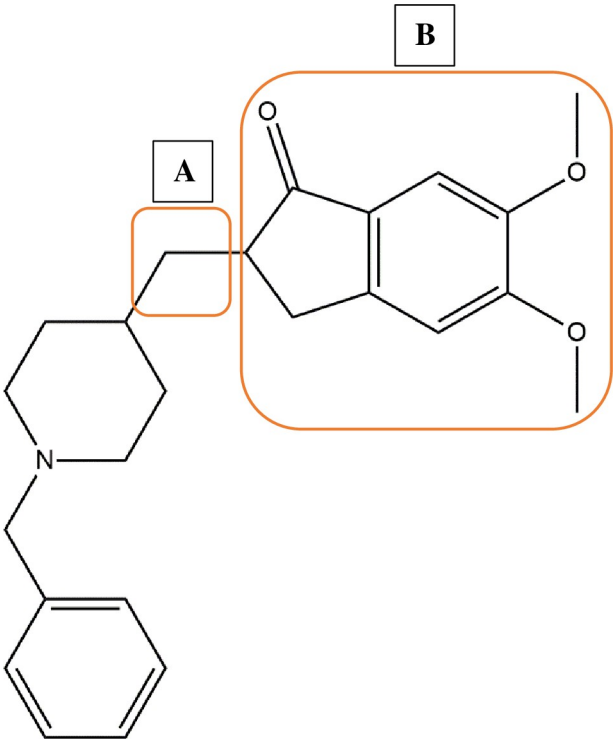


Fig 1. Chemical structure of donepezil showing two common sides of derivatization: A) indanone moiety and B) methyl linker.

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KwaZulu-Natal (South Africa) in 2005 and has shown resistance to pyrethroids and the organochloride, dichloro-diphenyl-trichloroethane (DDT) [19, 26]. On the other hand, COGS was collected in 2009 from Congo and displays resistance to multiple insecticides such as pyrethroids, organochlorides and carbamates [27, 28].

2.2. Larvicidal assay

The larval toxicity of novel donepezil analogues was assessed using the World Health Organization (WHO) bioassay for testing mosquito larvicides [29]. Briefly, batches of 20 third-instar larvae of each colony were transferred into the test cups into which 250 μL of a specific donepezil derivative was added. The incubation mixture was performed in a total volume of 250 mL of deionized water under 27˚C and humidity ≥78%. The test compounds were dissolved in DMSO and assessed for larvicidal activity at 10 times increasing concentrations from 0.0005 μM to 500 μM. Propoxur, a standard larvicide and an AChE agent [30, 31], was used as a positive control, while DMSO was employed for the negative vehicle control. Larval mortality was recorded in 24, 48 and 72 hours and larvae were fed with protein dog food at day 0 and after every mortality counting [32].

2.3. Brine shrimp lethality assay

Artificial seawater was prepared by dissolving 32 g of Tropic Marine® Sea salt in 1L of deionized water. The seawater was poured into an inverted plastic bottle after which the brine shrimp eggs were added for hatching. Regular airflow was supplied to the seawater to continually disperse the eggs and oxygenate the water. Moreover, a concentrated light was supplied from a lamp (220–240 V, 15W) to provide warmth to optimize hatching conditions for the 24

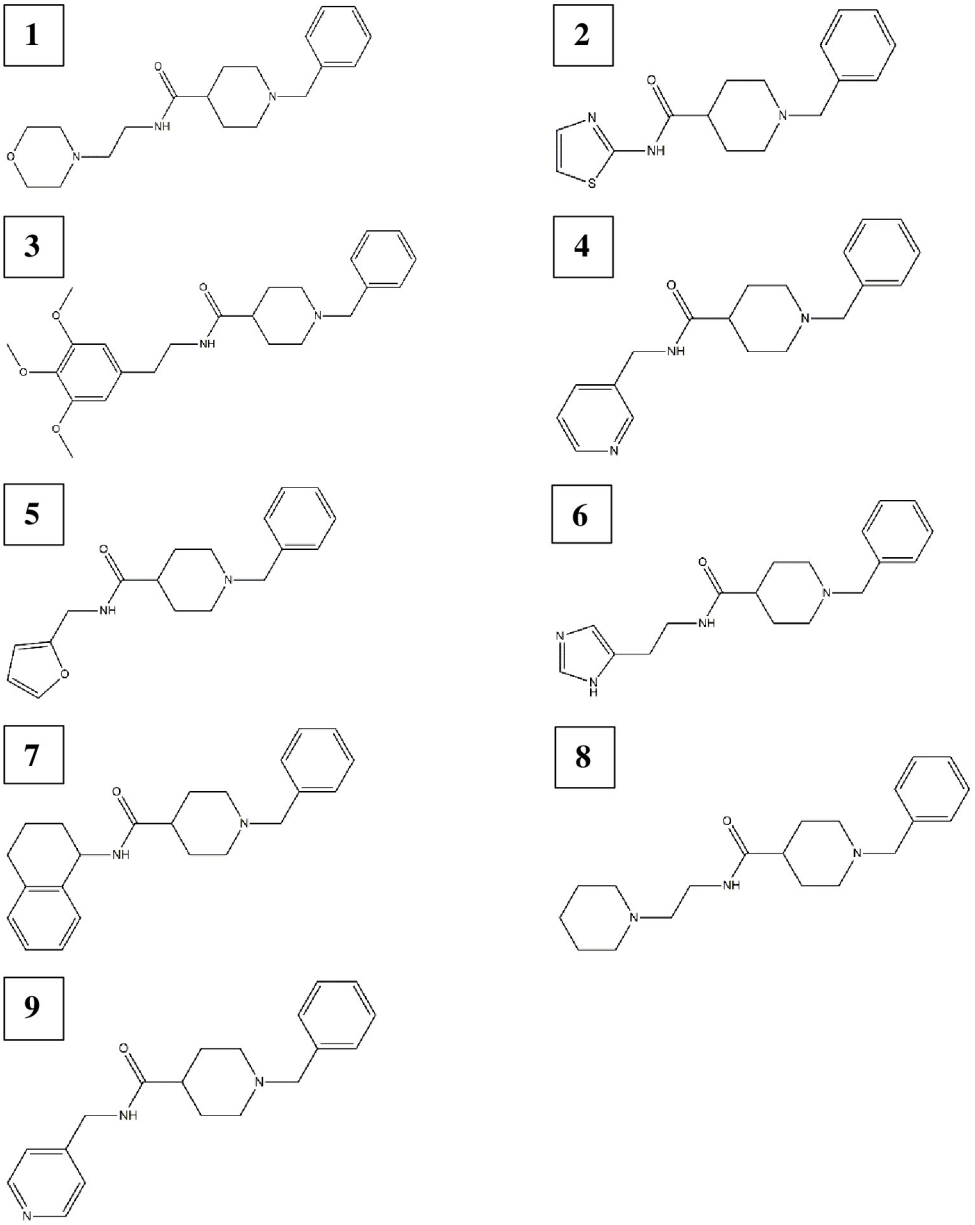


Fig 2. Chemical structures of the nine donepezil derivatives screened. 1-benzyl-*N*-(2-morpholinoethyl) piperidine-4-carboxamide **1**, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2**, 1-benzyl-*N*-(3,4,5-trimethoxyphenethyl) piperidine-4-carboxamide **3**, 1-benzyl-*N*-(pyridine-3-ylmethyl) piperidine-4-carboxamide **4**, 1-benzyl-*N*-(furan-2-ylmethyl) piperidine-4-carboxamide **5**, *N*-[2-(1H-imidazol-4-yl)ethyl]-1-benzylpiperidine-4- carboxamide **6**, 1-benzyl-*N*-(1,2,3,4-tetrahydro-naphthalen-1-yl) piperidine-4-carboxamide **7**, 1-benzyl-*N*-(2-(piperidin-1-yl)ethyl) piperidine-4-carboxamide **8**, and 1-benzyl-*N*-(pyridine-4-ylmethyl) piperidine-4-carboxamide **9** [3].

https://doi.org/10.1371/journal.pone.0277363.g002

h incubation time [33]. Following this, the cytotoxicity potential of the donepezil derivatives was evaluated by the brine shrimp lethality assay using *Artemia franciscana* [34]. The same concentrations used in the larvicidal assessment were also used for the toxicity evaluation. Inside 48-well plates, 50 μL of the test compound was incubated with 30–50 nauplii in 450 μL of the seawater. The wells were then observed under the stereo microscope (Olympus) at 10X magnification for dead nauplii and the induced mortality was recorded after 24 h. Where mortality was observed, the morphological changes were observed at 10X magnification using the

stereo microscope mounted with a Dino-Eye camera. For the negative control, DMSO was used in place of the test compound, while potassium dichromate was used as a positive control [35, 36].

2.4. AChE assay

The evaluation of AChE activity of the donepezil derivatives was conducted using the modified Ellman assay [11, 37]. Mosquitoes from the four *Anopheles* colonies were separately homogenized in Na_2HPO_4, 1% Triton X-100 (pH 8.0) and used as an enzyme source. Protein content was assessed using the standard Lowry protein assay [38]. The incubation mixture in a 96-well plate consisted of 20 μl of the enzyme in 132 μL of the assay buffer (Na_2HPO_4, 1% Triton X-100; pH 8.0). Twenty (20) μL of the donepezil derivatives at concentrations ranging from 0.0005 μM to 500 μM dissolved in DMSO were added. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), was freshly prepared in sodium phosphate buffer (dibasic; pH 7.0) and 8 μL (0.2 mM) added to the incubation mixture. To initiate the reaction, 20 μL (1 mM) of the AChE substrate, acetylthiocholine iodide was added making a total volume of 200 μL in each well and the absorbance readings were obtained using the UV-Visible spectrophotometer at 412 nm. Propoxur was kept as a positive control on the basis of having both AChE and larvicidal activities [31], while DMSO was used as a negative control. DMSO is known to exhibit AChE inhibition at concentrations above 1% [39], as such the highest DMSO concentration used in this study was 0.4%. For screening against electric eel AChE, donepezil was used as a positive control due to its known potent inhibitory activity against this target [3, 4]. However, the insecticide propoxur was also assessed against this target to determine AChE selectivity between electric eel and *Anopheles* and subsequent comparison with the test compounds.

2.5. *In silico* studies

2.5.1. Homology modelling. As a crystal structure of *An. arabiensis* was not available, it was elected to employ the use of homology modelling [40]. This approach involved four successive steps: (i) target amino acid sequence identification, (ii) template identification, (iii) sequence alignment between target and template, and (iv) model building and optimization [41, 42]. Homology modelling is considered the most accurate *in silico* approach to generate 3D models of proteins [42], however, certain minimum requirements had to be met. To produce a reliable protein structure, an existing sequence that matched at least 30% of the target sequence should be used as a template. These are known to display similar structures and interaction mechanisms [43–45]. Moreover, for the 30% identity cut-off, the length of the aligned sequences between the two should be >100 amino acid residues [46]. Sequence identity without taking into account the length of aligned amino acid residues, has been shown to result in less accurate models [47].

The amino acid sequence of *An. arabiensis* was retrieved from UniProt Knowledge Base (Accession number: A0A182HKN4). This accession number was submitted to the SWISS-MODEL database server for a reference 3D structure search and model building [48]. *An. gambiae* AChE (PDB: 5YDI) was selected as a template [49]. UCSF Chimera v1.16 was used for model optimization and visualization [50, 51]. Moreover, the correctness of the model was checked through ProQ webserver [52], and the model was validated with Verify3D and MolProbity [53, 54].

2.5.2. Molecular docking. Schrödinger Release 2018–2 molecular docking package, Maestro version 12.9, (Schrödinger LLC, New York) was used for ligand binding assessments [55]. The generated *An. arabiensis* AChE 3D structure and PDB sourced related proteins for comparative studies were prepared through Maestro's protein preparation function. This included

the AChE proteins from *An. gambiae* (PDB: 5YDI) [49]), electric eel (PDB: 1EVE [56]) and human (PDB: 4EY7 [57]). The preparation included optimization of H-bonds and removal of non-hetero groups and non-essential water molecules before minimization by OPLS3e force field [58]. The receptor grid was also generated using the OPLS3e force field to define the binding site [55]. Similarly, the ligands were prepared for docking using the LigPrep tool in which they were allowed to generate possible stereoisomers as well as ionization and tautomeric states at pH 7.0 (±2.0) [59, 60]. Finally, the extra precision mode was used for assessing the binding profiles of the prepared ligands to the receptor sites [61, 62].

2.6. Statistical analyses

All *in vitro* and *in vivo* assays were performed in triplicate. The 50% lethal concentration (LC_{50}) values were calculated from the probit analysis method using the SPSS Statistics v28 package (International Business Machines Corporation, NY, USA) and the IC_{50} values were determined by non-linear regression analysis using GraphPad Prism 9 (GraphPad Software, CA, USA) [30, 36].

3. Results and discussion

3.1. Larvicidal activity

Only derivatives **1**, **2**, **5** and **8** (Fig 2) showed larvicidal activities (Table 1). Specifically, derivatives **1**, **5** and **8** were active against *An. funestus* with lower LC_{50} values (2.65, 2.96 and 0.80 μM) compared to the positive control, propoxur (9.90 μM) (Table 1). On the other hand, derivative **2** was selective to *An. arabiensis*. This derivative was about 10-fold (LC_{50}: 0.88 μM) more potent than propoxur (LC_{50}: 8.77 μM) for *An. arabiensis* larval toxicity (Table 1). As a result, only derivatives **1**, **2**, **5** and **8** were selected for further analysis in the AChE inhibition assay.

Table 1. Larvicidal activities of the assessed compounds.

	***Anopheles* colonies Larvicidal LC_{50} (95% confidence interval range) (μM) [Chi square (X^2); degree of freedom (df); *p* value; intercept; standard error (SE)]**			
Donepezil derivative	***An. Funestus***	***An. arabiensis***	***An. gambiae***	***An. coluzzii***
1	2.65 (1.72–4.12) [X^2: 4.76; df: 5; $p = 0.45$[a]; intercept: -0.27; SE: 0.06]	> 100	> 100	> 100
2	> 100	0.88 (0.35–2.27) [X^2: 17.56; df: 5; $p = 0.004$[b]; intercept: 0.05; SE: 0.07]	> 100	> 100
3	> 100	> 100	> 100	> 100
4	> 100	> 100	> 100	> 100
5	2.96 (1.94–4.57) [X^2: 5.43; df: 5; $p = 0.37$[a]; intercept: -0.31; SE: 0.06]	> 100	> 100	> 100
6	> 100	> 100	> 100	> 100
7	> 100	> 100	> 100	> 100
8	0.80 (0.56–1.16) [X^2: 3.58; df: 5; $p = 0.61$[a]; intercept: -0.78; SE: 0.07]	> 100	> 100	> 100
9	> 100	> 100	> 100	> 100
Propoxur	9.90 (2.95–41.41) [X^2: 26.24; df: 5; $p < 0.001$[b]; intercept: -0.78; SE: 0.08]	8.77 (2.81–32.71) [X^2: 23.95; df: 5; $p < 0.001$[b]; intercept: -0.75; SE: 0.08]	63.99 (38.23–116.29) [X^2: 3.96; df: 5; $p = 0.56$[a]; intercept: -1.11; SE: 0.09]	62.68 (37.72–112.78) [X^2: 3.58; df: 5; $p = 0.61$[a]; intercept: -1.12; SE: 0.09]

[a] Since the significance level was greater than 0.15, a heterogeneity factor was not used in the calculation of confidence interval limits.

[b] Since the significance level was less than 0.15, a heterogeneity factor was used in the calculation of confidence interval limits.

https://doi.org/10.1371/journal.pone.0277363.t001

3.2. Brine shrimp lethality

None of the derivatives induced artemicidal effects (100% viability at 500 μM). This suggests relative safety of these novel compounds compared to the positive control, potassium dichromate that attained the LC_{50} value of 0.004 (0.002–0.007) μM (X^2: 8.10; df: 5; $p = 0.151$; intercept: 1.30; SE: 0.09) in agreement with a previous assessment [36]. This was a favourable outcome for the donepezil derivatives **1**, **2**, **5** and **8** which had potent larvicidal effects (Table 1), as it suggests that when used as larvicides, these derivatives would potentially be nontoxic to other aquatic lives.

3.3. AChE inhibition

Though the derivatives **1**, **5** and **8** showed potent larvicidal activity against *An. funestus*, none of these derivatives displayed AChE activities against this colony (Table 2). This suggests that these derivatives exert larvicidal activity through a different mechanism other than AChE inhibition. Surprisingly, derivatives **1**, **5**, **8** and the positive control, propoxur, showed moderate to low activity against the *in vitro An. gambiae* AChE target. This was in discordance to the *in vivo* larvicidal data where these molecules were inactive. It is common to have discrepancies between *in vitro* and *in vivo* data [63–65] and it has also been shown to occur with insecticide-resistant *Anopheles* larvae [66]. In fact, *An. gambiae* larvae have been shown to possess pharmacokinetic barriers such as thickened cuticle that play a role in preventing compound penetration at effective concentrations [66, 67]. Interestingly, derivative **2** showed potent AChE activity specifically against *An. arabiensis* (IC_{50} = 6.05 ± 2.21 μM; Table 2 (log-dose response curve shown in S1 Fig)), for which it also displayed larvicidal effects (Table 1).

Nevertheless, derivative **2** is known to have activity against electric eel AChE [3] and displayed an IC_{50} value of 55.70 ± 12.02 μM (Table 3). The calculated selectivity index (SI = IC_{50} electric eel AChE / IC_{50} *An. arabiensis* AChE) between the two AChE targets was 9.2. With the selectivity index less than 10, it may not be considered selective for *An. arabiensis* over electric eel AChE [68, 69]. However, in comparison to propoxur, which was essentially non-selective between *An. arabiensis* (IC_{50} = 0.78 ± 0.16 μM; Table 2) and electric eel (IC_{50} = 1.28 ± 0.35 μM; Table 3) in agreement with previous studies [13, 70], derivative **2** showed better potential for *Anopheles* selectivity. Furthermore, with good correlation between the larvicidal and AChE activity of derivative **2** consistently against *An. arabiensis*, presents it as a potential lead molecule for further derivatization into more potent and selective analogues. For this reason, the donepezil derivative **2**, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide, was selected as a lead molecule and assessed further for its AChE binding profile.

4. *In silico* studies

4.1. Homology modelling

The 3D AChE model for *An. arabiensis* was successfully built from the *An. gambiae* (PDB: 5YDI) template. Sequence identity between the two models was 100% with more than 500

Table 2. Comparison of the *Anopheles* AChE inhibitory potential of derivatives 1, 2, 5 and 8.

	Anopheles AChE inhibition IC_{50} ± SE (95% confidence interval range) (μM)			
Donepezil derivative	***An. funestus***	***An. arabiensis***	***An. gambiae***	***An. coluzzii***
1	> 100	> 100	48.85 ± 10.49 (25.77–71.93)	> 100
2	> 100	6.05 ± 2.21 (2.78–13.16)	> 100	> 100
5	> 100	> 100	31.42 ± 7.56 (14.78–48.06)	> 100
8	> 100	> 100	28.13 ± 6.89 (12.96–43.31)	> 100
Propoxur	0.89 ± 0.20 (0.45–1.33)	0.78 ± 0.16 (0.42–1.13)	26.08 ± 6.21 (12.42–39.74)	25.04 ± 6.14 (11.53–38.55)

https://doi.org/10.1371/journal.pone.0277363.t002

Table 3. Comparison of the electric eel AChE inhibitory potential of derivatives 1, 2, 5 and 8.

Donepezil derivative	*Electric eel* AChE inhibition IC_{50} ± SE (95% confidence interval range) (μM)
1	>100
2	55.70 ± 12.02 (29.24–82.16)
5	>100
8	88.29 ± 19.90 (44.49–132.10)
Propoxur	1.28 ± 0.35 (0.25–2.03)

https://doi.org/10.1371/journal.pone.0277363.t003

aligned amino acid residues (range 162–698) translating into a target structural coverage of 0.73. Global model quality estimate (GMQE) and model quality estimation QMEANDisCo Global were used to estimate the overall model quality [71, 72]. The GMQE score was 0.71, while the QMEANDisCo Global achieved 0.92 ± 0.05 indicating that the final model was analogous to the experimental crystal structures. The quality of the model was assessed by comparing it to the reference and non-redundant 3D structures from PDB (Fig 3A and 3B). Similarly, quaternary structure quality estimation (QSQE) assessed the accuracy of the generated target 3D structure in terms of the inter-chain contacts in accordance with the template and resulting alignment. A value above 0.7 indicates a reliable model [48, 73] and the final model in this study reached a score of 0.74.

The assessment of the correctness of the model through ProQ showed an LGscore of 11.077. This program analysed relative frequencies of intramolecular atomic interactions within the model where the LGscore >4 indicated an extremely good model [52]. In addition, the Verify3D suggested a model PASS with 97.11% of the amino acid residues a 3D/1D score of ≥0.2 (Fig 4A). This tool assessed how compatible a generated model was with its amino acid sequences. This is assessed based on the optimum environment for each amino acid residue such as the area of the residue buried deep in the protein, and hence inaccessible to the solvent, the area of the side-chains made of polar atoms, as well as the quality of the local secondary structure [53]. A favourable MolProbity score of 1.36 and 98th percentile was obtained for the model (Fig 4B) confirming the validity of the model [54]. The Ramachandran plot (Fig 4C) reported a score of 95.14% for amino acid residues in favoured regions, 99.8% in allowed regions and 0.0% for both Ramachandran outliers and Cβ deviations. This suggested a valid model with a stable backbone [74].

The new model was aligned with the existing *An. gambiae* AChE 3D structure (PDB: 5YDI) and visualized in UCSF Chimera. This program showed 100% alignment of the AChE catalytic sites of the modelled *An. arabiensis* and reference *An. gambiae* (Fig 5).

