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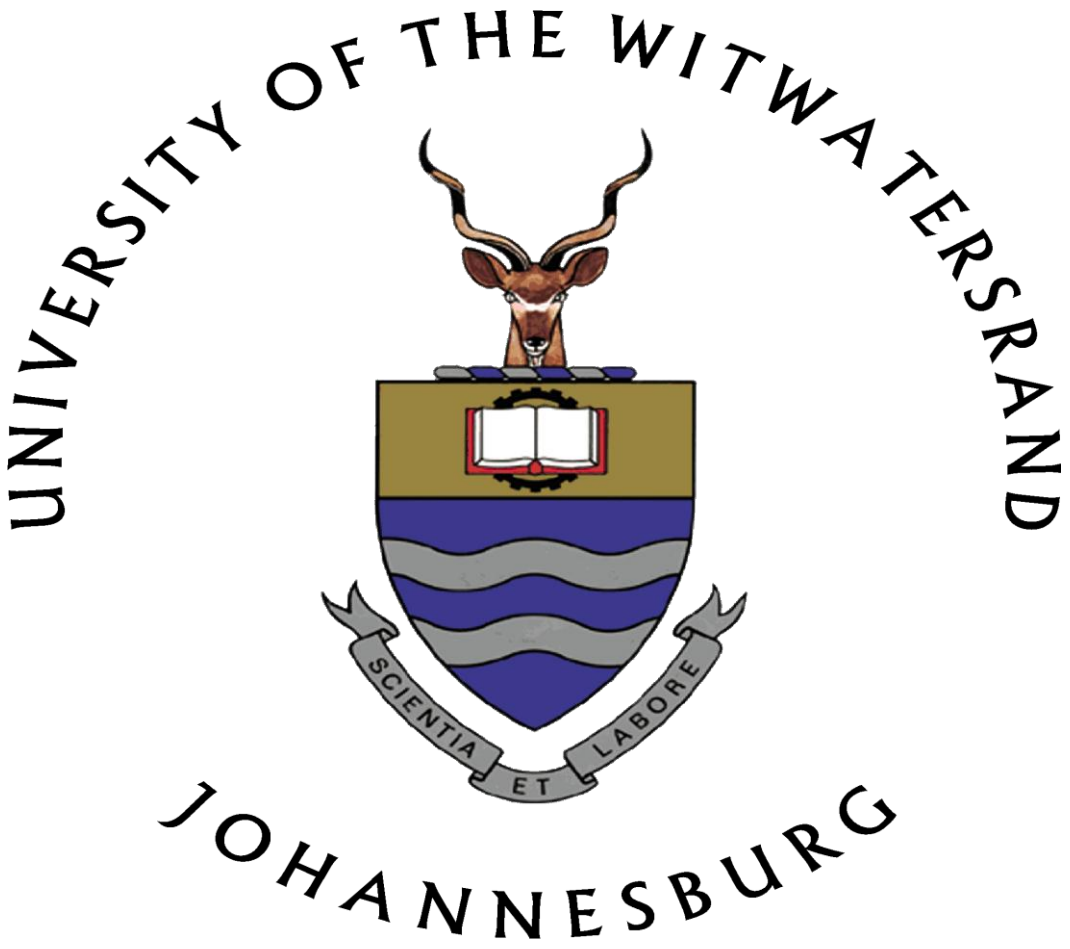
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The Antimicrobial Properties of The Major Compounds Found in South African Propolis



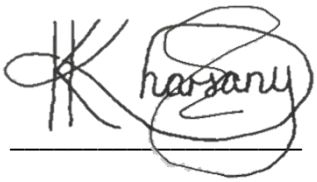
Khadija Kharsany

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Pharmacy

June 2019

DECLARATION

I, Khadija Kharsany, declare that this dissertation is my own work. It is being submitted in fulfilment for the degree of Master of Pharmacy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



Khadija Kharsany

Date: 02/06/2019

ABSTRACT

Propolis is a resinous substance produced by the *Apis mellifera* bee. The compounds predominantly found in South African propolis are the flavonoids pinocembrin, galangin, and chrysin. The aim of this study was to obtain an understanding of the antimicrobial activity of these compounds, both singularly and in combination, and to investigate the role of interactions between the compounds.