4.2. Molecular modelling

Assessment of molecular interactions with the AChE catalytic sites of electric eel (Fig 6A) and a built model of *An. arabiensis* (Fig 6B) showed some similar interactions with the targets. The thiazole group was bound by arginine residues (Arg^{289} (electric eel)/Arg^{233} (*An. arabiensis*), however with an additional aromatic stabilization by Tyr^{493} in the *An. arabiensis* model (Fig 6B). Several amino acid residues were involved in the interaction with the *N*-benzylpiperidine moiety. For the electric eel, these included two catalytic triad residues Glu^{199} through hydrogen bonding and stabilization of the *N*-benzylpiperidine ring through aromatic pi-pi interaction with His^{440}. This ring was held on the other side by another pi-pi interaction with Phe^{330}, while Trp^{84} and Tyr^{334} formed pi-cation interactions with the ionized nitrogen of *N*-benzylpiperidine. Similarly, this nitrogen was involved in pi-cation interactions with Trp^{245} and Tyr^{489} along with Glu^{359} in the *An. arabiensis* model, however, with no stabilization of the *N*-

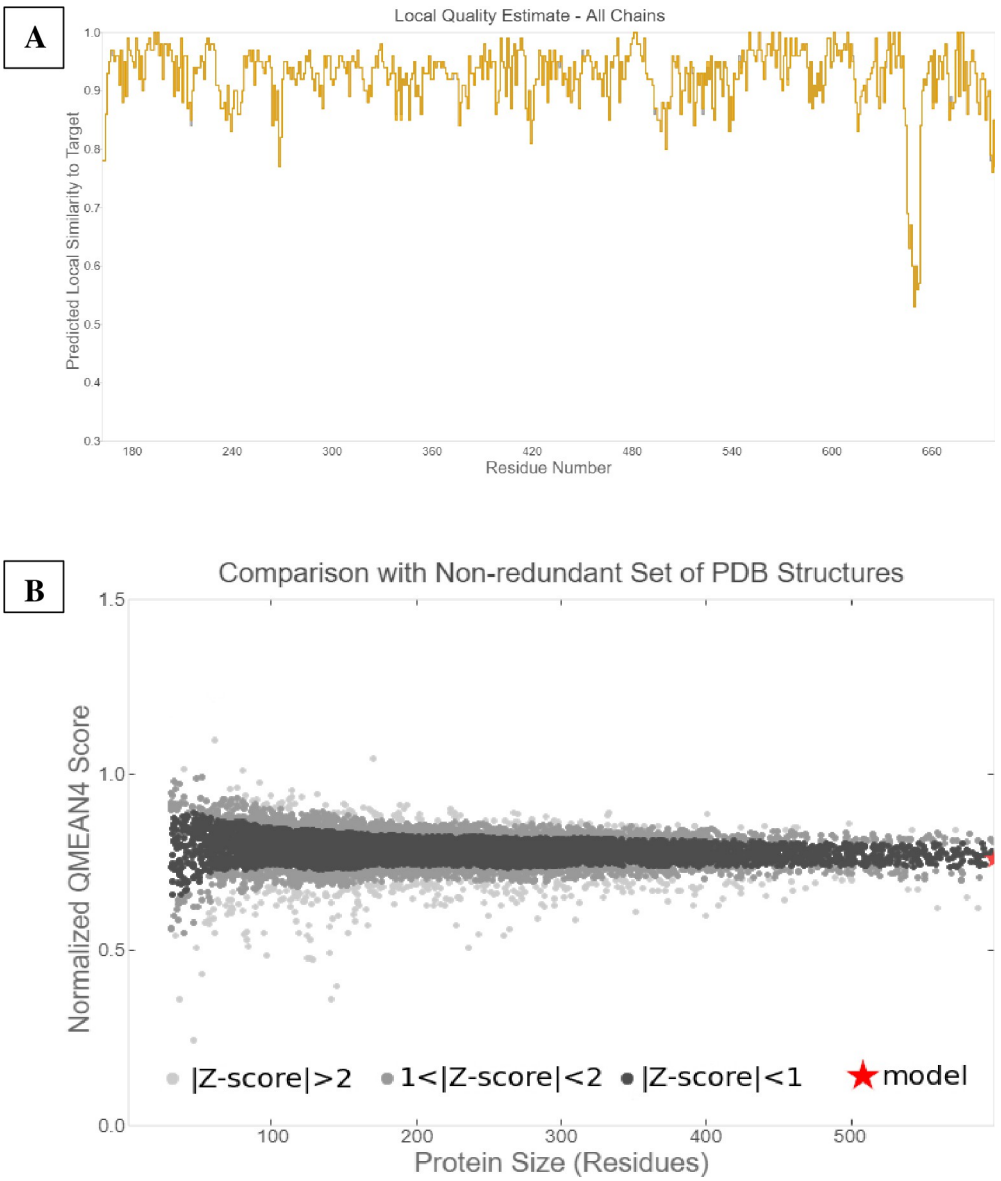


Fig 3. Quality of the *An. arabiensis* homology model. A) Shows the quality estimate of the generated model based on its similarity to the template and B) shows its comparison to non-redundant 3D structures.

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benzylpiperidine ring (Fig 6B). This caused the derivative to adopt a different orientation of the *N*-benzylpiperidine group when contrasted against the conformation observed in the electric eel AChE site. This possibly caused the observed lower binding score. To gain a better understanding, the differences in the binding sites of *Anopheles*, electric eel and human AChEs from PDB were assessed.

A comparison of electric eel, human and *Anopheles* AChE catalytic sites was performed by superimposing amino acid residues that represent the entrance to the catalytic site and the catalytic site amino acids through the Glide's Quick Align function. PDB 1EVE was used for electric eel [56], PDB 4EY7 for human [57], and PDB 5YDI for the *Anopheles* (*An. gambiae*) AChE target [49]. The key observable difference between *Anopheles* AChE and the two other proteins was the replacement of a larger phenylalanine (Phe^{288} (human)/Phe^{295} (electric eel)) with a

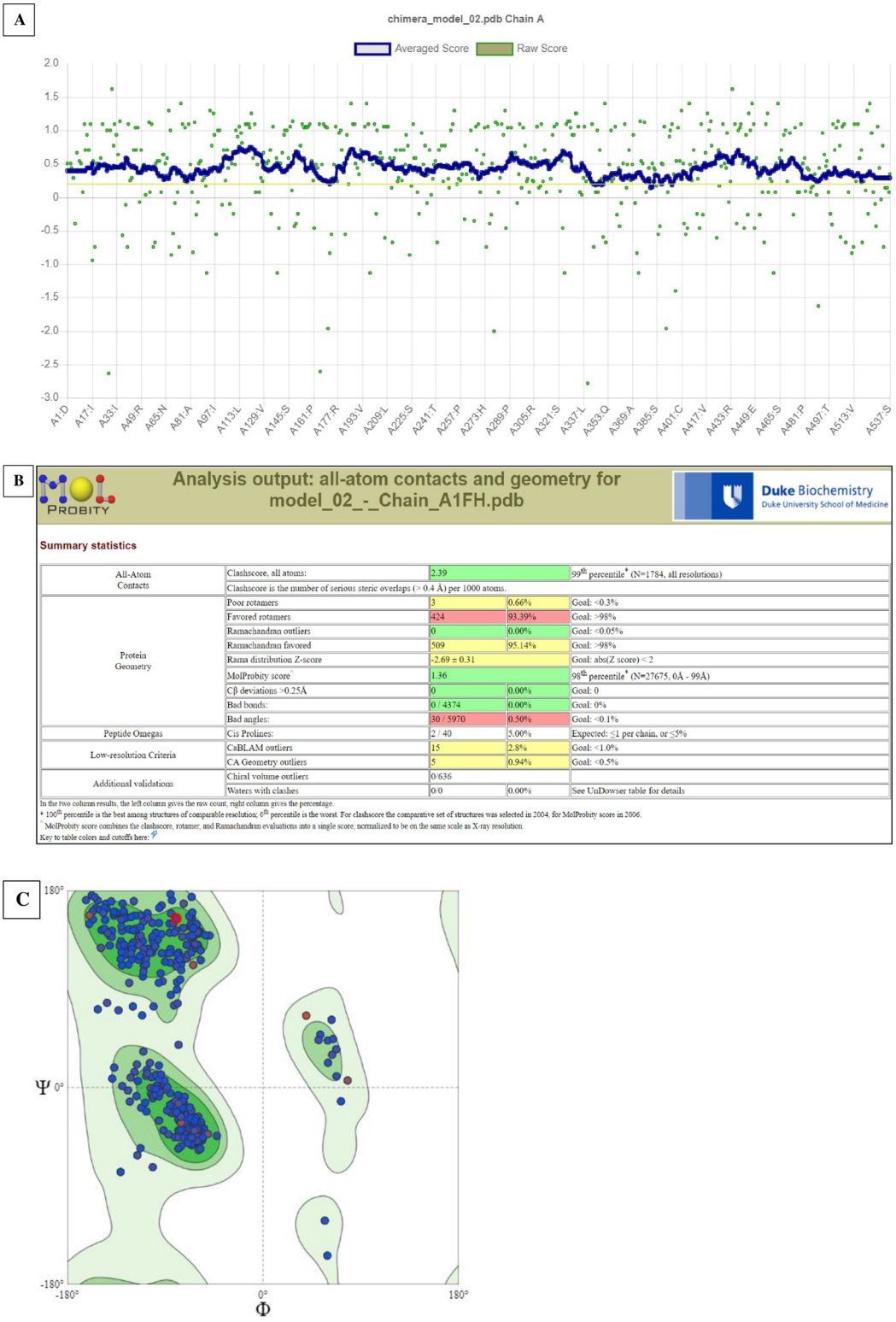


Fig 4. Validity of the model displayed by Verify3D tool (A) and scored by MolProbity (B) and the corresponding Ramachandran plot (C).

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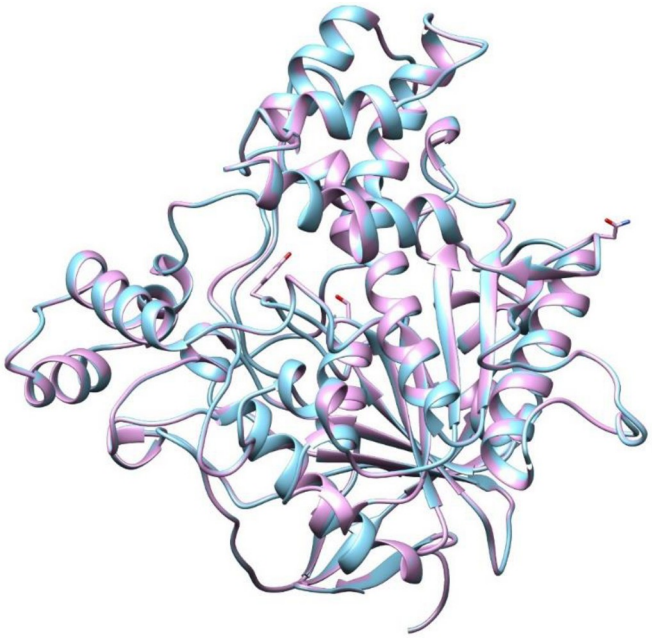


Fig 5. Alignment of the catalytic sites of the generated *An. arabiensis* AChE (sky blue) and reference *An. gambiae* (plum).

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smaller cysteine residue (Cys^{447}) at the entrance of the catalytic site. Similarly, a smaller aspartic acid residue (Asp^{602}) was identified at the base of the *Anopheles* AChE catalytic site, in place of the bulky tyrosine residues (Tyr^{442} (human)/Tyr^{449} (electric eel)) (Fig 7) in agreement with the reported literature [75].

To gain further insights into the influence of these differences in ligand binding, the potential differences between the binding poses of the known human and electric eel AChE inhibitor, donepezil [2, 3, 5], when bound to these three AChE proteins were assessed (Fig 8). Donepezil displayed a similar binding pose and interactions with both electric eel and human

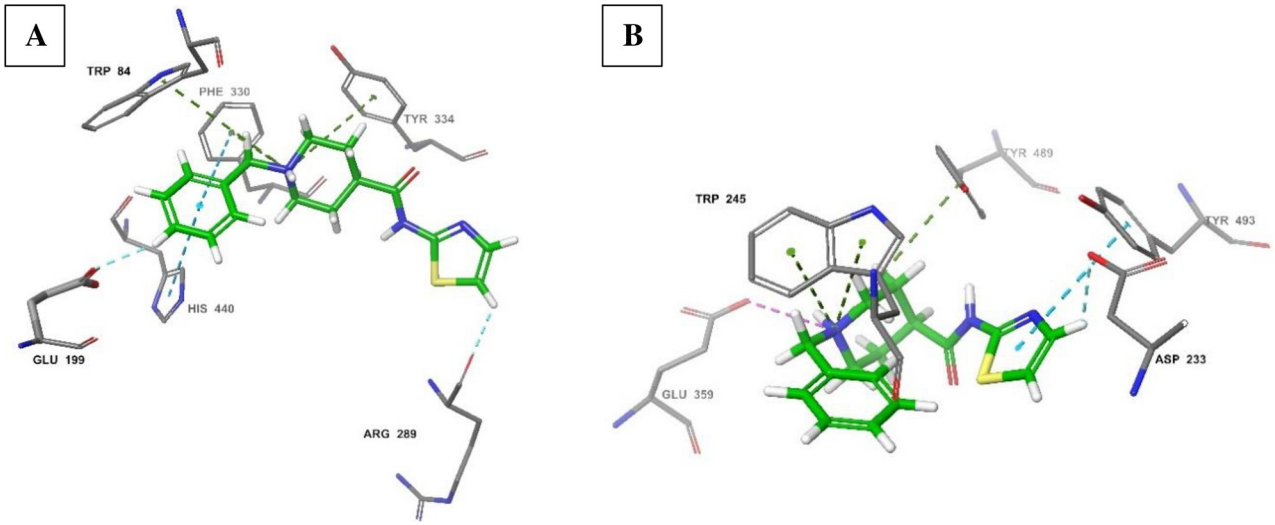


Fig 6. Molecular interactions of derivative **2** with electric eel AChE (A; Score: -11.8) and *An. arabiensis* (B; Score: -7.5).

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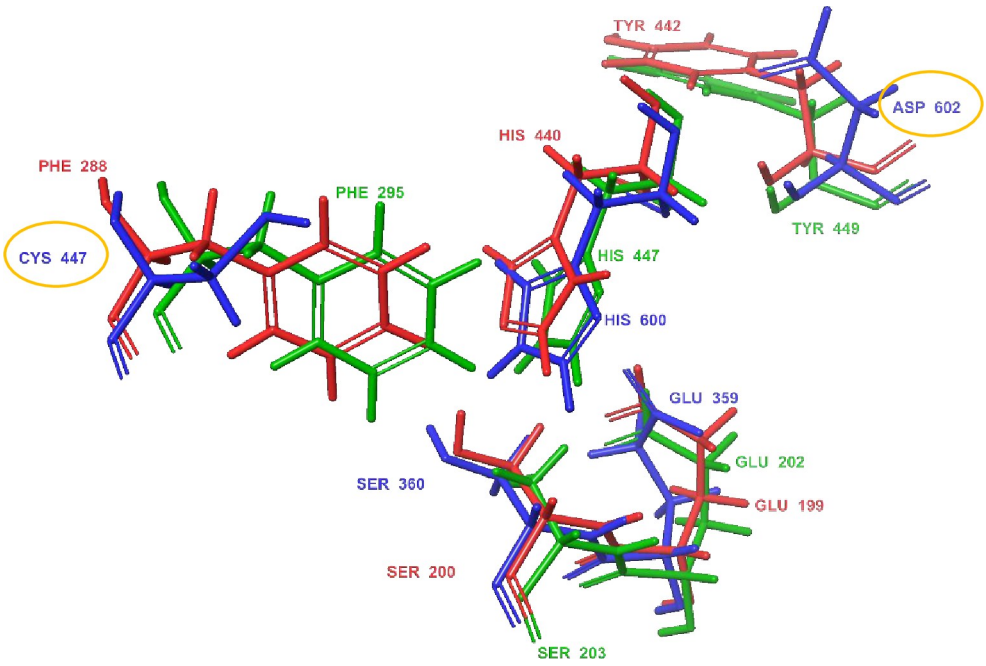


Fig 7. Superimposed amino acid residues showing the catalytic site entrance and the catalytic triad of electric eel (red), human (green) and *Anopheles* (blue) AChE. The distinct Cys^{447} at the *Anopheles* AChE catalytic entrance and Asp^{602} at the catalytic site base are shown by circles.

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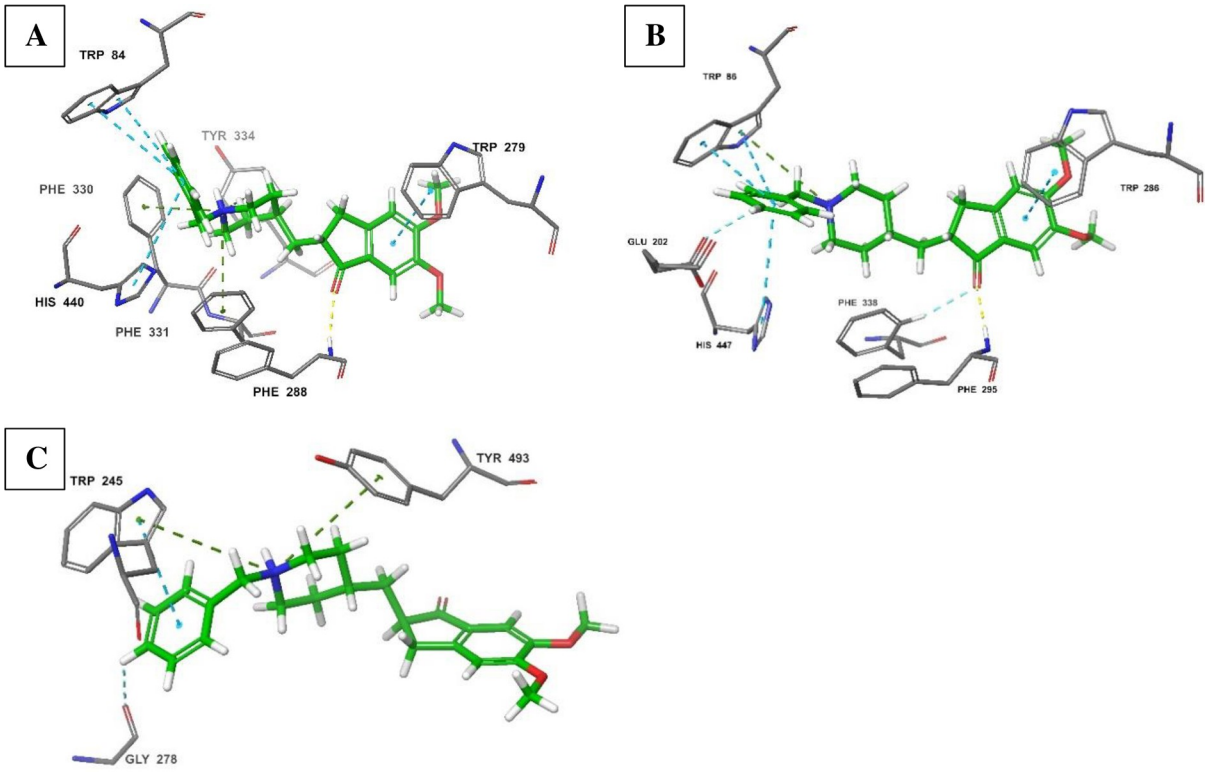


Fig 8. Comparison of the molecular interactions of donepezil with the AChE catalytic sites of electric eel (A; PDB: 1EVE; score: -15.0), human (B; PDB: 4EY7; score: -16.2) and *An. gambiae* (C; PDB: 5YDI; score: -10.2).

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AChE targets (Fig 8A and 8B). At both sites, donepezil was stabilized by aromatic interactions through the corresponding tryptophan residues. The donepezil indanone aromatic ring interacted with Trp^{279} and Trp^{286} in electric eel and human AChEs, respectively; while its *N*-benzylpiperidine ring established pi-pi stacking with Trp^{84} in electric eel and Trp^{86} in human target. Additionally, the catalytic site histidine residues (His^{440}; electric eel and His^{447}; human) also established the aromatic pi-pi interactions with the *N*-benzylpiperidine ring. However, in the human target, Glu^{202} was also involved in this interaction. The phenylalanine residues played a critical role in maintaining the crystal pose of donepezil through hydrogen bonding. This was achieved through Phe^{288} residue in electric eel and the comparable Phe^{295} in human AChE. Likewise, the pi-cation interactions between the nitrogen atom of *N*-benzylpiperidine and aromatic amino acids stabilized donepezil in the binding pose. In electric eel AChE, these interactions were generated from Phe^{330}, Phe^{331} and Tyr^{334}, while only Trp^{86} established this interaction in the corresponding human target (Fig 8A and 8B). In support of the binding similarity observed in this study, the binding potency of donepezil against electric eel and human AChE has also been reported to be similar (IC_{50} 0.035 μM and 0.030 μM, respectively) [76, 77].

On the other hand, donepezil generated different interactions with the *Anopheles* AChE from those obtained in electric eel and human targets (Fig 8C). There was no stabilization of the indanone moiety. However, the *N*-benzylpiperidine group was stabilized by Trp^{245} residues that are comparable to Trp^{279} and Trp^{286} in electric eel and human targets, respectively. The pi-cation interaction with the *N*-benzylpiperidine portion was established by the Trp^{245} and Tyr^{493} residues. This caused the aromatic ring of the *N*-benzylpiperidine to face down towards Gly^{278} residue and away from the catalytic triad amino acid histidine (His^{359}; *Anopheles*) that played a key role in the binding of donepezil in electric eel (His^{440}) and human (His^{447}) AChEs. For the first time, this study shows that donepezil adopts a different binding pose in the *Anopheles* target, lending support to previous studies that showed significant differences in selectivity for donepezil between human and *Anopheles* AChEs [16].