To observe the effects of the compounds against planktonic micro-organisms, the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) assays were undertaken. Combinations were tested at equal ratios using the MIC assay, the results of which were interpreted using the fractional inhibitory concentration (FIC) index. Varied ratio combinations using the MIC method were also undertaken and presented in isobolograms. Results from six bacterial strains and three yeast strains demonstrated that the singular compounds displayed moderate to weak activity, and that the activity of pinocembrin, galangin, and chrysin was improved when used in combination. When tested at 1:1 ratio, synergy occurred in six out of the 27 combinations (22%) whilst nine combinations (30%) were additive, and the remaining 12 combinations (44%) were non-interactive. No combination showed antagonism. The 1:1 combination of galangin and chrysin appeared to be the most effective, as it was synergistic against three of the nine micro-organisms (33%) tested, and additive against another three. Pinocembrin, galangin, and chrysin were then tested at varying ratio combinations against three micro-organisms, which produced nine isobolograms and a total of 81 ratios. From these 81 ratios, 32 ratios (40%) showed synergy, while 31 ratios (38%) were additive and the remaining 18 ratios (22%) were non-interactive. The combination of galangin and chrysin against *C. tropicalis* displayed the best activity, as all nine ratios of these two compounds in combination, showed synergy. The triple combination which used pinocembrin, galangin, and chrysin at a 1:1:1 ratio against nine pathogens, produced synergy against six of the nine micro-organisms (67%) tested and additivity against the remaining three (33%) micro-organisms. Selected combinations

possessed bactericidal activity, although the compounds on their own demonstrated no bactericidal activity at the concentrations tested. The most pronounced bactericidal activity was observed from the combination of galangin with chrysin against *Candida tropicalis*, with an MBC value of 0.16 mg/ml.

Anti-quorum sensing (QS) testing was undertaken using *Chromobacterium violaceum* as a monitor strain. The broth macrodilution method was used. In addition, an anti-QS broth microdilution method was tested for the first time in this study. Results from the broth macrodilution method showed that pinocembrin, galangin and chrysin, and their combinations, were capable of inhibiting violacein production from *C. violaceum*. When tested singularly, pinocembrin, galangin and chrysin all showed minimum quorum sensing inhibitory concentration (MQSIC) values of 0.31 mg/ml or less, and MBC values of 2.5 mg/ml or less. When tested together, the combinations of pinocembrin, galangin and chrysin showed MQSIC values of 0.16 mg/ml or less, and MBC values of 1.25 mg/ml or less. The combination of pinocembrin and galangin was the most promising, with a MQSIC value of 0.08 mg/ml and an MBC values of 0.31 mg/ml. Three out of four combinations (75%) demonstrated synergy through inhibiting QS, while the combination of pinocembrin and chrysin was additive. Results from the broth microdilution method corresponded with those of the macrodilution method qualitatively, although the quantitative results differed.

To assess the effects of pinocembrin, galangin and chrysin on biofilm prevention and disruption, the crystal violet (CV) assay was used. No clear pattern on antibiofilm activity was observed, although selected compounds and combinations were shown to be effective in inhibiting biofilms. Good biofilm inhibitory activity was noted for 18 out of 45 (40%) of the studies when the compounds were tested independently against the three micro-organisms at various timeframes. The best activity from a single compound was observed for chrysin against *Escherichia coli* at 24 hr (79.73% inhibition). When the compounds were tested in combination at a 1:1 ratio, only nine out of 60 (15%) combinations tested showed good inhibitory activity. However, the triple combination showed the best inhibitory activity of 100% when tested against *E. coli* at 24 hrs.

The toxicity of pinocembrin, galangin and chrysin was screened using the brine shrimp lethality assay (BSLA) after an exposure period of 24 hr. None of the compounds or combinations displayed

toxicity. When tested independently, the highest percentage mortality was noted from galangin, at 23.53%. When the compounds were tested in combination, the highest percentage mortality was observed for the combination of galangin and chrysin, at 6.17%. Three of the four combinations showed reduced toxicity.