Finally, as derivative **2** displayed selective inhibition of AChE for *An. arabiensis* over those from *An. gambiae*, *An. coluzzii* and *An. funestus*, the binding pose difference across the assessed *Anopheles* colonies was assessed. However, the only available crystal structures from PDB repository were from *An. gambiae* [49, 78]. Therefore, additional models were generated from *An. coluzzi* (UniProt accession number: A0A6E8V9T9) and *An. funestus* AChE amino acid sequences (UniProt accession number: A0A182RZ85). Using SWISS-MODEL, the template for *An. coluzzi* was identified as *An. gambiae* (PDB: 5YDH) with GMQE score of 0.81, GSQE score of 0.79, and 100% sequence identity with amino acid coverage from 162 to 699. The final QMEANDisCo Global was 0.93 ± 0.05. On the other hand, the best template identified for *An. funestus* was *An. gambiae* (PDB: 5YDI) with GMQE and GSQE scores of 0.80 and 0.74, respectively. This obtained 98.01% amino acid sequence identity with coverage from 160 to 696 and QMEANDisCo Global was 0.92 ± 0.05. The Ramachandran plots for *An. coluzzi* and *An. funestus* models obtained from the Ramachandran plot server [79] are reported in S2 and S3 Figs, respectively. Further, the local quality estimate results of the models and comparisons to non-redundant experimental crystal structures (obtained from SWISS-MODEL) are displayed in S4 and S5 Figs for *An. coluzzi* and *An. funestus*, respectively; followed by their validity verifications through Verify3D (S6 and S7 Figs, respectively). As a result, a comparison of the binding profile differences was performed using three new AChE models for *An. arabiensis*, *An. coluzzi*, and *An. funestus*, as well as two PDB sourced *An. gambiae* AChE targets; wild-type (PDB: 5YDI) [49] and resistant (PDB: 6ARY) through target site mutation (G280S) [78].

The observed stabilization of thiazole ring by Tyr^{493} in the *An. arabiensis* model (Fig 9A) could not be attained in the wild-type *An. gambiae* (Fig 9B). Similarly, the pi-cation stabilization by Tyr^{489} and Glu^{359} were also lost in the wild-type *An. gambiae* site. Instead, the

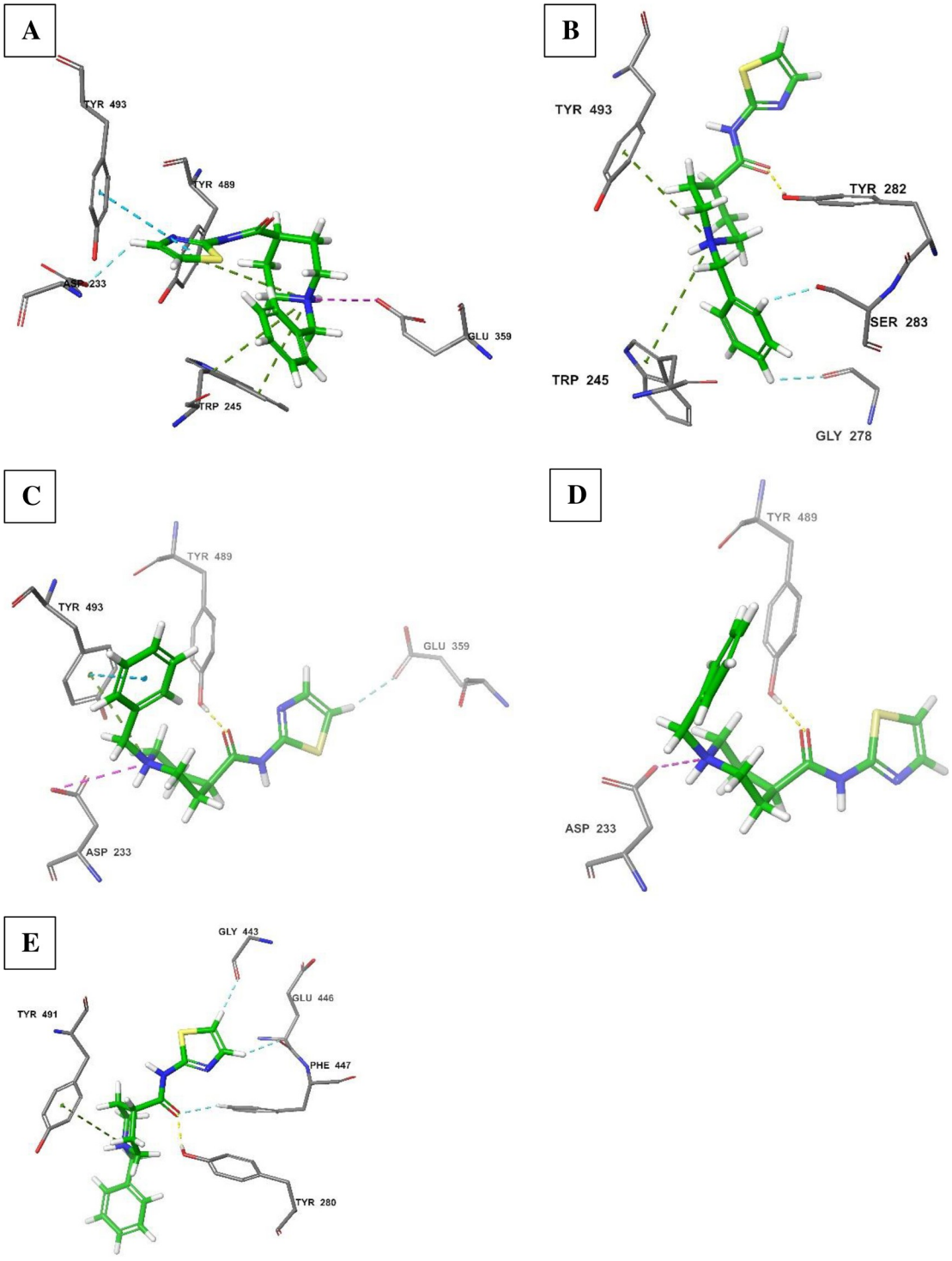


Fig 9. Comparison of the molecular interactions of derivative **2** with the AChE catalytic sites of *An. arabiensis* model (A; score: -7.5), wild-type *An. gambiae* (B; score: -9.2), target site mutated *An. gambiae* (C; score: -7.8), *An. coluzzii* model (D; score: -9.0) and *An. funestus* model (E; score: -9.1).

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interaction with the wild-type *An. gambiae* AChE showed aromatic hydrogen bonding between the *N*-benzylpiperidine ring and amino acid residues Gly^{278} and Ser^{283}. The

N-benzylpiperidine nitrogen atom was then involved in pi-cation interaction with aromatic amino acids, Trp^{245} and Tyr^{493}, while Tyr^{282} established the hydrogen interaction with an oxygen group of the amide linker (Fig 9B). The generated crystal pose at this site was clearly different from that obtained with the *An. arabiensis* model. At the mutated target site of *An. gambiae* target (Fig 9C), the orientation of derivative **2** was clearly the opposite of that generated in *An. arabiensis* model and this displayed comparatively fewer intermolecular interactions. At this target, Glu^{359} was involved in hydrogen bonding with the thiazole group and the *N*-benzylpiperidine moiety was stabilized by Tyr^{493} and Asp^{233}, in direct contrast to that observed with the *An. arabiensis* model where these Tyr^{493} and Asp^{233} stabilized the thiazole group. The Tyr^{489} held the molecule in space by establishing a hydrogen bond with the amide linker.

Least interactions were obtained against *An. coluzzii* target (Fig 9D). While the conformation of derivative **2** was similar to that obtained against mutant *An. gambiae* AChE (Fig 9C), where it only retained two interactions composed of Tyr^{489} H-bond and Asp^{233} pi-cation bond. Therefore, at both *An. gambiae* and *An. coluzzii* targets, derivative **2** assumed the orientation that was directly opposing that at *An. arabiensis* AChE catalytic site. Finally, at the *An. funestus* AChE site (Fig 9E), derivative **2** regenerated the crystal pose similar to that obtained at wild-type *An. gambiae* target (Fig 9B) with similar binding scores (-9.1 and -9.2 kcal/mol, respectively). In a similar fashion to wild-type *An. gambiae* AChE and different from *An. arabiensis* target, the pi-cation interaction with a tyrosine residue, Tyr^{491}, was established and supplemented by the linker H-bond with Tyr^{280} and Phe^{447}. At this target however, there was no further stabilization of the *N*-benzylpiperidine portion, but more H-bonds with the thiazole group from Gly^{443} and Glu^{446} residues (Fig 9E).

In general, derivative **2** generated a unique crystal pose against *An. arabiensis* model which may explain biological activity exclusively against this *Anopheles* species. On the other hand, this compound assumed a comparable pose between two targets, *An. funestus* wild-type and *An. gambiae* AChEs and established another conformation that was characteristic of *An. coluzzii* and mutant *An. gambiae* AChE.

As expected, this study showed great similarity between the AChEs from electric eel and human, in support of previous literature [4, 76, 77]. Interestingly, there were clear differences in ligand binding between either the electric eel or human AChE and *Anopheles* AChE, these differences potentially arising as a result of molecular differences around the catalytic site. In line with previous reports [75], the conserved Cys^{447} at the entrance to the catalytic site and Asp^{602} at the base of the active site were identified in this study. This molecular difference may bring about differences in ligand orientation, as well as interactions it establishes with surrounding amino acid residues. A smaller cysteine residue at the catalytic site entrance may create more volume available for a ligand to assume a different orientation as seen with a control, donepezil, and derivative **2**.

5. Conclusions

The current study identified a lead compound, 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide **2**, as an AChE-based larvicidal agent against *An. arabiensis* KWAG. Derivative **2** displayed more than 9-fold higher potency towards *An. arabiensis* AChE over that of electric eel AChE. Through molecular docking, this study has highlighted the close similarity between electric eel and human AChE, as well as their marked difference from *Anopheles* AChE. The differences in the binding of derivative **2** with electric eel and *An. arabiensis* AChE, respectively, were investigated at a molecular level through molecular modelling. Homology modelling was employed to generate a 3D AChE structure of *An. arabiensis* after which it was used

for comparative binding assessments. The conformation of the molecule and interactions with the protein amino acid residues was noticeably different between the two targets. This informed our study to assess the molecular differences between the AChE binding sites from electric eel, human and *Anopheles*. A critical distinction observed in *Anopheles* as opposed to the two mammal proteins, is a smaller cysteine residue in place of bulky phenylalanine groups at the entrance to the catalytic site and a smaller aspartic acid replacing larger tyrosine residues at the base of the catalytic site, in correlation with previous X-ray diffraction studies [75]. The influence of these amino acid residues on ligand binding and crystal pose generation needs to be further investigated.

Further, the selectivity of derivative **2** to *An. arabiensis* amongst other *Anopheles* species was evaluated by comparing binding profiles between the *An. arabiensis* model and *An. gambiae* AChE targets (wild-type and resistant (mutant)) from the PDB repository, as well as those of *An. coluzzii* and *An. funestus* generated through homology modelling. The molecule was better stabilized by a mixture of hydrogen bonds and pi-pi stacking in the *An. arabiensis* model than in the corresponding *Anopheles* targets. In conclusion, this study suggests that 1-benzyl-*N*-(thiazol-2-yl) piperidine-4-carboxamide presents as a lead compound for the design and synthesis of novel insecticides against the African malaria vector, *An. arabiensis*. Further derivatizations of this molecule may generate molecules with high potency against a wide range of *Anopheles* species and increased selectivity to *Anopheles* over mammal AChE.

Supporting information

S1 Fig. The *An. arabiensis* AChE inhibitory activity of 2.
(TIF)

S2 Fig. Ramachandran plot for *An. coluzzii* model (98.90% amino acid residues in the highly preferred region (green crosses); 1.10% in the allowed region (orange triangles) and 0.00% in the questionable region (red circles)).
(TIF)

S3 Fig. Ramachandran plot for *An. funestus* model (98.34% amino acid residues in the highly preferred region (green crosses); 1.66% in the allowed region (orange triangles) and 0.00% in the questionable region (red circles)).
(TIF)

S4 Fig. a. Local quality estimate of the An. coluzzii model. b. Local quality estimate of the An. funestus model.
(ZIP)

S5 Fig. a. Comparison of An. coluzzii model to the non-redundant experimental crystal structures. b. Comparison of An. funestus model to the non-redundant experimental crystal structures.
(ZIP)

S6 Fig. *An. coluzzii* model validity verification through Verify3D (98.98% of the amino acid residues obtained a score of $\geq$0.2 in the 3D/1D profiling).
(TIF)

S7 Fig. *An. funestus* model validity verification through Verify3D (98.88% of the amino acid residues obtained a score of $\geq$0.2 in the 3D/1D profiling).
(TIF)

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Writing – review & editing: Thankhoe A. Rants'o, C. Johan van der Westhuizen, Darren L. Riley, Jenny-Lee Panayides, Lizette L. Koekemoer, Robyn L. van Zyl.

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Appendix C.2: Supplementary information for Chapter 4 (published paper)

Title of the paper: The *in silico* and *in vitro* analysis of donepezil derivatives for *Anopheles* acetylcholinesterase inhibition

The data presented in this supplementary information covers the homology modelling for *An. funestus* and *An. coluzzii* AChE targets using UniProt database for amino acid sequences and SWISS-MODEL for homology models. It also shows the results from model validation through various tools such as Verify3D and MolProbity. Also presented here is the log-dose response curve for the most active donepezil analogue **2** against *An. arabiensis*. The general formatting and presentation of this data is aligned with the requirements of the journal in which it has been published.

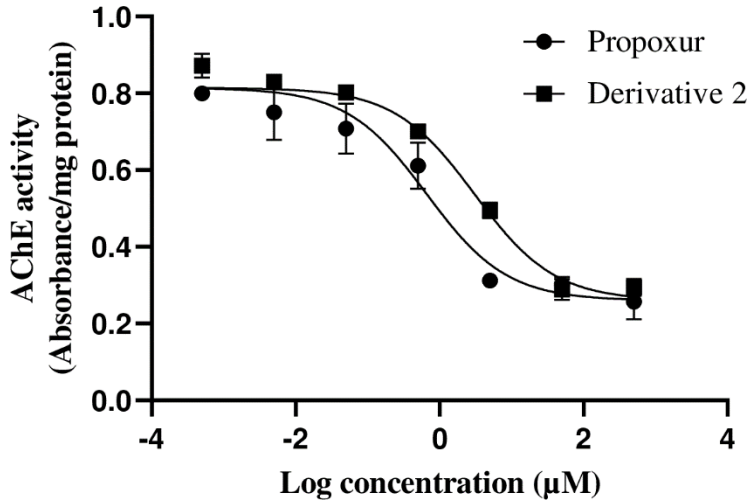


Fig S1: The *An. arabiensis* AChE inhibitory activity of **2**.

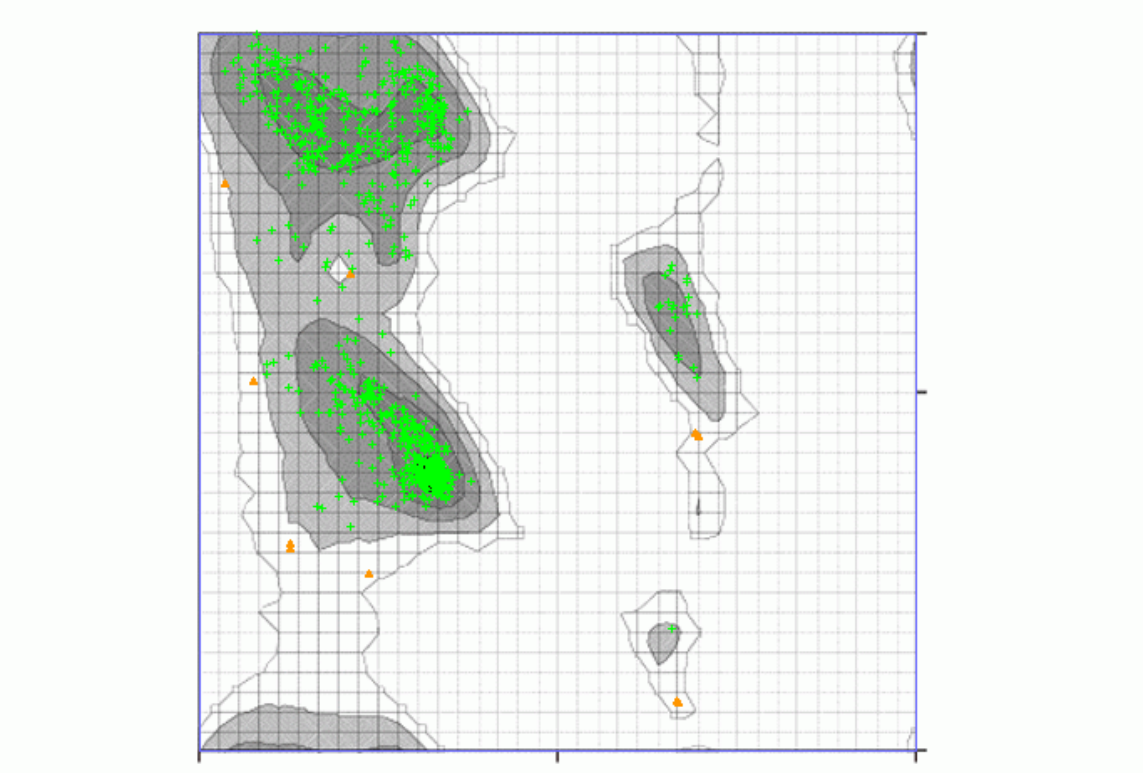


Fig S2: Ramachandran plot for *An. coluzzii* model (98.90% amino acid residues in the highly preferred region (green crosses); 1.10% in the allowed region (orange triangles) and 0.00% in the questionable region (red circles))

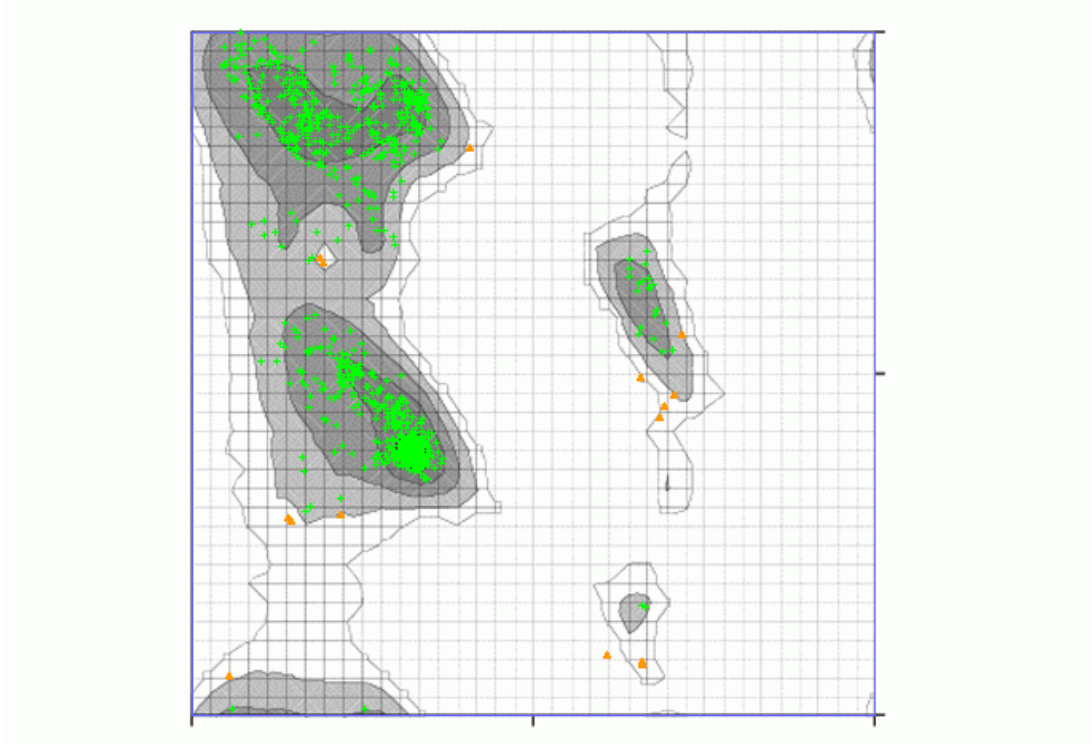


Fig S3: Ramachandran plot for *An. funestus* model (98.34% amino acid residues in the highly preferred region (green crosses); 1.66% in the allowed region (orange triangles) and 0.00% in the questionable region (red circles)).

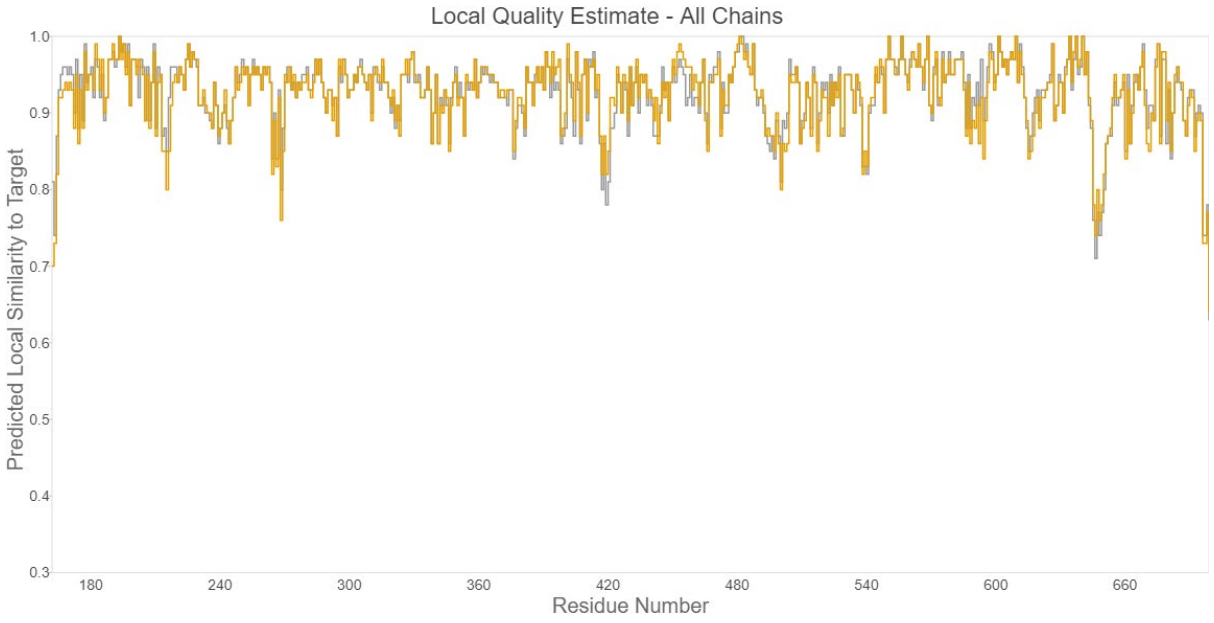


Fig S4a: Local quality estimate of the *An. coluzzii* model

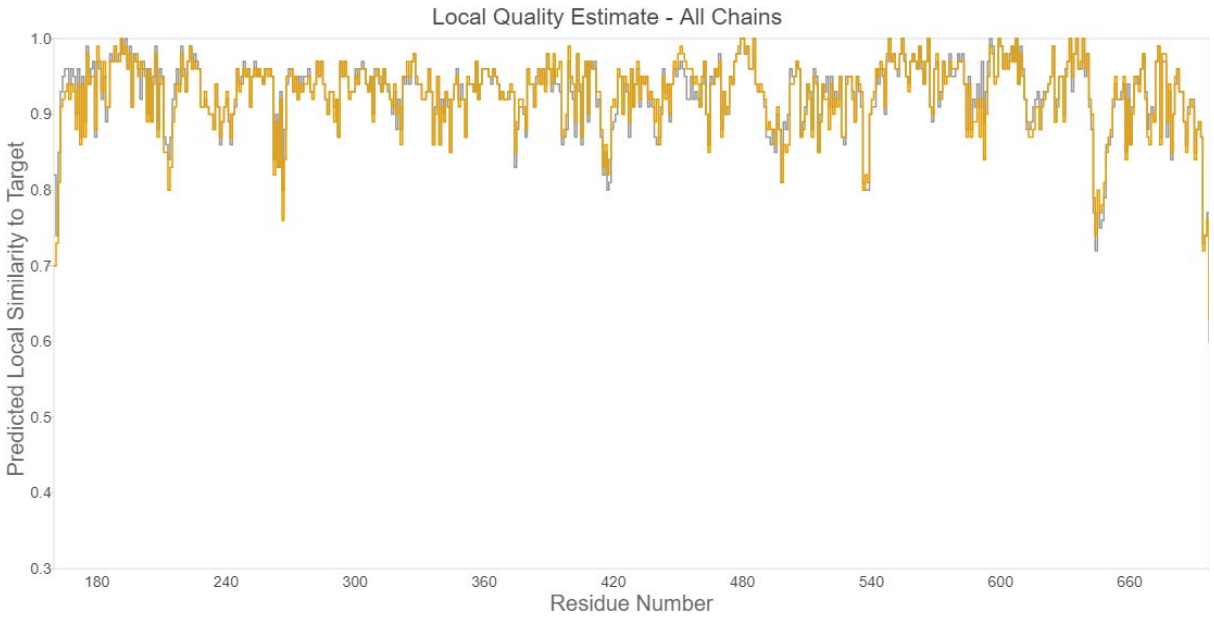


Fig S4b: Local quality estimate of the *An. funestus* model



Fig S5a: Comparison of *An. coluzzii* model to the non-redundant experimental crystal structures

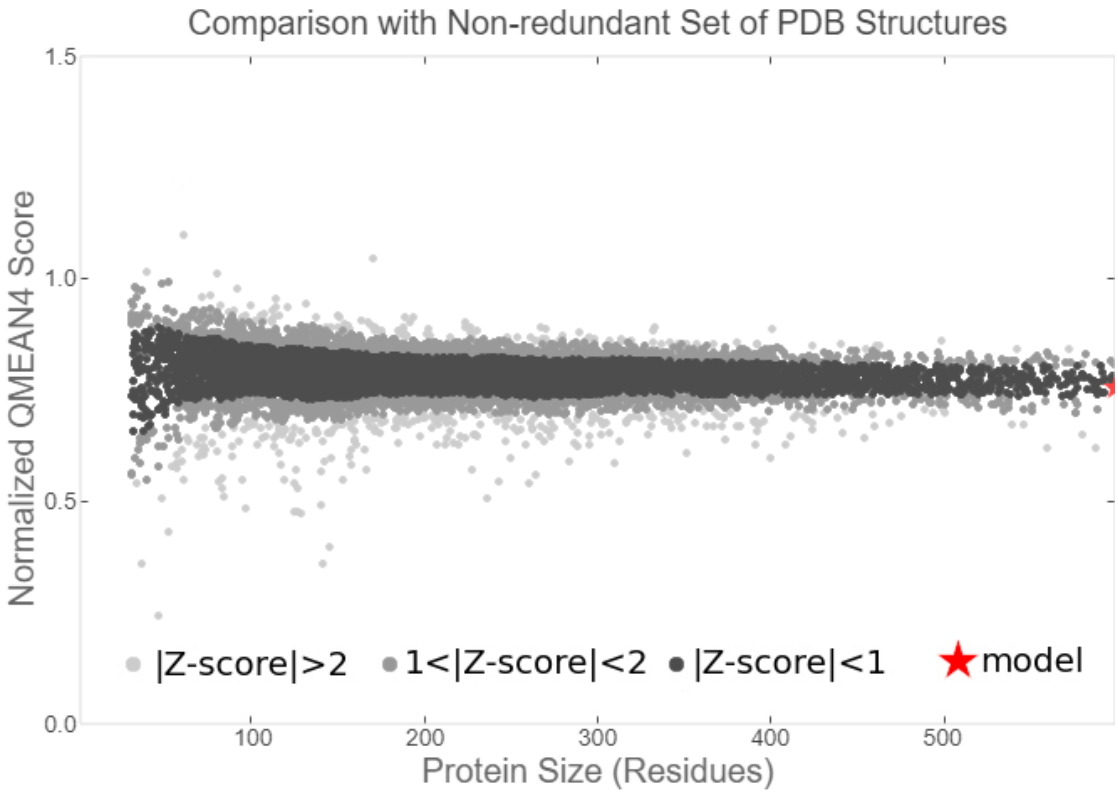


Fig S5b: Comparison of *An. funestus* model to the non-redundant experimental crystal structures

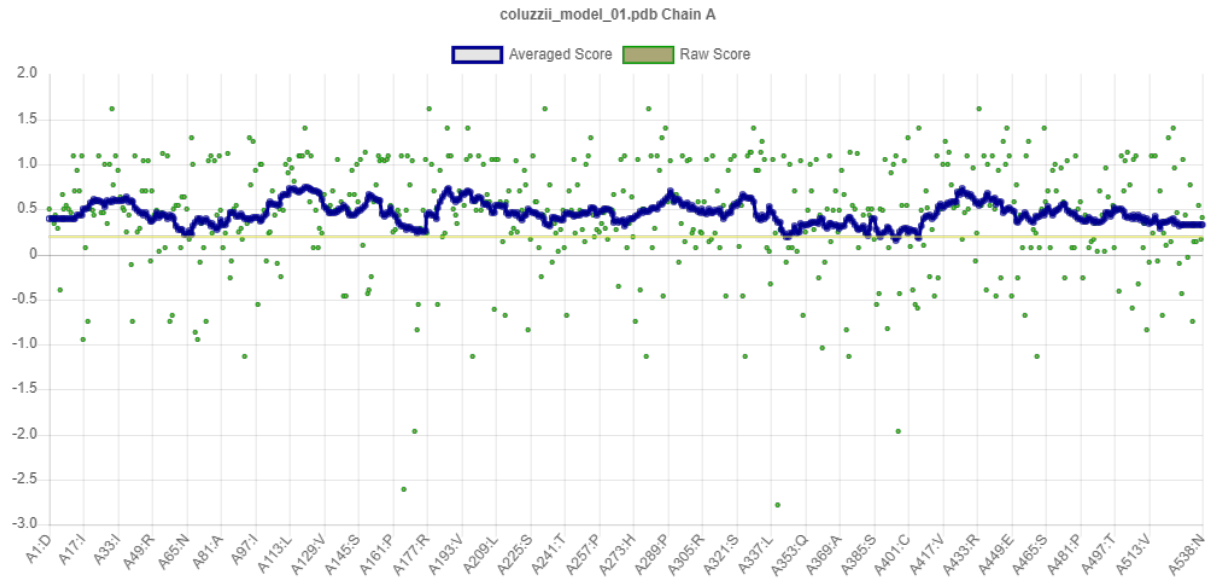


Fig S6: *An. coluzzii* model validity verification through Verify3D (98.98% of the amino acid residues obtained a score of ≥0.2 in the 3D/1D profiling).

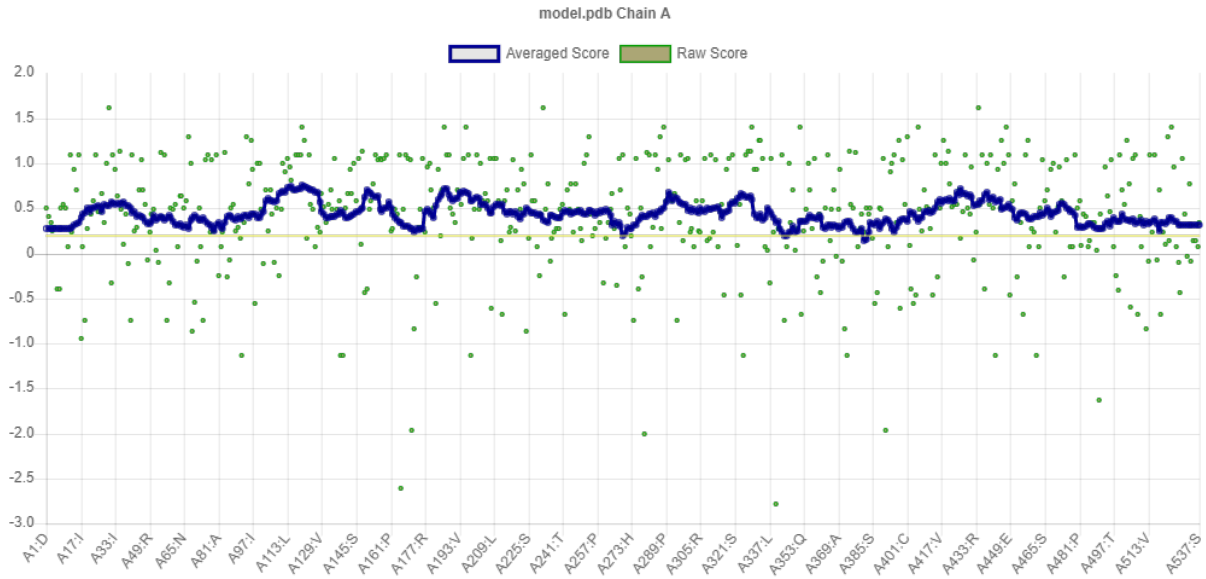


Fig S7: *An. funestus* model validity verification through Verify3D (98.88% of the amino acid residues obtained a score of ≥0.2 in the 3D/1D profiling)

Appendix D.1: *In vitro* and *in silico* analysis of the *Anopheles* anticholinesterase activity of terpenoids (Chapter 5; published paper)

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In vitro and *in silico* analysis of the *Anopheles* anticholinesterase activity of terpenoids

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ABSTRACT

Anopheles gambiae, An. coluzzii, *An. arabiensis*, and *An. funestus* are major vectors in high malaria endemic African regions. Various terpenoid classes form the main chemical constituent repository of essential oils, many of which have been shown to possess insecticidal effects against *Anopheles* species. The current study aimed to assess the bioactivity of terpenoids including four sesquiterpene alcohols, farnesol, (-)-α-bisabolol, *cis*-nerolidol, and *trans*-nerolidol; a phenylpropanoid, methyleugenol, and a monoterpene, (*R*)-(+)-limonene, using the larvicidal screening assay against the four *Anopheles* species. The mechanism of action was investigated through *in vitro* acetylcholinesterase inhibition assay and *in silico* molecular modelling. All six terpenoids showed potent larvicidal activity against the four *Anopheles* species. Insights into the mechanism of action revealed that the six terpenoids are strong AChE inhibitors against *An. funestus* and *An. arabiensis*, while there was a moderate inhibitory activity against *An. gambiae* AChE, but very weak activity against *An. coluzzii.* Interestingly, in the *in silico* study, farnesol established a favourable hydrogen bonding interaction with a conserved amino acid residue, Cys^{447}, at the entrance to the active site gorge. While (-)-α-bisabolol and methyleugenol displayed a strong interaction with the catalytic Ser^{360} and adjacent amino acid residues; but sparing the mutable Gly^{280} residue that confers resistance to the current anticholinesterase insecticides. As a result, this study identified farnesol, (-)-α-bisabolol, and methyleugenol as selective bioinsecticidal agents with potent *Anopheles* AChE inhibition. These terpenoids present as natural compounds for further development as anticholinesterase bioinsecticides.

1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) is a serine hydrolase enzyme belonging to the family of cholinesterases. It is located at the neuromuscular junctions and cholinergic brain synapses where it functions to maintain normal neuronal impulse transmission through hydrolysis of a neurotransmitter, acetylcholine. Particularly in insects, the cholinergic system is localized at the central nervous system [1,2]. Specialized insects, namely the female *Anopheles* mosquitoes, transmit malaria infections globally. >400 *Anopheles* species have been identified and about 30 these are the malaria vectors [3]. *Anopheles gambiae* s.l complex constitutes main African malaria vectors. The member species in *An. gambiae* complex include *An. gambiae* (formerly *An. gambiae* S-molecular form), *An. coluzzii* (previously *An. gambiae* M-molecular form) and *An. arabiensis* [4]. On the other hand, *An. funestus* group includes the *An. funestus* s.s., another major vectors in Sub-Saharan Africa [5]. Generally, these four species, *An. gambiae, An. coluzzii*, *An. arabiensis*, and *An. funestus*, are the dominant malaria vectors in Africa, as reported in recent World Health Organization (WHO) malaria report [6].

Malaria vector control programs mainly use insecticides to advance towards a goal of zero malaria transmissions. Insecticides have unique modes of action and acetylcholinesterase inhibitors are one of these. Insecticide classes such as organophosphates and carbamates specifically inhibit AChE. Unfortunately, insecticide resistance against these two classes has already been reported [6,7]. Resistance mechanisms have been attributed to target site mutation, as well as metabolic

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resistance resulting from overexpressed metabolic enzyme genes [8,9]. AChE target site mutation, known as G119S (substitution of glycine by a serine residue at position 199; *Torpedo californica* numbering), is the main diagnostic factor of organophosphate and carbamate cross-resistance [10]. Another challenge with the current anticholinesterase insecticides is low selectivity index between insect and mammal targets [11]. Given these, there is an urgent need to discover novel acetylcholinesterase inhibitors with better selectivity and able to cause mortality in insecticide resistant vector populations. AChE remains a target site in *Anopheles* mosquitoes since the cholinergic system, including the AChE enzyme, is an essential component of the insect nervous system [12]. Therefore, current research continues to focus on the identification of novel agents that have a high selectivity for all variants of *Anopheles* AChE compared to mammalian AChE.

Essential oils are potential natural sources of cost-effective and eco-friendly bioinsecticides [13]. Main constituents in essential oils are, among others, terpenoids such as sesquiterpenes, monoterpenes, and diterpenes as well as volatile phenolic compounds known as phenylpropanoids [14,15]. The sesquiterpene alcohols, farnesol and (-)-α-bisabolol, are main essential oil constituents in numerous aromatic plants including *Rosa x. damascene* Mill., *Polianthes tuberosa* L., *Matricaria chamomilla* L., *Cymbopogon nardus*, and *C. citratus* [16,17]. These essential oils have shown insecticidal activities from various studies. For example, *C. nardus* (citronella) and *C. citratus* (lemongrass) essential oils have shown toxic effects on *An. gambiae* adult mosquitoes [18]. The geometric isomers, cis-nerolidol and trans-nerolidol, are main constituents in many *Piper* species including *P. nigrum, P. umbellatum, P. capense*, and *P. clausseniaum* (Miq.) [19,20]. Nerolidol has previously shown larvicidal activity against *An. sinensis* larvae [21]. (*R*)-(+)-Limonene is a monoterpene obtained as a major constituent in aromatic plants such as *Citrus limon, Baccharis dracunculifolia* De Candole (1836) and *Schinus terebinthifolius* Raddi [22–25]. The achiral form of limonene has previously shown potent larvicidal activity against *An. sinensis* [21]. Similarly, the phenylpropanoid, methyleugenol, is a major phenolic constituent of many plants, including *C. nardus, Ocimum basilicum*, and *Colocasia antiquorum* [26,27]. The essential oil of *O. basilicum* has exhibited the larvicidal activity against *An. subpictus* while that of *C. nardus* possesses larvicidal effects against *An. gambiae* [18,27].

Jankowska et al. [28] highlighted that some essential oils exhibit insect neurotoxic effects marked by rapid paralysis and death. This is consistent with neurotoxicity caused by AChE inhibition and indeed various essential oils and their constituents have been reported to possesses insect anticholinesterase activity. The *in vitro* insect anticholinesterase activity can reliably be assessed by using an insect homogenate [29]. For improved activity profile, it is of paramount importance to evaluate the binding profiles of newly identified *Anopheles* AChE inhibitors in comparison to the current ones. Molecular modelling technology is well suited for this action to identify the binding modes, intermolecular interactions, and binding poses of compounds at the target site [30]. Terpenes consist of lipophilic cyclic or acyclic hydrocarbons that may result in hydrophobic interactions with target site [31]. Even though hydrogen bonding is recognized as a marker of molecular recognition and specificity at the binding site; van der Waals interactions play a critical role in the binding of organic molecules, however, these interactions are often neglected in molecular studies [32]. The protein-ligand hydrophobic interactions are better assessed through the solvent-accessible (SA) surface area method combined with generalized Born (GB) or Poisson-Boltzmann (PB) models. These include implicit solvation approaches such as molecular mechanics-GBSA (MM-GBSA) simulation [30]. In the Schrödinger's docking suite, the solvation model VSGB in MM-GBSA simulation considers the hydrophobic portions of the ligand and its interaction with the active site cavity of the protein by approximating the solvation and de-solvation energy [30]. As a result, the Schrödinger's Maestro docking program will be used to assess the binding profiles of these terpenoids as well as assessment of their affinities through MM-GBSA simulation.

The current study reports on the larvicidal activity of terpenoids including four sesquiterpene alcohols (farnesol, (-)-α-bisabolol, *cis*-nerolidol, and *trans*-nerolidol), a monoterpene ((*R*)-(+)-limonene) and a phenylpropanoid (methyleugenol) against the laboratory strains of *An. gambiae, An. coluzzii*, *An. arabiensis*, and *An. funestus*. The mechanism of action of the terpenoids was assessed through the *in vitro* acetylcholinesterase inhibition against the same strains. Following confirmation of AChE inhibition as the possible larvicidal mechanism of action, the anticholinesterase activity of these essential oil constituents (EOCs) was further investigated through *in silico* molecular modelling and molecular mechanics simulation studies.

2. Methods and materials

Acetylcholinesterase from *Electrophorus electricus* (electric eel) was bought from Sigma-Aldrich (South Africa). Propoxur (2-isopropoxyphenyl-*N*-methylcarbamate), acetylthiocholine iodide, Triton X-100, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dimethylsulfoxide (DMSO), sodium phosphate buffer (dibasic) (Na_2HPO_4), potassium dichromate and all terpenoids were bought from Sigma-Aldrich with a purity >90%.

2.1. Anopheles strains

This study obtained the animal research ethics waiver (Waiver Number: 07-11-2017-O) from Wits Animal Research Ethics Committee. Four *Anopheles* strains used in this study, *An. gambiae* (COGS), *An. coluzzii* (G3), *An. arabiensis* (KWAG), and *An. funestus* (FUMOZ), were maintained under the typical insectary conditions as described by Hunt et al. [9] and Zengenene and colleagues [33]. *An. gambiae* COGS is a laboratory resistant strain and was first established from Republic of the Congo in 2009 and is resistant to multiple insecticide classes including pyrethroids, organochlorines, and carbamates but susceptibility to organophosphates [34]. *An. coluzzii* G3 strain was colonized from The Gambia in 1970's [35] and has also shown resistance to pyrethroids and DDT [36]. The *An. arabiensis* KWAG was first colonized in 2005 from Kwazulu-Natal, South Africa, and *An. funestus* FUMOZ was established from Mozambique in 2000. Both KWAG and FUMOZ colonies are known to be resistant to pyrethroids and carbamates [9,37]; with KWAG showing resistance to an organochlorine, dichloro-diphenyl-trichloroethane (DDT) [38].

2.2. Larvicidal assay

The larval toxicity of these EOCs was evaluated using the World Health Organization (WHO) larvicidal bioassay [39]. Twenty (20) third instar larvae of each *Anopheles* species were transferred into the test cups containing deionized water. A specific test compound or positive control (250 μL) was then added, and the incubation mixture made up to 25 mL total volume. All test compounds and the positive control were dissolved in DMSO and evaluated for larvicidal activity at seven concentrations ranging from 0.0005 μM to 500 μM (10-fold serial dilutions). Propoxur, a known larvicide and anticholinesterase agent, was used as a positive control and DMSO was used for the negative control test [40,41]. The experiments were run in triplicate at 27 °C and humidity ≥78% simulating the insectary conditions. Larval mortality was documented at 24, 48 and 72 h. The larvae were fed with protein crushed dog biscuits mixed with brewer's yeast (3:1) at day 0 and after every mortality counting to prevent mortality related to starvation. The nutritional content of this meal includes fibre, protein, fat, vitamins and trace elements required for larval development [42]. The 50% lethal concentration (LC_{50}) values were determined by probit analysis method using the SPSS Statistics v27 software [40].

2.3. Artemia lethality assay

The cytotoxicity potential of the EOCs was assessed through the

Artemia lethality assay using *Artemia franciscana* [43]. The *Artemia* eggs were hatched in artificial sea water (32 g/L Tropic Marine® Sea Salt in deionized water) supplied with constant aeration to disperse the eggs under an optimal room temperature for 24 h. The same concentrations as used in the larvicidal assessment were used for the *Artemia* toxicity evaluation for direct comparison. Fifty microlitres of each test compound dissolved in DMSO was incubated in a 48-well plate with 30–50 nauplii in 450 μL of the sea water. The wells were observed under the stereomicroscope for any dead nauplii at the beginning of the experiment and the EOC-induced mortality was recorded over 24 h. For consistent comparison, propoxur was used as a positive control and DMSO as a negative control. The experiment was performed in triplicate and LC_{50} values were calculated with the probit analysis in SPSS Statistics v27 package [40].