This study has clearly demonstrated that the combination of the compounds pinocembrin, galangin and chrysin found in South African propolis synergistically enhances the antimicrobial activity of South African propolis on a number of levels whilst simultaneously further reducing the toxicity of the compounds. Furthermore, this study provides a convincing example of the need to examine compound interactions and to not always follow the reductionist approach of searching for a single active compound in natural product research.

DEDICATION

To my parents, Tahera and Ebrahim Kharsany, who have worked tirelessly to get me to where I am today. You have instilled in me the greatest values that any child could require: that of strength, perseverance and commitment. Thank you for your never-ending care and for the countless prayers and sacrifices that you have made for me.

Most of all, I have learned from you that nothing is possible without the mercy and grace of Allah s.w.t.

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LIST OF PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Presentations - Refer to Appendix A

Kharsany, K., Viljoen, A.M., Van Vuuren, S.F. The new buzz: proving synergy between propolis chemical compounds. The 2018 Indigenous Plant Use Forum (IPUF) conference – Oudtshoorn, South Africa, 1-4 July 2018. (Podium presentation).

Kharsany, K., Viljoen, A.M., Van Vuuren, S.F. Investigating the interactions between compounds found in South African propolis. School of Therapeutic Sciences Biennial Research Day – University of the Witwatersrand (Faculty of Health Sciences), Johannesburg, South Africa, 6 September 2018. (Poster presentation).

Kharsany, K., Van Vuuren, S.F., Viljoen, A.M. Exploring the role of synergy and additivity amongst the compounds found in South African propolis. Postgraduate Botany Symposium – University of Johannesburg (Department of Botany and Plant Biotechnology) Johannesburg, South Africa, 5 November 2018. (Podium presentation).

Publications - Refer to Appendix B

Kharsany, K., Viljoen, A.M., Leonard, C., Van Vuuren, S.F. (2019). The new buzz: Investigating the interactions between bioactive compounds found in South African propolis (Accepted in Journal of Ethnopharmacology, May 2019).

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LIST OF ABBREVIATIONS

%	Percent
°C	Degrees celsius
AHLs	N-acyl homoserine lactones
Anti-QS	Anti-quorum sensing
ATCC	American type culture collection
Ave	Average
BSLA	Brine shrimp lethality assay
CC	Culture control
CFU	Colony forming units
cidal	Bactericidal
CLSI	Clinical and Laboratory Standards Institute
conc	Concentration
DMSO	Dimethyl sulfoxide
EPS	Extra polysaccharide
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FBC	Fractional bactericidal concentration
FIC	Fractional inhibitory concentration
FPM	Fractional percentage mortality
FQSIC	Fractional quorum sensing inhibitory concentration
g	Grams
g/ml	Grams per millilitre
hrs	Hours
INT	*p*-iodonitrotetrazolium
l	Litre
LBA	Luria Bertani agar
LBB	Luria Bertani broth

MBC	Minimum bactericidal concentration
mg/ml	Milligrams per millilitre
MIC	Minimum inhibitory concentration
Min	Minute
ml	Millilitre
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCCLS	National Committee for Clinical Laboratory Standards
O.D	Optical density
pos	Positive
QQ	Quorum quenching
QS	Quorum sensing
QSI	Quorum sensing inhibitors
rpm	Revolutions per minute
SARs	Structure-activity relationships
spp.	Species
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
UPLC-MS	Ultra performance liquid chromatography-mass spectrometry
v/v	Volume per volume
vs.	Versus
WHO	World Health Organization
µg/ml	Micrograms per millilitre
µl	Microlitre
ΣFIC	Sum of the fractional inhibitory concentrations
ΣFBC	Sum of the fractional bactericidal concentrations
ΣFPM	Sum of the fractional percentage mortalities
ΣFQSIC	Sum of the fractional quorum sensing inhibitory concentrations