2.4. In vitro AChE assay

The anticholinesterase activity of the EOCs was assessed using the modified Ellman assay [44]. The *Anopheles* mosquitoes from four species were separately homogenized in assay buffer (dibasic sodium phosphate buffer, 1% Triton X-100 (pH 8.0)) and the homogenate used as an enzyme source. Protein content was determined using the standard Lowry protein assay and the mosquito homogenate standardized to 0.8 μg protein/mL for each experiment [45]. The incubation mixture in the 96-well plate was composed of the mosquito homogenate (20 μL) in 132 μL of assay buffer. For selectivity analysis, AChE from electric eel (0.02 U/mL) was used in place of the *Anopheles* homogenate. To this latter incubation mixture, 20 μL of test compounds (0.0005 μM to 500 μM) were added along with 8 μL (0.2 mM) of the freshly prepared Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB in Na_2HPO_4 (pH 7.0)). To initiate the enzymatic reaction, 20 μL (1 mM) of acetylthiocholine iodide (AChE substrate) was added and the absorbance measured at 412 nm with a UV–Visible plate spectrophotometer every 30 s. Propoxur was kept as a positive control since it is an anticholinesterase insecticide [41], while DMSO was used in the negative control wells. To note, DMSO is known to exhibit AChE inhibition at concentrations above 1% [46], as such, 0.4% was the highest DMSO concentration attained in the final incubation mixtures. The 50% AChE inhibitory concentration (IC_{50}) values were calculated with GraphPad Prism 9 (GraphPad Software, CA, USA).

2.5. Molecular docking and molecular mechanics simulations

2.5.1. Extra-precision docking

Schrödinger Release 2021-3 molecular modelling software, Maestro version 12.9, (Schrödinger LLC, New York) was used to assess the AChE target site binding profiles of terpenoids. This study intended to also evaluate the differences in binding poses of the EOCs across the four *Anopheles* species, however, this was hindered by the availability of only *An. gambiae* crystal protein structure [30].

The *An. gambiae* AChE 3D structure was sourced from PDB (PDB: 5YDI) and prepared by the Maestro's protein preparation function. This preparation included optimization of hydrogen bonds and removal of non-hetero groups as well as non-essential water molecules before minimization under the OPLS3e force field. The receptor grid was generated using the same force field to define the binding site. On the other hand, the EOCs were prepared with the LigPrep tool (Fig. 1) and were allowed to generate their possible stereoisomers as well as the tautomeric and ionization states at pH 7.0 (±2.0) [30]. The scaling factor for van der Waals interactions was set to 0.5 Å at a partial cut-off charge of 0.15, to ensure scaling of only nonpolar hydrogens [32]. Finally, the prepared ligands and receptors were used for molecular docking under the rigorous extra precision mode.

2.5.2. Molecular mechanics simulations

The binding affinities of the terpenoids were evaluated through the MM-GBSA simulations. The previously docked extra precision complexes were retrieved for redocking under the MM-GBSA conditions. The same hydrophobic interaction settings used in the extra precision docking mode were adopted; however, the permitted distance between the ligand and receptor was changed from 0 Å to 5 Å to allow receptor flexibility during the simulation. The effective solvation model VSGB along with the OPLS3e force field were used in the current molecular mechanics simulation [30].

3. Statistical analyses

The larvicidal and artemicidal assays as well as *in vitro* anticholinesterase studies were performed in triplicate. The 50% and 95% lethal concentrations (LC_{50} and LC_{95}) values were calculated from the probit analysis method using the SPSS Statistics v28 package (International Business Machines Corporation, NY, USA) and the IC_{50} values were determined by non-linear regression analysis using GraphPad Prism 9 (GraphPad Software, CA, USA) [40].

4. Results and discussion

4.1. Larvicidal activity

To identify terpenoids with larvicidal effects, the compounds were

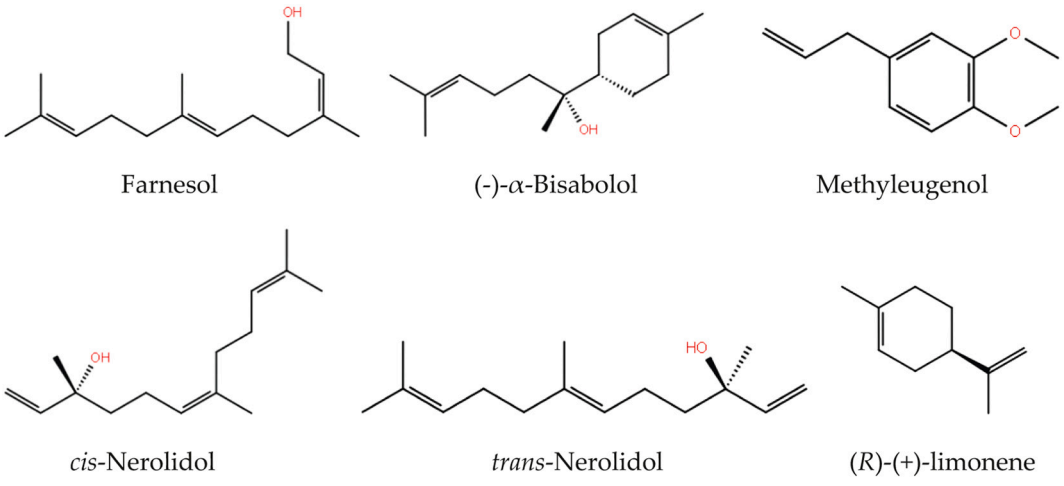


Fig. 1. Chemical structures of the terpenoids.

screened against four *Anopheles* species to assess their activity differences; and to assess cytotoxicity against other aquatic organisms, they were subsequently screened for artemicidal activity. Based on the LC_{50} values against *An. funestus*, farnesol and (-)-α-bisabolol were about time times more active than a positive control, propoxur, while *cis*-nerolidol and *trans*-nerolidol were five times more active than propoxur (Table 1). The phenylpropanoid methyleugenol and a monoterpene (*R*)-(+)-limonene, were only about two times more active than propoxur (Table 1). For *An. arabiensis*, two sesquiterpene alcohols, farnesol and (-)-α-bisabolol, were still the most active and about 150 times more active than propoxur. The monoterpene (*R*)-(+)-limonene displayed the lowest activity (LC_{50} = 6.32 μM) but was still significantly more active than propoxur (LC_{50} = 8.77 μM) (unpaired *t*-test: $P = 0.0008$). Though the terpenoids potency was relatively low in *An. gambiae*, a similar trend was observed where farnesol and (-)-α-bisabolol were most active (mean $LC_{50} \sim 7$ μM) followed by nerolidol geometric isomers (mean $LC_{50} \sim 10$ μM) and methyleugenol (LC_{50} = 21.16 μM) while (*R*)-(+)-limonene was rather inactive ($LC_{50} > 100$ μM). Farnesol and (-)-α-bisabolol were still the most active terpenoids against *An. coluzzii* with LC_{50} values <1 μM while *cis*-nerolidol, *trans*-nerolidol and methyleugenol attained similar potency with mean $LC_{50} \sim 2$ μM (one-way ANOVA: $P = 0.4419$). The standard carbamate, propoxur, showed low potency against *An. gambiae* and *An. coluzzii* with LC_{50} values of 63.99 and 62.68 μM, respectively. In terms of the LC_{95} values, farnesol and (-)-α-bisabolol were still the most active terpenoids attaining lethality values <100 μM against all *Anopheles* species except for *An. gambiae* (Table 1). In fact, all terpenoids attained LC_{95} values in the millimolar range (>1000 μM) against *An. gambiae*. (*R*)-(+)-Limonene was still suggested the lowest active terpenoid whereby it attained LC_{95} values in the millimolar range; along with the positive control, propoxur.

In general, *An. arabiensis* was more susceptible to the assessed terpenoids (Table 1). Taking into account the toxicity against *A. franciscana*, it was noted that farnesol and (-)-α-bisabolol were five to ten times more selective to *An. arabiensis* than *Artemia* (LC_{50} on *Artemia*/LC_{50} on *Anopheles* [47]). Interestingly, with relatively high LC_{50} against *Artemia* (42.79 μM), methyleugenol was highly selective (selectivity index >10 [47]) towards *Anopheles* species including *An. funestus*, *An. arabiensis*, and *An. coluzzii*. The monoterpene (*R*)-(+)-limonene was inactive against *Artemia* ($LC_{50} > 100$ μM) though it was active against only two *Anopheles* species, *An. funestus* and *An. arabiensis* with LC_{50} ranging from 5 to 7 μM. Although a positive control, propoxur, was also relatively active against *An. funestus* and *An. arabiensis* (LC_{50} range: 8–10 μM), it was found to be equally toxic to *Artemia* (LC_{50}: 9.08 μM). This carbamate has previously been reported to exhibit toxicity at LC_{50} range of 3.7–6.6 μg/mL (17.68–31.54 μM) against non-target aquatic species [48]. Moreover, using *A. salina*, carbamates have previously been shown to be highly toxic against this *Artemia* species with LC_{50} values as low as 1.74 μM [49].

4.2. In vitro AChE inhibition activity

The terpenoids mechanism of action was elucidated by screening them for *Anopheles* AChE inhibition. To assess the selectivity potential, the terpenoids were further screened against electric eel AChE as a comparable enzyme to human AChE [50]. In correlation to the larvicidal activities, the terpenoids were generally more inhibitory against AChE from *An. arabiensis* and *An. funestus* than *An. gambiae* and *An. coluzzii* (Table 2). Farnesol was highly active against *An. funestus* (IC_{50} = 0.127 μM); however, it was the only terpenoid that also possessed inhibitory activity against electric eel AChE. It was nonetheless approximately ten times more selective towards *An. funestus*. The selectivity indices between electric eel and other *Anopheles* colonies, especially *An. gambiae* and *An. coluzzii* were relatively low for farnesol, indicating non-selectivity [47]. In agreement to the literature, propoxur was non-

Table 1
Comparison of the larvicidal and artemicidal activities of the assessed terpenoids.

	Larvicidal *Anopheles* species				**Artemicidal *Artemia* species**
	LC_{50} (μM) (95% CI)				**LC_{50} (μM) (95% CI)**
	LC_{95} (μM) (95% CI)				**LC_{95} (μM) (95% CI)**
Compound	***An. funestus***	***An. arabiensis***	***An. gambiae***	***An. coluzzii***	***A. franciscana***
Farnesol	1.15 (0.39–3.60)	0.05 (0.03–0.07)	6.52 (2.84–16.37)	0.23 (0.16–0.34)	0.37 (0.13–1.03)
	85.21 (18.92–1555.01)	8.41 (4.30–19.40)	>1000	17.85 (9.81–37.54)	226.17 (44.38–3127.27)
(-)-α-Bisabolol	1.42 (0.43–5.08)	0.06 (0.04–0.10)	7.18 (3.18–17.62)	0.26 (0.18–0.37)	0.33 (0.12–0.94)
	89.02 (18.16–2490.30)	15.00 (7.46–35.80)	>1000	22.14 (12.04–47.12)	205.39 (39.44–3026.37)
cis-Nerolidol	2.37 (1.26–4.57)	1.21 (0.50–3.01)	10.29 (3.41–37.46)	2.08 (0.78–5.90)	1.26 (0.24–8.38)
	400.39 (137.93–1801.45)	279.94 (69.05–2544.15)	>1000	277.04 (62.26–3727.60)	512.45 (47.07–120,317.60)
trans-Nerolidol	2.11 (1.13–4.00)	1.43 (0.50–4.38)	10.87 (3.88–36.07)	2.15 (0.82–6.01)	1.31 (0.28–7.11)
	311.33 (110.51–1337.96)	576.73 (104.42–10,391.25)	>1000	365.47 (80.70–4800.78)	512.70 (54.85–59,841.34)
Methyleugenol	4.35 (1.26–17.50)	2.22 (0.72–7.59)	21.16 (7.18–84.95)	2.02 (0.92–4.57)	42.79 (18.56–120.01)
	617.12 (99.28–25,529.99)	690.51 (114.63–17,233.40)	>1000	560.78 (152.03–3954.66)	>1000
(*R*)-(+)-Limonene	5.41 (2.57–12.20)	6.32 (2.35–19.24)	>100	>100	>100
	>1000	>1000	>1000	>1000	>1000
Propoxur	9.90 (2.96–41.41)	8.77 (2.81–32.71)	63.99 (38.23–116.29)	62.68 (37.72–112.76)	9.08 (2.04–64.67)
	>1000	>1000	>1000	>1000	>1000

CI = confidence intervals

Table 2
Comparison of the *Anopheles* and electric eel anticholinesterase activity of the identified terpenoids.

	AChE inhibition $IC_{50} \pm SD$ (μM)				
Compound	***An. funestus***	***An. arabiensis***	***An. gambiae***	***An. coluzzii***	***E. electricus***
Farnesol	0.127 ± 0.001	0.710 ± 0.007	7.431 ± 0.195	69.055 ± 0.652	1.156 ± 0.059
(-)-α-Bisabolol	0.586 ± 0.137	0.721 ± 0.013	5.220 ± 0.517	61.120 ± 1.182	>100
cis-Nerolidol	0.244 ± 0.021	0.639 ± 0.004	3.534 ± 0.410	>100	>100
trans-Nerolidol	0.273 ± 0.005	0.537 ± 0.025	4.303 ± 0.404	>100	>100
Methyleugenol	0.488 ± 0.064	0.606 ± 0.058	6.883 ± 0.115	>100	>100
(*R*)-(+)-limonene	7.487 ± 0.161	0.622 ± 0.096	3.256 ± 0.004	>100	>100
Propoxur	0.893 ± 0.044	0.778 ± 0.004	28.060 ± 1.778	23.325 ± 4.379	1.283 ± 0.046

selective between the *Anopheles* and electric eel targets [51,52]. The nerolidol isomers were equally active against *An. funestus* AChE (unpaired *t*-test: $P = 0.0805$) and more active than (-)-α-bisabolol (one-way ANOVA: $P = 0.0035$) and methyleugenol (one-way ANOVA: $P = 0.0005$), while (*R*)-(+)-limonene was the least active and approximately ten times less active than propoxur. Propoxur was statistically more active against *An. arabiensis* than *An. funestus* AChE (unpaired *t*-test: $P = 0.0108$), which is in line with sensitivity pattern observed for these strains (Table 1).

Moreover, all terpenoids were similarly (IC_{50} range: 0.5–0.7 μM) potent against *An. arabiensis* in comparison to the IC_{50} values ranging from 3 to 7.5 μM against *An. gambiae* (Table 2); which were generally ten times higher than those obtained in the screening against *An. arabiensis* AChE. Notably, (*R*)-(+)-limonene showed potential activity against *An. gambiae* AChE as an *in vitro* target which did not correlate with the larvicidal data (Table 1). This is similar to the positive control (propoxur) that showed significantly higher activity against the extracted target than the whole organism (unpaired *t*-test: $P < 0.0001$) for both *An. gambiae* and *An. coluzzii*. This suggests the involvement of pharmacokinetic barriers in the whole larvae, of which decreased cuticle penetration has previously been shown to play a role [53]. Propoxur has formerly been reported as inactive ($IC_{50} > 100$ μM) against AChE from the *An. gambiae* and *An. coluzzii* larvae [54]. Perrier et al. [55] reported that propoxur was inactive even in the physiological studies with isolated *An. gambiae* neurons. *An. coluzzii* AChE proved to be the least sensitive to the assessed terpenoids where none of them attained an IC_{50} value <50 μM. This therefore rules out AChE inhibition as the mechanism of action for the observed larvicidal activities against the utilized *An. coluzzii* G3 strain.

4.3. In silico investigation of the receptor binding of terpenoids

To further elucidate the mechanism of action, the binding conformations of the terpenoids against the AChE targets were evaluated through molecular docking and molecular mechanics simulations. Since large structural portions of terpenoids are nonpolar (Fig. 2), the optimized non-polar interactions through MM-GBSA simulation were taken into consideration. However, since van der Waals interactions are naturally weak, and their energies are mainly considerable in combination [56], only the amino acid residues forming three or more of these interactions with the ligand after being optimized with MM-GBSA re-docking were considered and reported in Table 3 and Table 4. Lastly, due to MM-GBSA being more accurate in pose prediction and affinity ranking [30], only crystal poses from this molecular mechanics simulation were reported.

In correlation to the larvicidal and *in vitro* results, farnesol was still the most active terpenoid as suggested by the highest binding score and target interaction through various amino acid residues against *Anopheles* AChE (Table 3). The initial (extra precision) docking of farnesol suggested a similar binding score against *Anopheles* (−6.893 kcal/mol; Table 3) and electric eel (−6.518 kcal/mol; Table 4). However, in a more accurate molecular mechanics simulation, the results correlated with the *in vitro* data showing the binding affinity higher for *Anopheles* (−48.82 kcal/mol; Table 3) that the corresponding electric eel AChE (−41.53 kcal/mol; Table 4). Moreover, farnesol established H-bonds with Gly^{445}, Cys^{447} and Glu^{448} in the *Anopheles* target (Fig. 2A) while it could only form H-bonds with aromatic amino acid residues Phe^{288} and Phe^{331} in electric eel (Fig. 2B). Interestingly, farnesol preferentially interacted with a conserved amino acid residue Cys^{447} that is a current target in the design of *Anopheles* selective anticholinesterases [52].

Interestingly, the positive control, propoxur, generated a similar crystal pose against *Anopheles* and electric eel AChEs in support of the literature, where it is nonselective between these two targets (Fig. 3A and B) [51,52]. There was a correlation of binding scores at each docking level whereby the initial docking scores were − 5.683 kcal/mol (*Anopheles*) and − 5.809 kcal/mol (electric eel) and the subsequent MM-

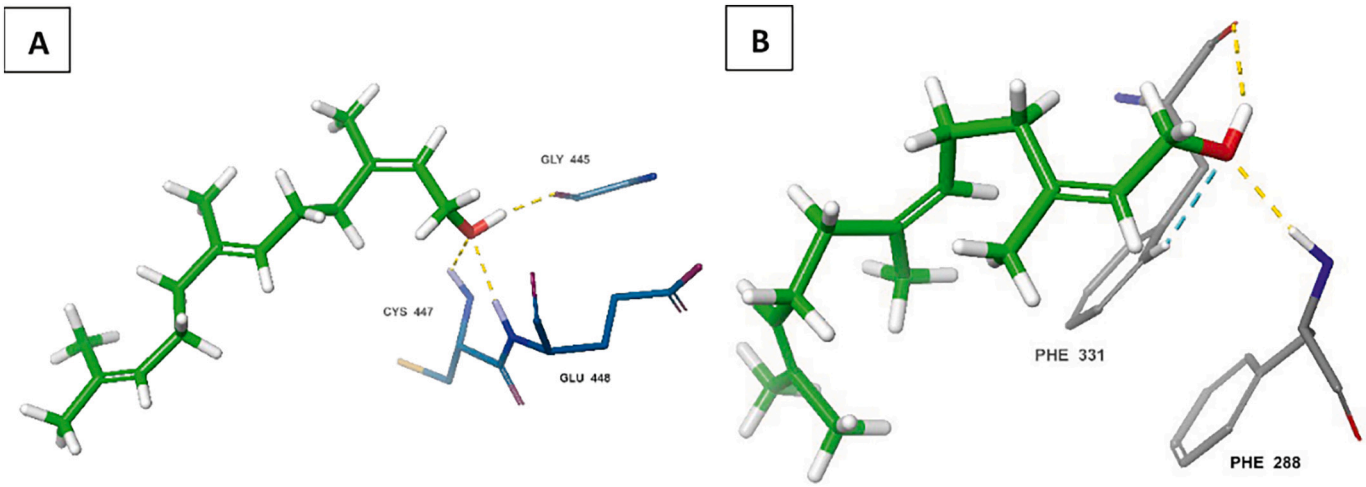


Fig. 2. Differences in farnesol poses against *Anopheles* AChE (A) and electric eel AChE (B).

Table 3
The binding scores and amino acid interactions of terpenoids and *Anopheles* AChE.

	An. gambiae AChE (PDB: 5YDI)			
	Docking score (kcal/mol)		Amino acid residues	
Compound	**Glide docking**	**MM-GBSA simulation**	**H-bonds and aromatic bonds**	**Nonpolar bonds**
Farnesol	−6.893	−48.82	Gly^{445}, Cys^{447}, Glu^{448}	Trp^{245}, Tyr^{282}, Phe^{449}, Tyr^{493}, Tyr^{489}, Phe^{490}
(-)-α-Bisabolol	−5.483	−30.36	Ser^{360}, Gly^{278}	Trp^{245}, Phe^{449}, Phe^{490}
cis-Nerolidol	−5.572	−40.41	Gly^{278}, Gly^{280}, Ser^{360}	Trp^{245}, Tyr^{282}, Phe^{449}, Tyr^{489}, Tyr^{493}, Phe^{490}
trans-Nerolidol	−5.518	−42.74	Gly^{278}, Gly^{280}, Ser^{360}	Trp^{245}, Tyr^{282}, Tyr^{489}, Tyr^{493}, Phe^{449}, Phe^{490}
Methyleugenol	−4.944	−38.59	Trp^{245}, Ser^{360}, Phe^{490}, His^{600}	Trp^{245}, Tyr^{282}, Phe^{449}, Tyr^{489}, Phe^{490}
(*R*)-(+)-limonene	−4.32	−25.03	–	Phe^{449}, Tyr^{489}, Tyr^{493}, Tyr^{494}, Phe^{490}
Propoxur	−5.683	−32.03	Gly^{280}, Ser^{360}, Ala^{361}, Phe^{490}, His^{600}	Trp^{245}, Tyr^{282}, Phe^{449}, Tyr^{489}, Tyr^{493}

Table 4
The comparison of the docking scores and amino acid interactions of farnesol and propoxur against electric eel AChE.