CHAPTER 1

General Introduction

1.1. Propolis and its medicinal uses

Propolis, otherwise known as bee glue due to its stickiness and pliability, is a resinous substance produced by the *Apis mellifera* bee (Marcucci, 1995; Kuropatnicki et al., 2013). Bees collect the original substance from buds and other parts of plants in the vicinity of the hive and then mix it with beeswax and β-glucosidase to form propolis (Lotfy, 2006; Probst et al., 2011). The mechanical strength that propolis imparts makes it useful in blocking holes and cracks in the hive or to repair or strengthen combs (Bankova et al., 2014). Bees use this substance as a building insulating material and as a defence to close off and protect the beehive from external invaders; hence the word "propolis" being formed from the words 'pro' and 'polis' meaning 'defence' and 'the city' respectively (Burdock, 1998; Bankova et al., 2014). Propolis is also used to preserve bee carcasses, and to embalm any other foreign objects that have entered the hive, thus preventing decomposition and the spread of infection (De Castro, 2001; Probst et al., 2011). Use of propolis has been documented back to early civilizations and has been shown to be used as early as the year 300 B.C (Burdock, 1998). Examples of civilizations who took advantage of the biological activities include the early Egyptians who used it as an embalming agent due to its anti-putrefaction properties. The Incas used propolis to reduce fever due to its antipyretic properties, and the Arabs used it as a mouth disinfectant (Suleman et al., 2015; Tiveron et al., 2016). Other historical medicinal uses include propolis as an antiseptic by Arab physicians in the middle ages, and as a treatment for sores and ulcers in ancient Egypt (Castaldo and Capasso, 2002; Suleman et al., 2015). It has also been reported that Hippocrates employed propolis for the treatment of sores and ulcers (De Castro, 2001). Use of propolis has since been widespread, extending to treatment of burns, wound healing, skin conditions such as psoriasis, neurodermatitis and pruritis ani, herpes, as an anaesthetic in dental procedures, in dental care for gingivitis, for stomatitis and cheilitis, and for

the treatment of rheumatic disorders and sprains (Burdock, 1998). It is found incorporated into many formulations such as lotions, creams, solutions, mouthwashes, throat lozenges, tablets, capsules, powder, toothpastes, shampoos, and even in chewing gum, candies and chocolate bars (Burdock, 1998; Castaldo and Capasso, 2002; Ramos and Miranda, 2007). Despite all of its uses, propolis has not been accepted into conventional medicine due to its varying chemical composition and lack of standardization (Silva-Carvalho et al., 2015).

1.2. Antimicrobial activity of propolis

The antimicrobial properties of propolis have been long recognized. A review by Elnakady et al. (2017) on the biological activities of propolis has reported that propolis possesses antibacterial, antifungal, cytostatic, wound healing, antitumor and anti-inflammatory properties. As it has been hypothesized that bees produce propolis to provide phytochemical protection against pathogens, it is possible to infer that it also possesses antimicrobial properties that may be medicinally useful (Siheri et al., 2016). A number of studies on the antimicrobial effects of propolis have shown that propolis is more effective against fungi and Gram-positive pathogens than against Gram-negative pathogens (Grange and Davey, 1990; Fernandes et al., 2001; Lotfy, 2006; Yaghoubi et al., 2007). To illustrate, a study by Grange and Davey (1990), showed that propolis completely inhibited the growth of Gram-positive pathogens *Staphylococcus aureus* (including the multiple resistant *Staphylococcus* (MRSA) strains), *Staphylococcus epidermidis*, *Enterococcus* spp., *Corynebacterium* spp., *Branhamella catarrhalis* and *Bacillus cereus*. The study demonstrated that propolis partially inhibited Gram-negative pathogens *Pseudomonas aeruginosa* and *Escherichia coli*, and had no effect on the Gram-negative pathogen *Klebsiella pneumoniae*. A study undertaken by Ugur and Arslan (2004) on the antibacterial properties of propolis samples from Turkey reported that the antimicrobial activity of propolis depends on a number of factors including the type of propolis sample, the dosage of propolis and the solvent selection for extraction. The same study ran a comparison between the antimicrobial effects of antibiotics and propolis samples against *Streptococcus mutans*, *Salmonella typhi*, *Shigella sonnei*, *Candida albicans* and *P. aeruginosa*, using the MIC method, and discovered that propolis samples displayed an equal or greater inhibitory effect than standard antibiotics on these organisms, thus supporting the use of propolis as an antimicrobial agent (Ugur and Arslan, 2004).