	E. electricus AChE (PDB: 1EVE)			
	Docking score (kcal/mol)		Amino acid residues	
Compound	**Glide docking**	**MM-GBSA simulation**	**H-bonds and aromatic bonds**	**Nonpolar bonds**
Farnesol	−6.518	−41.53	Phe^{288}, Phe^{331}	Tyr^{84}, Tyr^{121}, Phe^{290}, Phe^{330}, Tyr^{334}
Propoxur	−5.809	−38.16	Gly^{118}, Gly^{119}, Ser^{200}, Ala^{201}, Tyr^{121}	Trp^{70}, Trp^{279}, Phe^{330}, Phe^{331}, Tyr^{334}

GBSA attained −32.03 kcal/mol (*Anopheles*) and − 38.16 kcal/mol (electric eel). This covalent inhibitor was stabilized by aromatic pi-pi interaction through its aromatic ring and the phenylalanine residue (Phe^{490}; *Anopheles*/Phe^{331}; electric eel) while held tightly by the catalytic serine (Ser^{360}; *Anopheles*/Ser^{200}; electric eel). The H-bonds were formed with amino acid residues adjacent to the catalytic serine including the Ala^{361}, His^{600} and Gly^{280} (Fig. 3A). The latter amino acid is of great concern due to its involvement in mutation and resistance in *Anopheles* AChE [57]. This is a similar binding profile that has been reported for organophosphates where they also interact with Gly^{280} [30]. This supports the idea that substitution of this smaller amino acid with a relatively larger serine residue (G280S) in resistant strains, confers steric hindrance to the optimal binding of organophosphates and carbamates [52,58]. In the electric eel target, propoxur established the comparable H-bonds with Ala^{201}, Gly^{118}, and Gly^{119}, as well as aromatic H-bonding with Tyr^{121} (Fig. 3B).

(-)-α-Bisabolol established only two H-bonds with critical amino acids including the catalytic Ser^{360} and adjacent Gly^{278} utilizing it hydroxyl group (Fig. 4A). The nerolidol geometric isomers established same interactions with the target site amino acids (Fig. 4B and C). Notably, they formed a H-bond with a mutable Gly^{280} among others that included Gly^{278} and the catalytic serine^{360}. The influence of mutated Gly^{280} to Ser^{280} on the binding and effectiveness of these isomers should be directly evaluated by the biological and *in silico* studies. In comparison, the geometric isomers of nerolidol obtained similar scores (−5.572 kcal/mol and − 5.518 kcal/mol for *cis*-nerolidol and *trans*-nerolidol, respectively) in both extra precision docking and MM-GBSA re-docking (−40.41 kcal/mol and − 42.74 kcal/mol for *cis*-nerolidol and *trans*-nerolidol, respectively). Finally, methyleugenol was stabilized by pi-pi stacking through its aromatic ring by Trp^{245}, Phe^{490} and His^{600} as it formed H-bonds with both oxygen atoms from its methoxy groups and catalytic Ser^{360} residue (Fig. 4D).

The nonpolar monoterpene, (*R*)-(+)-limonene, could not form any H-bonds and obtained the lowest affinity as per the MM-GBSA prediction (−25.03 kcal/mol) (Table 3). This supported the larvicidal results (Table 1), whilst conflicting with the *in vitro* data that indicated that (*R*)-(+)-limonene was a highly potent AChE inhibitor against *An. gambiae* (Table 2).

Generally, the terpenoids interacted with the catalytic active site through H-bonds and established nonpolar interactions with aromatic amino acid residues localized at the entrance to the active site gorge. A free cysteine, Cys^{447}, is a conserved nucleophilic amino acid residue at the entrance to the active site gorge [57] and farnesol formed a H-bond

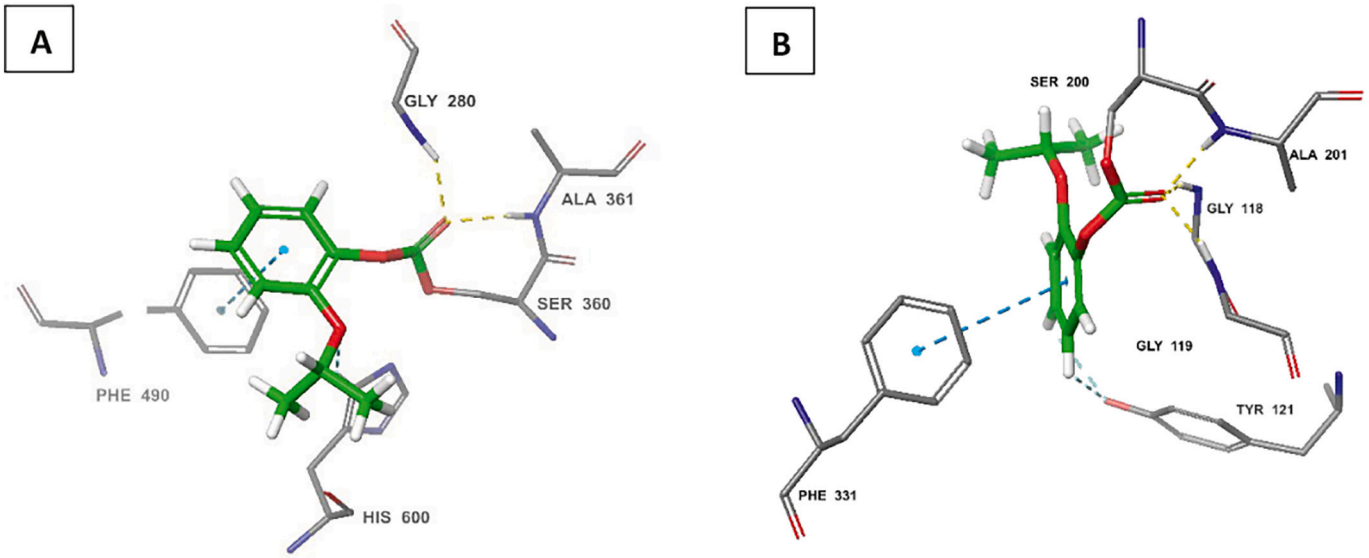


Fig. 3. Comparison of propoxur binding pose against *Anopheles* (A) and electric eel (B).

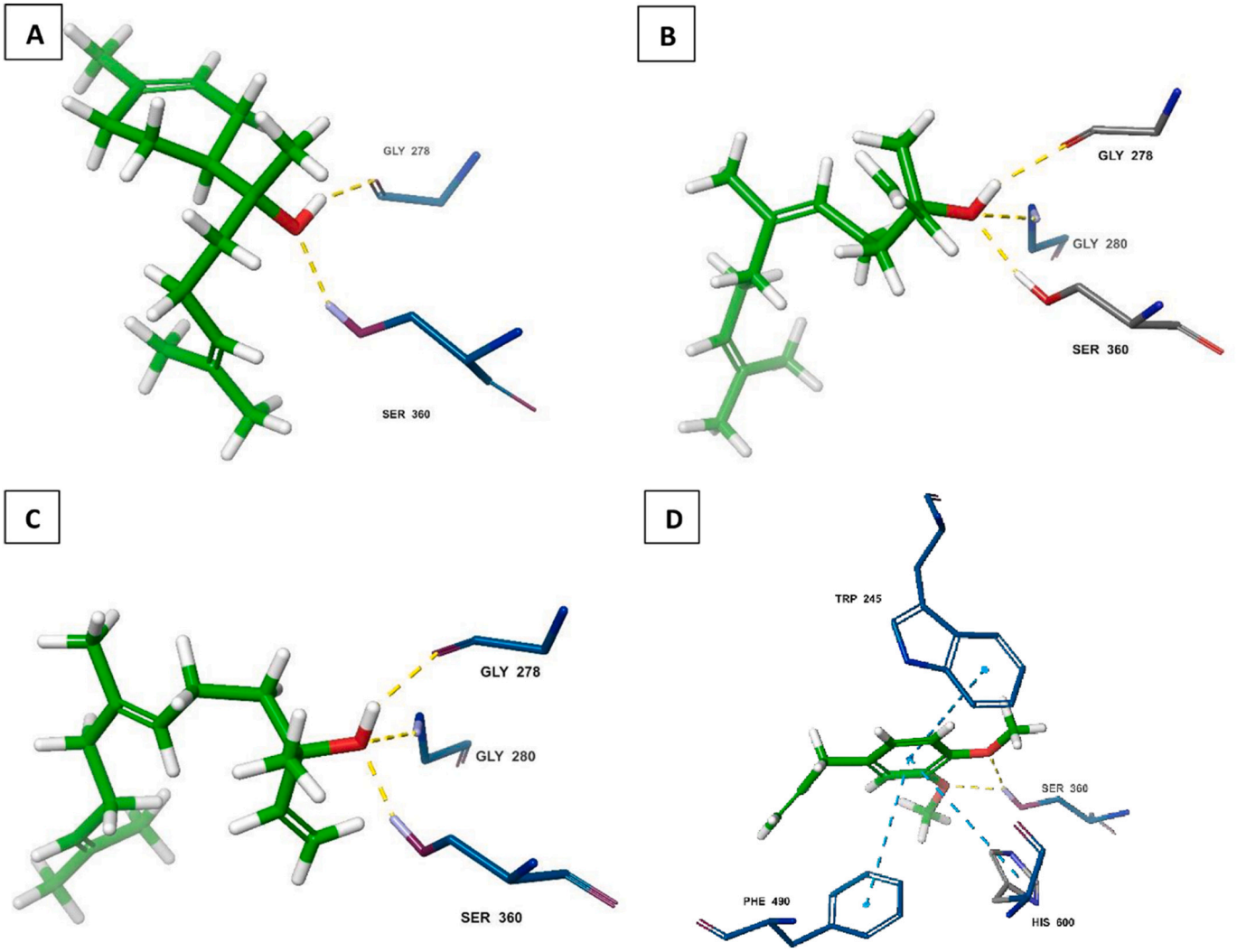


Fig. 4. Crystal poses of (-)-α-bisabolol (A), *cis*-nerolidol (B), *trans*-nerolidol (C) and methyleugenol (D) in the *Anopheles* AChE binding site.

with this amino acid. The aromaticity of this entrance is caused by Phe^{449}, Tyr^{493}, and Tyr^{489}, which were the common amino acids involved in the terpenoid van der Waals interactions. It has been reported that these aromatic amino acids can establish the aromatic and van der Waals interactions, useful in ligand trafficking [57]. The (*R*)-(+)-limonene chemical structure has no aromatic ring or polar groups (Fig. 1), hence it could only interact with the target through van der Waals interactions. As a result, this monoterpene assumed a preferred position at the entrance to the active site gorge and formed similar van der Waals interactions as other terpenoids, as indicated in Table 3. The superimposition of (*R*)-(+)-limonene with other terpenoids is displayed in Fig. 5 showing that it assumed a similar binding pose but was lodged in the entrance due to higher capacity for nonpolar interactions at this site. Highly nonpolar molecules commonly display nonspecific binding and often show exaggerated high potencies in *in vitro* assays [59,60]. This study suggests that the observed high potency of (*R*)-(+)-limonene (Table 2) in the *in vitro* screening and inconsistency with the larvicidal (Table 1) and *in silico* (Table 3) findings is due to nonspecific binding at the entrance to the AChE active site that may block access to this site by the substrate.

5. Conclusions

This study identified terpenoids for potential application as anti-cholinesterase bioinsecticides. In this work, the *in vitro* AChE inhibition of terpenoids was integrated to the larvicidal effects, and their mode of action was further elucidated at the molecular level through the *in silico* models. Farnesol, (-)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, (*R*)-(+)-limonene, and methyleugenol proved to be potent larvicidal agents against major African malaria vectors *An. funestus*, *An. arabiensis*, *An. gambiae*, and *An. coluzzii*. Two sesquiterpene alcohols, farnesol and (-)-α-bisabolol, were the most active followed by *cis*-nerolidol and *trans*-nerolidol while methyleugenol and (*R*)-(+)-limonene were the least active agents. Interestingly, farnesol, (-)-α-bisabolol, methyleugenol, and (*R*)-(+)-limonene were more selective to *Anopheles* than *Artemia* in comparison to a positive control, propoxur, or the nerolidol isomers. The evaluation of these terpenoids against AChE samples extracted from the four *Anopheles* species indicated them as strong AChE inhibitors against *An. funestus* and *An. arabiensis* with moderate inhibitory activity against *An. gambiae* but very weak activity against *An. coluzzii.* This eliminated AChE inhibition as a possible mode of action for the observed terpenoid-induced *An. coluzzii* larval toxicity. Further elucidation of the mechanism of action using molecular modelling and molecular mechanics simulations technology identified farnesol as the most active terpenoid, in correlation to the *in vitro* and larvicidal results. In general, the terpenoids interacted with the catalytic site through hydrogen bonding and with the entrance to the active site through van der Waals interactions, utilizing the polar environment of the active site and high density of the aromatic amino acid residues localized at the entrance, respectively. Most fascinating was the favourable hydrogen bonding interaction of

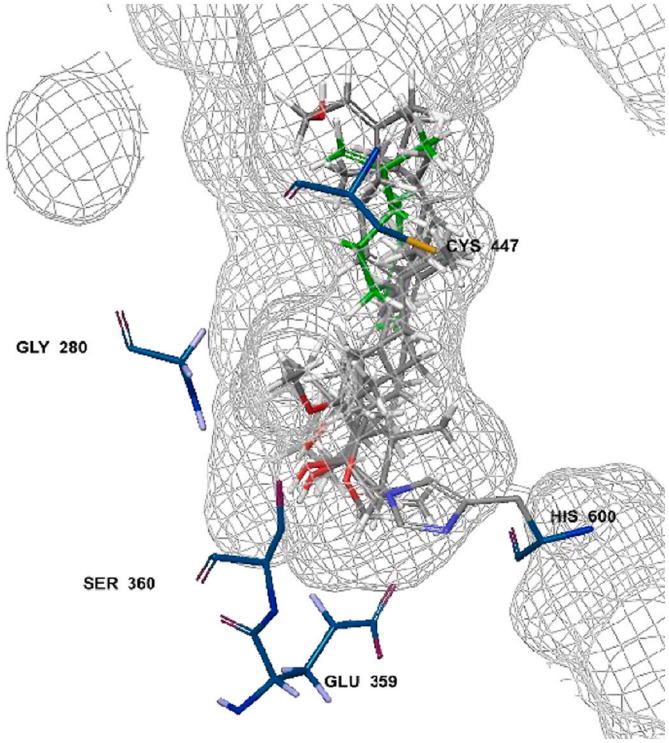


Fig. 5. Superimposition of terpenoid poses on the active site of *Anopheles* AChE, with (*R*)-(+)-limonene lodged at the active site entrance (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

farnesol and a conserved amino acid residue, Cys^{447}, at the entrance to the active site gorge. Farnesol generated completely different binding poses between *Anopheles* and electric eel AChE targets where a higher binding score and more H-bonds were formed in the *Anopheles* target in support of the larvicidal and *in vitro* data. Another highly potent terpenoid, (-)-α-bisabolol, formed H-bonds with the catalytic Ser^{360} and adjacent Gly^{278} which differed to the nerolidol isomers that in addition, established the undesirable interaction with the mutable Gly^{280} residue that is capable of conferring resistance to the AChE inhibitors. The phenylpropanoid methyleugenol also displayed a strong interaction with the *Anopheles* AChE where its aromatic ring attracted aromatic stabilization from Trp^{245}, Phe^{490} and His^{600} placing its methoxy groups in a best position to interact with the catalytic Ser^{360} residue. Therefore, this study has identified farnesol, (-)-α-bisabolol, and methyleugenol as potent inhibitors of AChE in the *in vitro* and *in silico* studies with their biological activity proven in the larvicidal assay. These three EOCs present as terpenoids of interest for further development as anticholinesterase bioinsecticides.

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Declaration of Competing Interest

Authors declare no conflict of interest in this study.

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Appendix E.1: The insecticidal activity of essential oil constituents against pyrethroid-resistant *Anopheles funestus* (Diptera: Culicidae) (Chapter 7; published paper)

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The insecticidal activity of essential oil constituents against pyrethroid-resistant *Anopheles funestus* (Diptera: Culicidae)

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ABSTRACT

Malaria vector control relies on the use of insecticides for indoor residual spraying and long-lasting bed nets. However, insecticide resistance to pyrethroids among others, has escalated. *Anopheles funestus,* one of the major African malaria vectors, has attained significant levels of resistance to pyrethroids. Overexpressed P450 monooxygenases have been previously identified in pyrethroid resistant *An. funestus.* The escalating resistance against conventional insecticides signals an urgent need for identification of novel insecticides. Essential oils have gained recognition as promising sources of alternative natural insecticides. This study investigated six essential oil constituents, farnesol, (−)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, methyleugenol, santalol (α and β isomers) and essential oil of sandalwood, for the adulticidal effects against pyrethroid-resistant *An. funestus* strain. The susceptibility against these terpenoids were evaluated on both pyrethroid-susceptible and resistant *An. funestus*. Furthermore, the presence of overexpressed monooxygenases in resistant *An. funestus* was confirmed. Results showed that both the pyrethroid-susceptible and resistant *An. funestus* were susceptible to three EOCs; *cis*-nerolidol, *trans*-nerolidol and methyleugenol. On the other hand, the pyrethroid-resistant *An. funestus* survived exposure to both farnesol and (−)-α-bisabolol. This study however does not show any direct association of the overexpressed *Anopheles* monooxygenases and the efficacy of farnesol and (−)-α-bisabolol. The enhanced activity of these terpenoids against resistant *An. funestus* that has been pre-exposed to a synergist, piperonyl butoxide, suggests their potential effectiveness in combination with monooxygenase inhibitors. This study proposes that *cis*-nerolidol, *trans*-nerolidol and methyleugenol are potential agents for further investigation as novel bio-insecticides against pyrethroid-resistant *An. funestus* strain.

1. Introduction

Malaria is a life-threatening disease caused by the *Plasmodium* parasites and is transmitted through infected female mosquito bites [1]. There were approximately 247 million malaria cases worldwide in 2021 [2]. In addition, malaria-related deaths were estimated at 619000, 76% of which were children under the age of five. Ninety-five (95) percent of these cases and 96% deaths were attributed to the African region [2]. Anopheline mosquitoes are responsible for transmitting the malaria parasites. Among these, *Anopheles funestus* Giles (Diptera: Culicidae) is one of the major malaria vector species in Africa [3,4].

Insecticides are the core interventions used for malaria vector control. Pyrethroid-based insecticides have a long history of use in the malaria vector control programmes. Historically, pyrethrum extracted from the flowers of *Chrysanthemum cinerariifolium,* was used against indoor *Anopheles* mosquitoes [5]. Currently, pyrethroid insecticides are still utilized in the form of insecticide-treated nets as well as indoor residual sprays [6–8]. Pyrethroids are the only insecticides recommended by World Health Organization (WHO) for use in the insecticide-treated nets [8,9]. Malaria cases have however risen despite the use of insecticides and is most likely due to the increased level of insecticide resistance [7]. Insecticide resistance have been reported in all the main African malaria vector species, including *An. funestus*. The pyrethroid metabolic resistance mechanisms are well studied in this species since it

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was first described by Hargreaves et al. [10].

Biochemical and synergist bioassays have suggested the elevated levels of detoxifying monooxygenase as the major mechanism of pyrethroid resistance in *An. funestus* populations in Sub-Saharan Africa [7,11]. Specific P450 genes like *CYP6P9a* and *CYP6P9b* are usually upregulated in pyrethroid-resistant *An. funestus* [12–15]. These overexpressed genes convey the cross-resistance among the type I and type II pyrethroids, invalidating any possible substitutions in these groups [16,17]. The same mechanism also results in cross resistance to carbamate resistance [7,18]. The importance of the monooxygenases in detoxifying insecticides was confirmed using piperonyl butoxide (PBO), a monooxygenase (P450) inhibitor. PBO has also been incorporated into bed nets and these are currently being evaluated by the WHO [8,19,20]. In addition, biochemical assays are used to identify the elevated enzyme activities responsible for conveying resistance [21–23]. This is achieved by measuring the levels of heme-containing P450 enzymes in mosquito populations. The peroxidase activity of the inherent heme group is directly proportional to the P450 levels, hence useful in establishing enzyme activity differences between resistant and susceptible colonies [22]. Brogdon et al. [22] and Namountougou et al. [23] highlighted the importance of evaluating the monooxygenase enzyme levels upon detection of resistance in disease vectors. This assay has previously been used to complement synergist studies in confirming the P450-based resistance mechanism in *An. gambiae* [24].

Insecticide resistance reports have significantly increased and have signalled the urgency for the identification of novel insecticides to combat malaria. Plants often naturally biosynthesize constituents to protect themselves against insect pests, including mosquitoes [25,26]. Due to this, plant constituents and essential oils serve as potential sources of effective chemical agents against insect species including *Anopheles* mosquitoes. The essential oils, hydrophobic extracts from aromatic plants, along with their constituents, have attracted recognition as potential alternative insecticides [9,26,27]. In a recent study by Balboné et al. [28], it has been shown that when used in combination, essential oils have improved bio-insecticidal activity and may be used as alternative insecticides against pyrethroid resistant malaria vectors. Isman [29] stated that the current trend shows great promise towards manufacturing, approval and commercialization of botanical insecticides.

The essential oil constituents (EOCs) are mainly terpenoids and phenylpropanoids [30]. Rants'o et al. [31] reported the larvicidal effects of various EOCs including farnesol, (−)-α-bisabolol, *cis-nerolidol, trans-nerolidol, and* methyleugenol, against *An. funestus*, *An. arabiensis*, *An. gambiae*, and *An. coluzzii*. The sesquiterpene alcohols farnesol and (−)-α-bisabolol are naturally found as major phytoconstituents in the essential oils of various plants including *Rosa x. damascene* Mill., *Polianthes tuberosa* L., *Matricaria chamomilla* L., *Cymbopogon citratus*, *Cymbopogon nardus* [32,33]. *The C. citratus (lemongrass) and C. nardus (citronella) essential oils have shown adulticidal effects against An. gambiae* [34]. *Nerolidol geometric isomers, cis-nerolidol and trans-nerolidol, are also* sesquiterpene alcohols *that have been identified as main constituents in the essential oils of Piper species such as P. clausseniánum* (Miq.), *P. umbellatum, P. nigrum* and *P. capense* [35,36]. Similarly, methyleugenol, a phenylpropanoid, has been identified in the essential oils of >20 plant species including *C. nardus, Ocimum basilicum and Colocasia antiquorum* [37,38] *and while the essential oil of C. nardus has shown adulticidal activity against An. gambiae, that of O. basilicum (basil) has exhibited larvicidal effects against An. subpictus* [34,38]. *Interestingly,* Balboné et al. [28] *reported that a* combination of the essential oils of *C. nardus* and *Ocimum americanum* (90:10) induced adulticidal effects against pyrethroid resistant *An. gambiae* with LC_{50} value of 0.32% *v*/v. *Additionally,* sandalwood oil is an essential oil of medicinal interest with diverse biological activities including insecticidal effects. It is obtained from *Santalum album* L. [39,40]. Interestingly, sandalwood oil has shown larvicidal and adulticidal effects against *An. stephensi* and *An. gambiae*, respectively [34,41]. Major phytoconstituents in sandalwood oil are the sesquiterpene alcohols α- and β-santalol isomers [42,43]. This study aimed to assess farnesol, (−)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, methyleugenol, sandalwood oil, and santalol (α and β) for the adulticidal effects against pyrethroid-resistant *An. funestus*.

2. Materials and methods

The current study was granted the animal research ethics waiver (Waiver Number: 07-11-2017-O) from Wits Animal Research Ethics Committee. Two laboratory reared colonies of *An. funestus*, namely FANG (insecticide susceptible) and FUMOZ-R (pyrethroid-resistant) were used in this study, and these were maintained under standard insectary conditions as reported by Hunt et al. [4] and Zengenene et al. [44]. All EOCs including santalol (76% α-santalol and 6% β-santalol; GC–MS; Catalog No. 75831) and the EO sandalwood oil, deltamethrin, cytochrome C from equine heart and PBO were purchased from Sigma Aldrich (South Africa) with >90% purity. PBO was further diluted to 4% *v*/v in olive oil:acetone (50:50) solvent [7,45].

2.1. P450 biochemical analysis

To confirm that increased P450 activity was still present in FUMOZ-R colony as per previous studies [4,7], the biochemical analyses were conducted [24]. The unexposed samples from both FANG and FUMOZ-R were anesthetized on ice and homogenized in sodium phosphate buffer (pH 7.2) [22]. The protein content was assessed using the standard Lowry protein assay [46]. The P450/monooxygenase assay was conducted with slight modifications as reported by Brogdon et al. [22] as well as Penilla et al. [21]. The mosquito homogenate (20 μL) was added to 80 μL sodium phosphate buffer (pH 7.2). The freshly prepared substrate, 3,3′,5,5′-tetramethylbenzidine (*TMB;* 2% (*w/v*) in absolute methanol), was diluted in 18 mL of 0.25 M sodium acetate buffer (pH 5.0) before 200 μL of this was added to the homogenate incubation mixture. The reaction was then initiated by the addition of 25 μL of 3% (*v*/v) hydrogen peroxide. Standard curves for TMB peroxidation were assessed with equine heart cytochrome C oxidase in place of mosquito homogenate. Cytochrome C oxidase is an electron-carrying protein found in the mitochondria and is bound to two heme groups and carries out cellular oxidation biological processes making it a suitable positive control for P450 peroxidase activity [21,47,48]. Absorbances were read at 650 nm using the Multiskan Go™ microplate spectrophotometer (Thermo Fisher Scientific) at 5 min intervals. The absorbance values were compared with a standard curve of absorbance for known concentrations of cytochrome C oxidase and values reported as equivalent units of cytochrome P450/mg protein. The relative P450 activity was calculated using the P450 absorbance/mg protein.

2.2. WHO bioassays

Susceptibility tests were conducted using the WHO standard bioassay [49]. Batches of 25–30 non-blood fed female mosquitoes aged 2–5 days were assessed in WHO exposure tubes. The mosquitoes were exposed to WHO 0.05% deltamethrin (positive control) test papers for 1 h after which they were transferred into the holding tubes. The control batch was exposed to the WHO pyrethroid control paper. The knockdown was then recorded, and mosquitoes were provided with 10% (*w/v*) sugar solution. The final mortality was recorded 24 h post-exposure. Mortalities on FANG and FUMOZ-R were compared. For each of the experiments, four replicates were conducted. The testing room conditions were maintained at 27 °C and ≥ 75% relative humidity, as well as a 12-h light and 12-h dark photoperiod. Mortality rate was calculated using the formula: % Mortality = (Mean number of dead mosquitoes / Mean number of treated mosquitoes) x 100 [49,50]. Furthermore, pyrethroid resistance was identified using the WHO criteria [49].

2.2.1. Essential oil constituent susceptibility

Whatman Grade 1 (12 × 15 cm^2) filter paper was impregnated with 2 mL of the EOC diluted in acetone [9]. The mosquitocidal activities were assessed at seven EOC concentrations (2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.0313% *v*/v) [51,52]. Deltamethrin (0.05%) was used as a reference insecticide paper and the EOC control included Whatman filter papers impregnated with 2 mL HPLC grade acetone. The standard susceptibility assay was conducted as done for the reference insecticides with four replicates. Both FANG and FUMOZ-R colonies were assessed, and 24-h mortality rates compared. The resultant data afforded the calculation of the mean lethal concentrations for 50% and 99% mortality (LC_{50} and LC_{99}, respectively) with 95% confidence intervals by probit analysis using the SPSS Statistics 27.0 (IBM, New York, USA). The diagnostic concentration for susceptibility tests was defined as twice the LC_{99} obtained with the susceptible colony, FANG, in line with WHO guidelines [49,53]. Furthermore, the resistance ratio (RR) between the pyrethroid susceptible and resistant strains was computed by dividing the LC_{50} (RR_{50}) or LC_{99} (RR_{99}) the FUMOZ-R strain by that of the FANG strain. The RR < 5 suggested low resistance, RR 5–10 indicated moderate resistance, while the RR > 10 was suggestive of high resistance against the tested EOC [28,54]. These EOC susceptibility assays were conducted following the same procedure as for deltamethrin susceptibility.

2.3. Synergist bioassay

The synergist assay was carried out to determine the involvement of metabolic P450 resistance against deltamethrin and identified EOCs [23,24,55]. For this assay, the mosquitoes were pre-exposed to 4% PBO for an hour before being exposed to 0.05% deltamethrin or EOC at the determined diagnostic dose. Synergized batches were assessed concurrently with nonsynergized cohorts for direct comparison. The control assays included the exposure to the synergist alone, exposure to the WHO pyrethroid control paper, to acetone and to olive oil:acetone (50:50). Four test replicates were generated, and the results were discarded and repeated should any of the controls induce >20% mortality [51]. The potentiation of the efficacy of deltamethrin or EOC in the presence of PBO was assessed.

Additionally, the synergistic relationship between EOCs and PBO was characterized using the co-toxicity factor calculated with the following equation [56,57]:

$$Co-toxicity\ factor = \frac{(observed\%mortality - expected\%mortality)}{(expected\%mortality)} \times 100$$

Where expected mortality is the sum of percentage mortalities obtained by PBO alone and EOC alone. The co-toxicity factor values less than −20 indicated an antagonistic relationship, values between −20 and 20 specified an additive interaction, and those >20 proposed synergism [57].

3. Results

3.1. Pyrethroid susceptibility status of Anopheles funestus

Prior to the analysis with EOCs, pyrethroid susceptibility of the two *An. funestus* colonies, FANG and FUMOZ-R, was investigated. Results confirmed that FANG was fully susceptible to deltamethrin, while FUMOZ-R was resistant with 29.5% mortality 24 h post exposure (Table 1). This mortality difference was statistically significant [58] between the two colonies (unpaired *t*-test: $P < 0.0001$; t-value: 17.38; df: 6) and in agreement with previous studies [4,7,59].

Table 1
WHO susceptibility analysis of *An. funestus* colonies, FANG and FUMOZ-R, to % deltamethrin.

An. funestus colony (n)	Insecticide	24 h % Mortality ± SD
FANG (104)	Deltamethrin	100.0 ± 0.0
FANG (100)	Pyrethroid control	0.0 ± 0.0
FUMOZ-R (100)	Deltamethrin	29.5 ± 3.5
FUMOZ-R (102)	Pyrethroid control	1.0 ± 1.0

3.2. Anopheles funestus monooxygenase levels

The FUMOZ-R resistance has been associated with elevated P450 activity [4,7,59], this increased activity was assessed using the monooxygenase assay on the current colonies, FANG and FUMOZ-R [21]. The monooxygenase levels on FANG were significantly lower than that on FUMOZ-R (unpaired *t*-test: $P < 0.0001$; t-value: 95.95; df: 4) (Fig. 1). In addition, the P450 activity on FUMOZ-R samples was significantly higher (~ 4-fold), in comparison to FANG (unpaired *t*-test: $P < 0.0001$; t-value: 70.49; df: 4) (Fig. 1).

3.3. Anopheles funestus terpenoid susceptibility

The two colonies, FANG and FUMOZ-R were subsequently used to evaluate *An. funestus* susceptibility to the EOCs. The EOCs were screened at concentrations 0.03, 0.06, 0.13, 0.25, 0.50, 1.00, and 2.00% which span from 30 to 2000 μg/mL. The LC_{99} value for each EOC against the susceptible strain, FANG, was noted [49]. This concentration was then doubled and compared to that of a positive control, deltamethrin. The nerolidol isomers, *cis*-nerolidol and *trans*-nerolidol obtained the lowest LC_{50} values (lethal concentrations at which 50% female mosquitoes died) of 0.14% (140 μg/mL) and 0.17% (170 μg/mL) followed by methyleugenol at 0.24% (240 μg/mL). These along with (−)-α-bisabolol (LC_{50}: 320 μg/mL), santalol (LC_{50}: 350 μg/mL) and sandalwood oil (LC_{50}: 360 μg/mL) showed LC_{50} values in the moderate *Anopheles* toxicity range (100–500 μg/mL) while farnesol exhibited low insecticidal activity (LC_{50}: >500 μg/mL) [60,61]. Considering the concentrations at which 99% female mosquitoes died (LC_{99} values) and the estimated 2× LC_{99} concentrations, three EOCs *cis*-nerolidol, *trans*-nerolidol, and methyleugenol consistently showed relatively lower values, however, higher than that of deltamethrin (Table 2; one-way ANOVA: $P = 0.0001$). In correlation to previous studies, a positive control deltamethrin obtained the LC_{50} and LC_{99} concentrations of 0.007% and 0.05%, respectively [62,63].

Following this, the baseline susceptibility of *An. funestus* to the terpenoids was evaluated by screening them at 2× LC_{99} concentrations against both FANG and FUMOZ-R and compared with deltamethrin [28]. However, farnesol, (−)-α-bisabolol, santalol (α and β), and sandalwood oil could not be screened at the double LC_{99} concentrations due to requirements for high concentrations at which solubility was lost. Since the double LC_{50} values have also been used in previous studies [64], these were used to assess the baseline activity of farnesol, (−)-α-bisabolol, santalol (α and β), and sandalwood oil. Interestingly, *cis*-nerolidol, *trans*-nerolidol, and methyleugenol induced 100% mortality in both FANG and FUMOZ-R, where no <100 females were assessed against each EOC (Fig. 2a). Differently, deltamethrin showed a significantly higher mortality in the susceptible colony (100%) than the resistant colony (29.5%) (unpaired *t*-test: $P = 0.0013$; t-value: 28.20; df: 2) in agreement to the literature [4,7,62].

Similar to deltamethrin, there was a significant difference in the susceptibility of FANG and FUMOZ-R to farnesol, (−)-α-bisabolol, santalol (α and β) and sandalwood oil (Fig. 2b). However, deltamethrin could reach 100% 24-h mortality against FANG ($n = 104$) at double LC_{99} concentration, while none of these EOCs reached this mortality rate. On the other hand, this pyrethroid could only attain 21.8% against FUMOZ-R at this concentration ($n = 106$) (unpaired *t*-test: $P < 0.0001$; t-value: 54.59; df: 6). Farnesol induced 65% ($n = 103$) mortality after 24 h in the pyrethroid susceptible colony, FANG, and this was significantly different from the 6% (n = 106) mortality recorded for the pyrethroid resistant colony, FUMOZ-R (unpaired *t*-test: $P < 0.0001$; t-value: 22.90; df: 6).

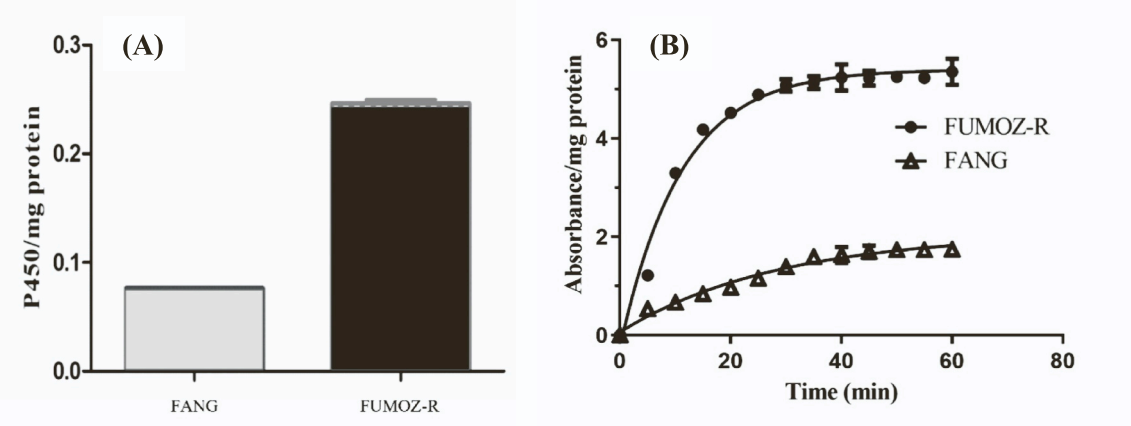


Fig. 1. Monooxygenase activity in *An. funestus*. (A) Comparison of the equivalent units of cytochrome P450/mg protein in *An. funestus* FUMOZ-R and *An. funestus* FANG; (B) Comparison of the P450 activity in *An. funestus* FUMOZ-R and *An. funestus* FANG.

Table 2
The lethality concentrations of EOCs against pyrethroid-susceptible *An. funestus*.

	An. funestus (FANG)		
Essential oil constituent	LC_{50} (%) (95% CI)	LC_{99} (%) (95% CI)	2× LC_{99} concentration (%)
cis-Nerolidol	0.14 (0.08–0.23)	1.65 (0.71–12.42)	3.30
trans-Nerolidol	0.17 (0.10–0.29)	1.92 (0.79–18.97)	3.84
Methyleugenol	0.24 (0.15–0.37)	2.47 (1.16–12.32)	4.94
Farnesol	0.74 (0.54–1.11)	30.31 (11.88–146.13)	60.62
(−)-α-Bisabolol	0.32 (0.27–0.37)	8.69 (5.75–14.69)	17.38
Santalol (α and β)	0.35 (0.30–0.41)	9.83 (6.34–17.28)	19.66
Sandalwood oil	0.36 (0.27–0.50)	8.90 (4.35–28.20)	17.80
Deltamethrin	0.007 (0.005–0.008)	0.05 (0.04–0.10)	0.10

Similarly, for (−)-α-bisabolol, 78.3% ($n = 100$) mortality was recorded for FANG 24 h after exposure, and this was statistically different from the 39.5% ($n = 108$) mortality recorded for FUMOZ-R (unpaired *t*-test: $P < 0.0001$; t-value: 9.80; df: 6). Santalol (α and β) showed 76.5% mortality against FANG versus 28% FUMOZ-R mortality (unpaired *t*-test: $P < 0.0001$; t-value: 9.32; df: 6). Likewise, sandalwood oil recorded 79% FANG mortality and only 27% mortality against FUMOZ-R (unpaired *t*-test: $P = 0.0003$; t-value: 7.40; df: 6). Moreover, the activity levels of sandalwood oil and its main constituent, santalol (α and β), were comparable for FANG (76.5%; santalol (α and β) and 79%; sandalwood oil) (unpaired *t*-test: $P = 0.7347$; t-value: 0.36; df: 6) and showed no statistical difference in their intensity against FUMOZ-R (28%; santalol (α and β) and 27%; sandalwood oil) (unpaired *t*-test: $P = 0.8533$; t-value: 0.19; df: 6). This suggested that santalol (α and β) is a constituent responsible for the insecticidal activity in the essential oil of sandalwood.

Given the low activity observed for farnesol, (−)-α-bisabolol, santalol (α and β), and sandalwood oil against FUMOZ-R, where they respectively reached 6%, 39.5%, 28%, and 27% mortality at baseline screening (Fig. 2b), these compounds could not generate accurate LC_{50} and LC_{99} values. However, the LC_{50} and LC_{99} values of three most active EOCs were determined against FUMOZ-R (Table 2). Similar to their screening against FANG (Table 2), these EOCs still maintained moderate toxicity (LC_{50} range between 100 μg/mL and 500 μg/mL) against FUMOZ-R (Table 3).

To confirm similar effects against pyrethroid-susceptible and resistant *An. funestus*, it was worth comparing the LC_{50} and LC_{99} values of these three active terpenoids obtained against FUMOZ-R to those acquired in screening against FANG. *cis*-Nerolidol had similar LC_{50} values against these two colonies (Table 3) (unpaired *t*-test: $P = 0.7646$; t-value: 0.30; df: 198). Comparably, *trans*-nerolidol as well showed similar LC_{50} values against FANG and FUMOZ-R (unpaired *t*-test: $P = 0.7711$; t-value: 0.29; df: 198). Finally, methyleugenol also attained similar LC_{50} values against the susceptible and resistant colonies (unpaired *t*-test: $P = 0.8403$; t-value: 0.20; df: 198). Notably, deltamethrin displayed significantly higher LC_{50} values against FOMOZ-R, in comparison to the susceptible colony (unpaired *t*-test: $P < 0.0001$; t-value: 0.5832; df: 198). In terms of LC_{99}, deltamethrin still had a significant decline in potency against FUMOZ-R ($LC_{99} = 1.74$) in comparison to FANG ($LC_{99} = 0.05$) (unpaired *t*-test: $P < 0.0001$; t-value: 7.41; df: 198) (Table 3). These potency differences suggested that its activity has dramatically been reduced in pyrethroid resistant *An. funestus* (FUMOZ-R). This was also evident from the calculated RR values in which deltamethrin attained the RR > 10 indicating high resistance level of FUMOZ-R against it [28]. On the contrary, the RR values for all the three active EOCs were less than five, suggesting insignificant resistance against them by the FUMOZ-R strain (Table 3).

3.4. Synergist assays

Since they generally showed similar activity levels in FANG and FUMOZ-R (Fig. 2a and Table 3), the three most active EOCs, *cis*-nerolidol, *trans*-nerolidol and methyleugenol were not included in the synergy study. On the other hand, due to the 24-h mortality profile that showed statistically significant differences between the two colonies when exposed to deltamethrin (Fig. 2b), the role of P450 monooxygenases was investigated. The colonies are no longer under pyrethroid selection pressure [4], therefore the role of monooxygenases was confirmed when PBO pre-exposed FUMOZ-R *An. funestus* females restored deltamethrin susceptibility (Table 4) in line with recent studies [11,65]. In a similar fashion, the impact of synergising monooxygenases prior to farnesol and (−)-α-bisabolol exposure on pyrethroid resistant *An. funestus* was investigated. Their assessment in the synergist assay revealed that, in the presence of a P450 inhibitor, PBO, 100% mortalities were recorded 24 h after exposure for both farnesol and (−)-α-bisabolol (Table 4). On the other hand, the combination of either santalol (α and β) or sandalwood oil with PBO did not result in a 100% 24-h mortality in FUMOZ-R, despite having increased mortality rates (Table 4). This mortality increase was not statistically significant for santalol (α and β) (unpaired *t*-test: $P = 0.3480$; t-value: 1.02; df: 6) but significant in the case of sandalwood oil (unpaired *t*-test: $P = 0.0167$; t-value: 3.29; df: 6).

In addition to the results from the synergist assay, the calculated co-toxicity factor [57] confirmed that deltamethrin, farnesol and (−)-α-bisabolol exhibit a synergist character when in combination with PBO (Table 5). Moreover, synergism was still responsible for the 10–15-fold increase in FUMOZ-R toxicity when santalol (α and β) or

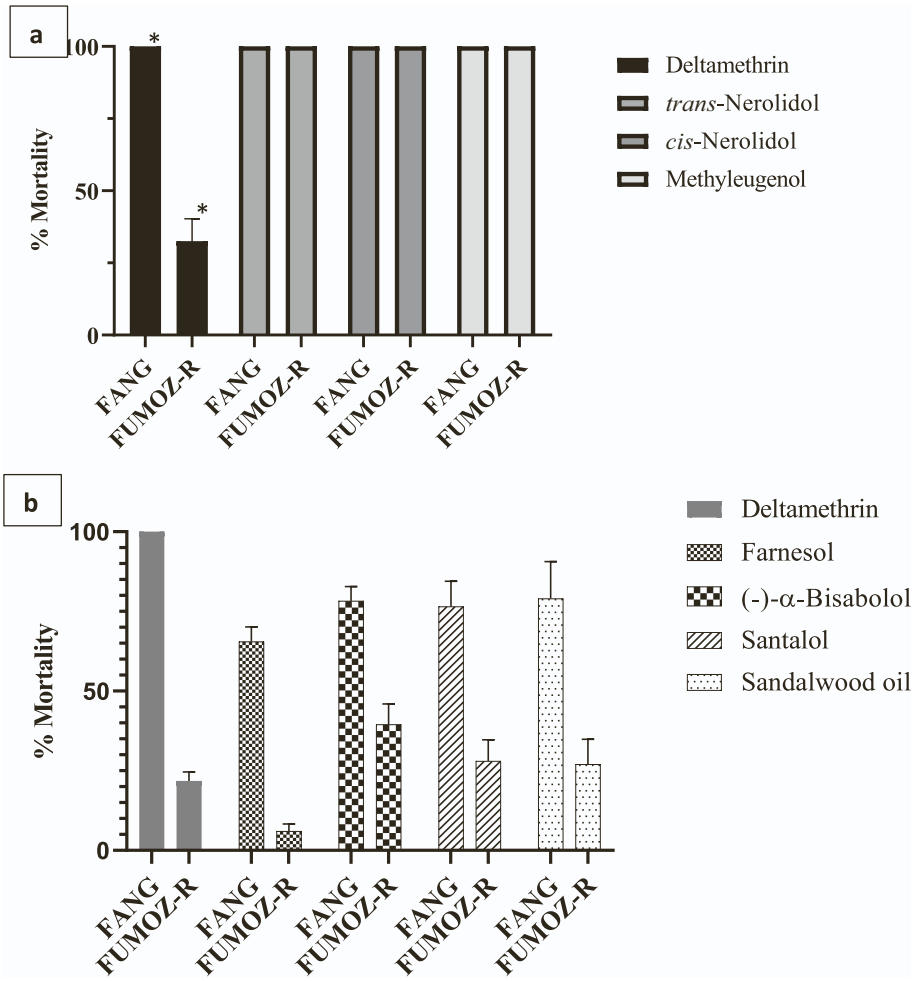


Fig. 2. The susceptibility status of FANG and FUMOZ-R against three most active EOCs assessed at their double LC_{99} concentrations (a). The comparison of FANG and FUMOZ-R susceptibility to three less active EOCs and one EO (b). The terpenoid-induced mortalities were compared to deltamethrin. *Only deltamethrin in (a) showed significantly different mortality rates between FANG and FUMOZ-R strains ($p < 0.05$) while all test compounds in (b) showed significant difference in the mortality against FANG versus FUMOZ-R ($p < 0.05$) [58].