South African propolis became popular when it was employed by doctors during the Anglo-Boer war to disinfect and heal wounds (Lotfy, 2006). More recently, websites have promoted the use as a natural antibiotic (Serfontein, 2015). Very little research has been undertaken on the antimicrobial activities of propolis from South Africa. Suleman (2015) conducted a study on the antimicrobial activity of South African propolis and found that all six micro-organisms tested showed susceptibility to the 39 propolis samples tested (with MIC values ranging from 6 µg/ml to 6250 µg/ml), thus validating its use as an antimicrobial agent.

1.3. Chemical composition of propolis

The chemical composition of propolis is dependent on a number of factors including geographical region, botanical origin, the producing bee species and the season during which it was produced (De Castro, 2001; Huang et al., 2014). The chemical composition has been found to be complex, with over 300 chemical constituents identified (Huang et al., 2014). Propolis is characteristically made up of flavonoids, aromatic acids, diterpenoid acids and triterpenoids (Buchta et al., 2011; Elnakady et al., 2017). From these, the most common secondary metabolites found in propolis are the flavonoids, which are also found in many plants (Kosalec et al., 2003). The flavonoids found in propolis often represent the plants from which the bees collect the exudate to make the propolis (Kosalec et al., 2003). This varies according to geographical origin thus making it difficult to standardize propolis worldwide (Burdock, 1998). A number of predominant flavonoids have been commonly identified in propolis globally, as shown in Figure 1.1. Raw propolis is made up of 50% resin and vegetable balsam and 30% wax while the remaining 20% is made up of aromatic oils, essential oils, pollen, and organic debris (Burdock, 1998; Kosalec et al., 2003; Silva-Carvalho et al., 2015).

A study on the antimicrobial and chemical properties of South African propolis conducted by Suleman et al. (2015), found the presence of pinobanksin, quercetin, caffeic acid, and p-coumaric acid, with the major compounds identified as pinocembrin (5,7-dihydroxyflavanone), galangin (3,5,7-trihydroxyflavone) and chrysin (5,7-dihydroxyflavone). Burdock (1998) reported that the characteristic yellow tinge of comb wax is imparted to it by chrysin which, when extracted, appears

as a yellow powder. After studying the chemical composition of propolis, both Suleman et al. (2015) and Zhang et al. (2014) reported that South African propolis samples have a similar chemical profile to temperate region polar propolis. This is supported by Huang et al. (2014), who discovered that propolis from temperate regions are typically made up of flavonoids without the β-ring substituents, such as chrysin, galangin, pinocembrin and pinobanksin. These findings may explain why the three major compounds (namely, pinocembrin, galangin and chrysin) found in South African propolis are also commonly found in propolis samples from other countries (Figure 1.1) It should be noted that propolis from non-temperate regions differ vastly in chemical composition. Brazilian propolis, for example, was found to be rich in the compound artepillin C but does not contain any of the main compounds found in South African propolis (Suleman et al., 2015). This is not surprising as Bankova et al. (2014) have reported that temperate region propolis is often made from bud exudates sourced from poplar trees (of the Populus species), which are not found in tropical areas.

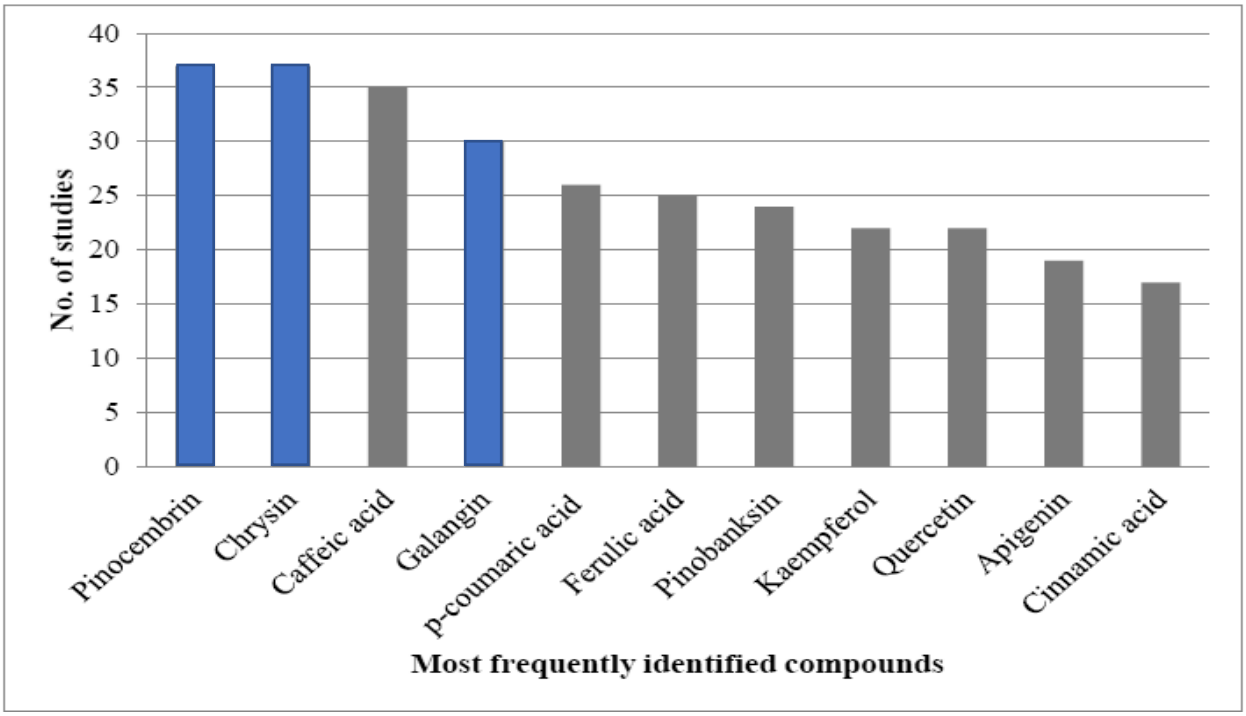


Figure 1.1: The compounds most frequently identified in propolis globally. Taken from Suleman (2016). The blue bars show the compounds predominant in South African propolis.

Propolis analyzed from areas in South America and China were found to consist of mainly acacetin and apigenin; South Bulgaria propolis contains mostly pinocembrin; and Canadian propolis also is made up mainly of pinocembrin, galangin and chrysin (Kosalec et al., 2003). High concentrations of pinocembrin, galangin and chrysin indicate that the propolis was collected from poplar plants (Kosalec et al., 2003). Illustrated in Figure 1.1 is a graph displaying the most frequently identified compounds found in propolis worldwide (Suleman, 2016).

1.3.1. Flavonoids

South African propolis is rich in flavonoids (Suleman et al., 2015). Flavonoids are secondary metabolites derived from plant sources (Cao et al., 2016). They are a large group of polyphenolic benzo-γ-pyrone derivatives compounds, found commonly in plants, fruits and vegetables (Heijnen et al., 2002; Liu et al., 2014). Flavonoids are responsible for imparting colours to fruits and flowers and are responsible for the growth and development of plants (Di Carlo et al., 1999; Bylka et al., 2004). These compounds are described as possessing numerous health benefits including positive effects in cardiovascular and pulmonary diseases, which has been attributed to their anti-oxidant properties (Heijnen et al., 2002). They have also been said to lower the risk of cancer, chronic inflammation, and other degenerative diseases (Narayana et al., 2001; Cao et al., 2016). Amongst the properties of flavonoids are the anti-inflammatory, anti-allergic, antiviral and antihepatotoxic activities (Narayana et al., 2001; Cao et al., 2016).

Flavonoids are low molecular weight compounds with C6-C3-C6 structures, in which the two C6 groups are substituted benzene rings, and the C3 is an aliphatic chain which contains a pyran ring (Bylka et al., 2004). Flavonoids can be further sub-categorized as flavones, flavanols, flavanones, chalcones, xanthones, isoflavones, and biflavones according to substitutions of the aromatic rings in its structure and according to the degree of benzo-γ-pyrone ring saturation, with flavones being the largest subclass (Di Carlo et al., 1999; Bylka et al., 2004; Liu et al., 2014). The effects of individual flavonoids are linked to their structure-activity relationships (SARs). For example, the anti-oxidant effects of flavonoids are attributed to its aromatic OH-group, with postulations that the greater the number of OH-groups in a flavonoid structure, the higher its potency (Heijnen et al., 2002).