Table 3
Comparison of terpenoid potencies between pyrethroid-susceptible and resistant *An. funestus* strains.

	Lethality concentrations							
	LC_{50} (%) (95% CI)			LC_{99} (%) (95% CI)			Resistance ratio (RR)#	
Compound	(FANG)	(FUMOZ-R)	*p*-value	(FANG) LC_{99}	FUMOZ-R)	*p*-value	RR_{50}	RR_{99}
cis-Nerolidol	0.14 (0.08–0.23)	0.23 (0.16–0.34)	0.7646	1.65 (0.71–12.42)	2.09 (1.08–7.64)	0.1443	1.64	1.27
trans-Nerolidol	0.17 (0.10–0.29)	0.26 (0.19–0.37)	0.7711	1.92 (0.79–18.97)	2.18 (1.19–6.76)	0.4009	1.53	1.14
Methyleugenol	0.24 (0.15–0.37)	0.27 (0.23–0.31)	0.8403	2.47 (1.16–12.32)	5.03 (3.57–7.74)	< 0.0001*	1.13	2.04
Deltamethrin	0.007 (0.005–0.008)	0.14 (0.11–0.18)	< 0.0001*	0.05 (0.04–0.10)	1.74 (1.06–3.75)	< 0.0001*	20.00	34.80

* Statistically significant difference ($p < 0.05$) [58].
\# $RR_{50} = LC_{50}$ FUMOZ-R/ LC_{50} FANG, $RR_{99} = LC_{99}$ FUMOZ-R/ LC_{99} FANG [54].

sandalwood oil were combined with PBO (Table 5). The mortality levels of synergized santalol (α and β) (37.3%) and sandalwood oil (42.5%) were still statistically similar (unpaired *t*-test: $P = 0.8358$; t-value: 0.24; df: 2).

4. Discussion

This study reports for the first time the potential insecticidal activity of EOCs on pyrethroid-resistant *An. funestus*. The current and increasing insecticide resistance has rendered many conventional insecticides ineffective and is a threat for vector control programs. Resistance to pyrethroids, the only insecticides used for insecticide-treated nets, poses a major challenge in the future of malaria vector control programs [6]. Due to this high occurrence of resistance, the use of pyrethroids is reported to have decreased by 53% in the recent 10-year WHO insecticide use survey [66]. The *An. funestus*, a major African malaria vector, possesses significant pyrethroid resistance mainly through rapid detoxification of insecticides by P450 enzymes [4,7]. Identification of novel insecticides from natural products is a relatively cost-effective alternative [27]. Isman [26] suggested that commercialization of essential oil-

Table 4
Evaluation of *An. funestus* FUMOZ-R susceptibility to deltamethrin and EOCs with and without a synergist.

Deltamethrin synergy		
An. funestus colony (n)	Insecticide	24 h Mortality (% ± SD)
FUMOZ-R (106)	Deltamethrin	21.8 ± 2.9
FUMOZ-R (101)	PBO	0.0 ± 0.0
FUMOZ-R (101)	Deltamethrin + PBO	100 ± 0.0
FUMOZ-R (104)	Pyrethroid control	1.0 ± 1.0
FUMOZ-R (101)	PBO control	0.0 ± 0.0
EOCs synergy		
FUMOZ-R (106)	Farnesol	6.0 ± 2.3
FUMOZ-R (106)	Farnesol + PBO	100 ± 2.0
FUMOZ-R (100)	(−)-α-Bisabolol	39.5 ± 0.7
FUMOZ-R (106)	(−)-α-Bisabolol + PBO	100 ± 0.0
FUMOZ-R (101)	Santalol (α and β)	28.0 ± 3.4
FUMOZ-R (101)	Santalol (α and β) + PBO	37.3 ± 6.4
FUMOZ-R (100)	Sandalwood oil	27 ± 3.9
FUMOZ-R (100)	Sandalwood oil + PBO	42.5 ± 5.2
FUMOZ-R (101)	PBO	0.0 ± 0.0
FUMOZ-R (102)	Acetone	0.0 ± 0.0
FUMOZ-R (99)	PBO control*	0.0 ± 0.0

All the control tests including PBO alone did not cause any mortality.
* Olive oil:acetone (50:50).

based insecticides will be highly facilitated by the exemption of some of them from normal lengthy regulatory processes, given their known safety profiles and long history of use. Many studies have proven that essential oils are promising sources of alternative natural insecticides [9,52] and some have shown their potential in reversing pyrethroid resistance in mosquito species such as *An. gambiae* [28]. The current identification of EOCs with potent insecticidal activity against pyrethroid-resistant *An. funestus* has potential for greater benefits for malaria vector control.

In this study, six EOCs farnesol, (−)-α-bisabolol, *cis*-nerolidol, *trans*-nerolidol, methyleugenol, and santalol (α- and β) along with one EO, sandalwood oil, were assessed for this potential. The WHO insecticide susceptibility assay has confirmed the pyrethroid susceptibility of the *An. funestus* colony, FANG, and pyrethroid resistance in the second *An. funestus* colony, FUMOZ-R. Further, the synergist assay confirmed that the monooxygenase-based mechanism of resistance in FUMOZ-R is still present, as described in previous studies [4,7]. This was also confirmed by increased monooxygenase levels in FUMOZ-R compared to the fully susceptible FANG strain using the P450 biochemical assay. Brooke et al. [7] showed that the monooxygenase levels on the resistant *An. funestus* colony could reach >100 times higher than the susceptible colony. At a transcriptional level, Riveron et al. [17] reported that the specific genes; *CYP6P9a* and *CYP6P9b*, were significantly overexpressed (~50-fold) in pyrethroid-resistant *An. funestus* from Mozambique and Malawi. Similarly, Wondji et al. [67] and Christian et al. [53] reported that the *CYP6P9* gene was highly overexpressed in FUMOZ-R as opposed to FANG.

Following confirmation of the monooxygenase-based pyrethroid resistance, FUMOZ-R colony as well as a pyrethroid susceptible colony, FANG, were investigated for susceptibility to six EOCs and one EO. Insecticide susceptible *Anopheles funestus* females were used as a reference for determining the double LC_{99} concentrations for test compounds [28]. The three lowest double LC_{99} concentrations were attained with *cis*-nerolidol (3.30%), *trans*-nerolidol (3.84%) and methyleugenol (4.94%). These three EOCs showed similar LC_{50} values regardless of whether pyrethroid resistant or susceptible *An. funestus* were used. Additionally, the RR values for these three EOCs were all less than five, which is an indication of low resistance level against them. This appears to indicate that the efficacies of these EOCs are not negatively impacted by the pyrethroid resistant genotype of *An. funestus*.

On the other hand, four other test compounds; farnesol, (−)-α-bisabolol, santalol (α and β), and sandalwood oil showed statistically lower activity against pyrethroid resistant *An. funestus* with <50% 24-h mortality. The association of overexpressed monooxygenases in the pyrethroid *An. funestus* on the activity of these essential oils was assessed through standard synergist assays. When the monooxygenases were inhibited by PBO, both farnesol and (−)-α-bisabolol, showed 100% 24-h mortality in pyrethroid resistant *An. funestus*. However, there is no direct indication in this study that overexpressed *Anopheles* monooxygenases reduce the efficacy of farnesol and (−)-α-bisabolol. The enhanced activity of farnesol and (−)-α-bisabolol against resistant *An. funestus* when synergized by PBO, rather indicates their potential effectiveness in combination with P450 monooxygenase inhibitors. As an example, the combination of pyrethroids with PBO has already shown great impact in reversing resistance in the field applications [19,20].

On the other, sandalwood oil and its major constituent, santalol (α and β), could still not attain 100% 24-h mortality in pyrethroid resistant *An. funestus,* even when combined with PBO. Notably, the LC_{50} values of santalol (α and β) and sandalwood oil were similar. Furthermore, the statistical similarity of efficacies of santalol (α and β) and sandalwood oil against either insecticide susceptible or pyrethroid resistant *An. funestus*, suggests that santalol (α and β) may serve as the main active component in this essential oil.

In this study, nerolidol geometric isomers obtained the lowest LC_{50} and LC_{99} values against pyrethroid-susceptible and resistant *An. funestus*. The nerolidol-rich essential oil of *Lantana camara* has previously induced adulticidal effects against *An. gambiae* (susceptible 'Kisumu' strain), with the LC_{50} value of 0.24% [52], comparable to this study. Nerolidol is currently registered by Food and Drug Administration as a food flavouring agent [68,69]. It has been proven nontoxic to mammals at concentrations higher than those reported here for insecticidal activity [69]. It is commercially used at concentrations as high as 20% in human fragrance products and showed the LD_{50} at about 10 g/kg dose in mice [69,70]. Therefore, nerolidol is considered safe and for that reason, it may be further investigated for field application as a novel bio-insecticide in the form of a residual spray. Another EOC that showed comparable LC_{50} value to the nerolidol isomers is a phenylpropanoid methyleugenol. This EOC also showed similar potencies between the pyrethroid-susceptible and resistant *An. funestus*.

5. Conclusion

The findings of this study suggest that both the pyrethroid-susceptible and resistant *An. funestus* colonies are susceptible to *cis*-nerolidol, *trans*-nerolidol, and methyleugenol. This study has also identified two essential oil constituents, farnesol and (−)-α-bisabolol,

Table 5
Co-toxicity effects of four select EOCs in combination with PBO against *An. funestus* FUMOZ-R.

		% Mortality					
Synergist	EOC	Synergist alone	EOC alone	Expected	Observed	Co-toxicity factor	Co-toxicity type
PBO	Farnesol	1.0	6.0	7.0	99.0	1314.3	Synergism
PBO	(−)-α-Bisabolol	1.0	39.5	40.5	100.0	146.9	Synergism
PBO	Santalol (α and β)	0.0	28.0	28.0	38.0	35.7	Synergism
PBO	Sandalwood oil	0.0	27.0	27.0	42.5	57.4	Synergism
PBO	Deltamethrin	0.0	21.8	21.8	100.0	358.7	Synergism

that have a potential to successfully kill the pyrethroid resistant *An. funestus*, when combined with P450 synergists such as PBO. Therefore, this study reports for the first time, *cis*-nerolidol, *trans*-nerolidol and methyleugenol as potential candidates for further investigations as novel entities for development as bioinsecticides against *An. funestus*. These EOCs should further be evaluated against other malaria vectors with known insecticide resistance phenotypes. The nerolidol geometric isomers, *cis*-nerolidol and *trans*-nerolidol were the most active EOCs and given the well-established mammal safety record of nerolidol, these terpenoids are recommended for further investigations as resistance-breaking bioinsecticides. Furthermore, these sesquiterpene alcohols may serve as templates for further Declaration of Competing Interest

Authors declare no conflict of interest.

structural modification for generating more active insecticides against pyrethroid resistance.

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Appendix F.1: Author declarations

Appendix F.1.1: Chapter 2: Potential of essential oil-based anticholinesterase insecticides against *Anopheles* vectors: A review

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Thankhoe Abram Rants'o, student number [1841103], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis.

Signature of Student ..... **Date**.....20-Sept-2022.....

Name of Primary Supervisor.....Prof Robyn van Zyl.....

Signature of Primary Supervisor .....**Date**.....20-Sept-2022.....

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his Thesis. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his degree purposes.

Article 1: Title: ...Potential of essential oil-based anticholinesterase insecticides against *Anopheles* vectors - A review

Journal name, year, volume and page numbers: ...Molecules, 2022;27(20): 7026.....

Authors	Name	Signature	Date
1st author	Thankhoe A Rants'o		20-Sept-2022
2nd author	Lizette L. Koekemoer		20-Sept-2022
3rd author	Jenny-Lee Panayides		20-Sept-2022
4th author	Robyn L. van Zyl		20-Sept-2022
5th author			
6th author			

Comments by primary supervisor:

.....

.....Student is primary author to prepare paper, declaration of contribution by co-authors in the paper.....

.....

1

Appendix F.1.2: Chapter 3: Optimization of the *in silico* approach to assess the acetylcholinesterase inhibitory potential of chemical entities using molecular modelling and molecular mechanics simulations

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Thankhoe Abram Rants'o, student number [1841103], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis.

Signature of Student …… **Date**……20-Sept-2022……

Name of Primary Supervisor……Prof Robyn van Zyl……

Signature of Primary Supervisor ……**Date**……20-Sept-2022……

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his Thesis. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his degree purposes.

Article 2: Title: …Optimization of covalent docking for organophosphates interaction with *Anopheles* acetylcholinesterase

Journal name, year, volume and page numbers: …Journal of Molecular Graphics and Modelling, 2022, 110: 108054……

Authors	Name	Signature	Date
1st author	Thankhoe A. Rants'o		20-Sept-2022
2nd author	C. Johan van der Westhuizen		20-Sept-2022
3rd author	Robyn L. van Zyl		20-Sept-2022
4th author			
5th author			
6th author			

Comments by primary supervisor:
All research undertaken by student and article prepared by student with input from CJVDW and RLVZ

……

Appendix F.1.3: Chapter 4: The *in vitro* and *in silico* assessment of donepezil derivatives for the *Anopheles* acetylcholinesterase inhibition

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Thankhoe Abram Rants'o, student number [1841103], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis.

Signature of Student ..... **Date**.....20-Sept-2022.....

Name of Primary Supervisor.....Prof Robyn van Zyl.....

Signature of Primary Supervisor .....**Date**.....20-Sept-2022.....

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his Thesis. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his degree purposes.

Article 3: Title: ...The *in silico* and *in vitro* analysis of donepezil derivatives for *Anopheles* acetylcholinesterase inhibition...

Journal name, year, volume and page numbers: ...PLoS One, 2022;17(11): e0277363...

Authors	Name	Signature	Date
1st author	Thankhoe A. Rants'o		20-Sept-2022
2nd author	Divan G. van Greunen		20-Sept-2022
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4th author	Darren L. Riley		20-Sept-2022
5th author	Jenny-Lee Panayides		20-Sept-2022
6th author	Lizette L. Koekemoer		20-Sept-2022
7th author	Robyn L. van Zyl		20-Sept-2022

Comments by primary supervisor:

All lab work done by student, declaration of contribution by co-authors in the paper

.....

1

Appendix F.1.4: Chapter 5: The *in vitro* and *in silico* assessment of select terpenoids for the *Anopheles* acetylcholinesterase inhibition

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Thankhoe Abram Rants'o, student number [1841103], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/Dissertation/Research Report.

Signature of Student ..... **Date**.....26-Sept-2022.....

Name of Primary Supervisor.....Prof Robyn van Zyl.....

Signature of Primary Supervisor .....**Date**.....26-Sept-2022.....

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/Dissertation/Research Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 4: Title: *In vitro* and *in silico* analysis of the *Anopheles* anticholinesterase activity of terpenoids

Journal name, year, volume and page numbers: Parasitology International, 2023;93: 102713.

Authors	Name	Signature	Date
1st author	Thankhoe A Rants'o		26-Sept-2022
2nd author	Lizette L. Koekemoer		26-Sept-2022
3rd author	Robyn L. van Zyl		26-Sept-2022
4th author			
5th author			
6th author			
7th author			

Comments by primary supervisor:

All lab work and paper preparation done by student, declaraion of contributions by co-authors in the publication

1

Appendix F.1.5: Chapter 6: The activity of terpenoids against multiple stages of *Anopheles* life cycle

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Thankhoe Abram Rants'o, student number [1841103], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/Dissertation/Research Report.

Signature of Student …… **Date**……20-Oct-2022……

Name of Primary Supervisor……Prof Robyn van Zyl……

Signature of Primary Supervisor ……**Date**……20-Oct-2022……

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/Dissertation/Research Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 5: Title: Bioactivity of select essential oil constituents against life stages of *Anopheles arabiensis* (Diptera: Culicidae)

Journal name, year, volume and page numbers: Experimental Parasitology (Manuscript ID: EXPARA-D-22-00337)

Authors	Name	Signature	Date
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Comments by primary supervisor:

Declaration in the paper - with student undertaking research and drafting publication

……

Appendix F.1.6: Chapter 7: The insecticidal activity of essential oil constituents against insecticide resistant *Anopheles* with overexpressed P450 monooxygenases

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Thankhoe Abram Rants'o, student number [1841103], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/Dissertation/Research Report.

Signature of Student …… **Date**……26-Sept-2022……

Name of Primary Supervisor……Prof Robyn van Zyl……

Signature of Primary Supervisor ……**Date**……26-Sept-2022……

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/Dissertation/Research Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 4: Title: The insecticidal activity of essential oil constituents against pyrethroid-resistant *Anopheles funestus* (Diptera: Culicidae)

Journal name, year, volume and page numbers: Parasitology International, 2023;95: 102749

Authors	Name	Signature	Date
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Comments by primary supervisor:
All lab work and paper preparation done by student, declaraion of contributions by co-authors in the publication

Appendix G.1: Approval of change of title

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Private Bag 3 Wits, 2050
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Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

06 April 2022
Person No: 1841103
TAA

Mr TA Rants'o
100 Putney Road
Brixton
2092
South Africa

Dear Mr Thankhoe Rants'o

Doctor of Philosophy: Change of title of research

I am pleased to inform you that the following change in the title of your Thesis for the degree of **Doctor of Philosophy** has been approved:

From:	**Novel acetylcholinesterase inhibitors against the life cycle of Anopheles and Plasmodium falciparum**
To:	**In vitro and in silico characterization of the anticholinesterase activity of select terpenoids against Anopheles vectors**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

Appendix H.1: Human Research Ethics waiver

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Innovation)

29/08/2018

Ref: W-CBP-180829-03

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Mr T Rants'o
Student No. (if appropriate): 1841103
Staff No. (if appropriate):

Supervisor: Professor R van Zyl

School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University

Project title: *In vitro and in silico characterisation of the anticholinesterase activity of select terpenoids against Anopheles vectors*
Change of study title noted on 01/07/2022

Reason: In vitro study
No human participants will be involved in the study

CB Penny

Professor CB Penny
Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
Third Floor, Phillip Tobias Building, corner of St Andrews and York Roads, Parktown, Johannesburg 2193
Postal address: Private Bag 3, Wits 2050
Tel Nos: +27 (0)11 717 1234/1252/2656/2700
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za
Website:
https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/

Appendix I.1: Biosafety Ethics certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE (IBC)
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20181001**

BRIEF DESCRIPTION OF APPLICATION:

Novel acetylcholinesterase inhibitors against the life cycle of anopheles and plasmodium falciparum

APPLICANT: Mr TA Rants'o

SUPERVISOR: Prof RL van Zyl

SCHOOL/DEPARTMENT : Pharmacy and Pharmacology

DATE CONSIDERED: Adhoc

EXPIRY DATE: 22 October 2022

DECISION OF COMMITTEE: Approved unconditionally
Approval granted from 22 October 2018 (retrospective approval cannot be granted under South African Law)

1. This clearance certificate expires on 22 October 2022 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form
4. Unreported changes to the application may invalidate the clearance given by the IBC

DATE: 22 October 2018

CHAIRPERSON: _____
(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed in duplicate and **one copy** returned to the Research Office, Phillip Tobias Building, Room 301, 3rd Floor, 29 Princess of Wales Terrace, Parktown, Faculty of Health Sciences, University.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, *e.g.* suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
   a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
   b. that I will not begin work until such approvals are obtained

Signed: _____ **Date:** 22-10-18

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix J.1: Animal Research Ethics waiver

Appendix J.1.1: *Anopheles* waiver

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

7 November 2017

To whom it may concern,

Re: Waiver from Animal Ethics Screening Committee of the University of the Witwatersrand

This letter serves to confirm that Associate Professor Robyn van Zyl (Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, University of the Witwatersrand) does not require full animal ethics clearance for her studies which use *Anopheles* mosquito species to evaluate known or novel synthetic and natural compounds as potential insecticides against *Anopheles* species.

Reasons: Mosquito eggs and larva are used in the study. No animals or animal products are used in the study.

Conditions: All approved biosafety and biosecurity SOPs should be adhered to.

Should you require any further information, do not hesitate to contact me.

Yours sincerely,

Kennedy Erlwanger

(Chairman: Animal Research Ethics Committee, University of the Witwatersrand)

Reference: R van Zyl –Anopheles Waiver 07-11-2017-O

Assoc Prof Kennedy H. Erlwanger
School of Physiology
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Email: Kennedy.Erlwanger@wits.ac.za

Appendix J.1.2: *Artemia*

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE (AREC)

Registration number: AREC0101210-002

Solomon Mahlangu House
10th Floor, Room 10004
Jorissen Street
Braamfontein, Johannesburg

7 November 2017

To whom it may concern,

Re: Waiver from Animal Ethics Screening Committee of the University of the Witwatersrand

This letter serves to confirm that Associate Professor Robyn van Zyl (Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, University of the Witwatersrand) does not require full animal ethics clearance for her studies which use *Artemia* (Brine shrimp) as a toxicological model to evaluate known or novel synthetic and natural compounds.

Reasons: *Artemia* eggs and *nauplii* are used in the study. No animals or animal products are used in the study.

Conditions: All approved biosafety and biosecurity SOPs should be adhered to.

Should you require any further information, do not hesitate to contact me.

Yours sincerely,

Kennedy Erlwanger

(Chairman: Animal Research Ethics Committee, University of the Witwatersrand)

Reference: R van Zyl Waiver (Artemia) 07-11-2017-O

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