1.3.1.1. Pinocembrin

Pinocembrin (5, 7-dihydroxyflavanone) is a natural flavonoid compound found in propolis (Lan et al., 2016). Pinocembrin is the highest contributor towards the total flavonoid content of South African propolis (Kasote et al., 2014; Suleman et al., 2015). It has the molecular formula $C_{15}H_{12}O_4$, with a molecular weight of 256.257 g/mol, and a 2D structure as shown in Figure 1.2 (National Center for Biotechnology Information, 2005a). It belongs to the flavanone class (Bylka et al., 2004). It was first extracted from the plant species *Eriodictyon californicum* (Hook. & Arn.) Torr., in 1992 and has since been found in several other plant species including *Eucalyptus globulus* Labill., *Mutisia ilicifolia* Cav., *Eucalyptus sieberi* L.A.S.Johnson, *Boesenbergia pandurate* Schltr, *Pinus banksiana* Lamb., *Pinus strobus* L., *Lippia graveolens* Kunth., *Piper hostmannianum* C.DC., *Dalbergia louvelii* R.Vig., *Sparattosperma leucanthum* K.Schum., and the *Populus* and *Euphorbia* genera (Napal et al., 2009; Rasul et al., 2013; Lan et al., 2016). When extracted, pinocembrin produces crystals with a slightly yellowish tinge (Lutskii et al., 1968). Zhang et al. (2016) have pointed out that since pinocembrin is a small molecular weight natural compound also found in honey, which is consumed by humans, its use should be considered safe. Pinocembrin can also be biosynthesized using a recombinant form of *E. coli* (Lan et al., 2016). Pinocembrin, as a therapeutic agent, was found to have antimicrobial, anti-inflammatory, anti-oxidant, antiviral, vasorelaxant, and anticancer activities (Napal et al., 2009; Rasul et al., 2013). Furthermore, it has been shown to exhibit good metabolism and absorption after oral ingestion (Lan et al., 2016). Pinocembrin has also been shown to have strong antifungal activity. In a study undertaken by Peng et al. (2012), in which they measured the inhibition of mycelial growth, it was shown that pinocembrin inhibited the fungus *Penicillium italicum* in a dose dependent manner. This was studied further by Yang et al. (2011) who reported that pinocembrin's activity against *P. italicum* is due to the presence of hydroxyl groups. The antifungal activity of pinocembrin is confirmed by Quiroga et al. (2006) who tested the MIC and hyphal inhibition of pinocembrin and found that it possesses activity against a wide range of fungi (*Aspergillus niger*, *Penicillium notatum, Saccharomyces carlsbergensis, Phomopsis* spp., *Fusarium* spp., *Trichoderma* spp., and *Rhodotorula* spp.) at values ranging from 20 to 50 µg/ml. A study by Drewes and van Vuuren (2008) tested the antibacterial effects of the dibenzyloxy derivative of pinocembrin using the MIC method and found it to have noteworthy activity against a number of pathogens including

Enterococcus faecalis, S. epidermidis, S. aureus, methicillin resistant *S. aureus* (MRSA), glycopeptide-resistant *S. aureus* (GRSA), *B. cereus, E. coli, K. pneumoniae* and *P. aeruginosa* at values less than 64 µg/ml. A study conducted by Ruddock et al. (2011) found that pinocembrin has activity against both susceptible and resistant forms of *Neisseria gonorrhoea* and is inhibitory to these strains at concentrations of 64 µg/ml or less, and thus has potential as a treatment for gonococcal infections.

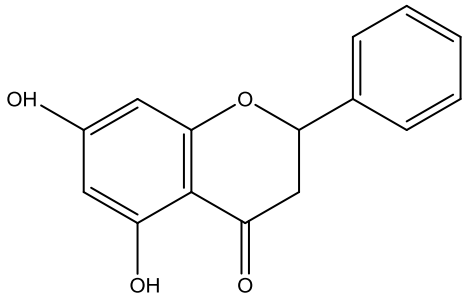


Figure 1.2: The chemical structure of pinocembrin.

1.3.1.2. Galangin

Galangin (3,5,7-trihydroxyflavone) is another major flavonoid found in propolis. It belongs to the flavanol subclass (Lu et al., 2011). It has a molecular formula of $C_{15}H_{10}O_5$, a molecular weight of 270.24 g/mol and its 2D structure is as illustrated in Figure 1.3 (National Center for Biotechnology Information, 2005b). It is also found in honey, fruits and vegetables (Nuttaporn, 2007). Galangin was first isolated and extracted from the root of *Alpinia officinarum* Hance in 1881 and has since been found in other members of the Zingiberaceae family such as *Alpinia nigra* (Gaertn) Burtt and *Alpinia galanga* (L.) Willd, and in the plant species *Helichrysum auronitens* Sch.Bip., *Plantago major* L., *Scutellaria galericulata* L, *Achyrocline satureioides*, and *Alnus pendula* Matsum (Ferraro et al., 1981; Afolayan and Meyer, 1997; Nuttaporn, 2007; Wen et al., 2012). Galangin has been found to have antimutagenic, anticlastogenic, anti-oxidative, radical scavenging and metabolic enzyme modulating activity, and has also been cited as a potential treatment for cancer, obesity, arthritis, and Alzheimer's disease (Guo et al., 2010; Lu et al., 2011; Patel et al., 2012; Goktas et al., 2014). *Alpinia officinarum* Hance, otherwise known as lesser galangal, is used as a spice and also as a traditional medicine for centuries, especially in China and neighbouring Asian

countries (Lu et al., 2011). Lu et al. (2011) have reported that the exceptional anti-oxidant effect of *A. officinarum* is due mainly to the constituent galangin. Like pinocembrin, galangin possesses antifungal activity against *A. niger*, *P. notatum*, *S. carlsbergensis*, *Phomopsis* spp., *Fusarium* spp., *Trichoderma* spp., and *Rhodotorula* spp. (Quiroga et al., 2006). Chabot et al. (1992), in their study on the activity of flavonoid compounds concluded that galangin has an inhibitory effect on fungal growth after noting that it reduced the total hyphal growth of germinating spores of *Gigaspora margarita* Becker and Hall by 2.8 times. Galangin also possesses strong antibacterial activity. A study conducted by Pepeljnjak and Kosalec (2004) determined that galangin has activity against several multi-resistant bacterial strains, and that the higher the content of galangin in propolis, the lower the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the sample. Interestingly, galangin has shown not only to have activity against resistant *Staphylococci* strains but has also been shown to reverse the resistance of these strains to conventional antibiotics such as, penicillin, ceftazimide, methicillin, cloxacillin, amoxicillin, ampicillin and cefotaxime when used in combination, thus indicating synergism between galangin and the antibiotics used (Eumkeb and Richards, 2003). Cushnie and Lamb (2005) found that galangin causes a reduction of 1000-fold or more in viable counts of *S. aureus*, thus indicating that it may have bactericidal activity. Nishino et al. (1987) tested the activity of twenty-six flavonoids against the skin condition causing pathogen *S. epidermidis* and found galangin to exhibit the strongest activity out of all the flavonoids, with an MIC value of 6.3 μg/ml. The authors also attempted to investigate the structure-activity relationships (SARs) of flavonoids and found that although it is impossible to attribute precise SARs to the flavonoids, a planar A/C ring in flavanols and the presence of O-H groups in flavonoids are essential for their antimicrobial activity (Nishino et al., 1987).

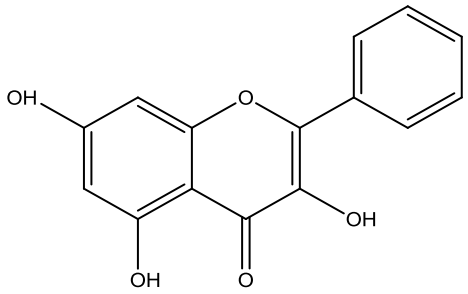


Figure 1.3: The chemical structure of galangin.

1.3.1.3. Chrysin

Chrysin (5,7-dihydroxyflavone), is another major flavonoid found in propolis and other plants such as the *Scutellaria* and *Pelargonium* genera, in the passionflower *Passiflora caerulea* L., in *Oroxylum indicum* Vent., *Potentilla evestita* Th.Wolf, *Desmos cochinchinensis* Lour., and from the root of *Scutellaria baicalensis* Georgi (Popova et al., 1976; Medina et al., 1990; Nabavi et al., 2015; Rauf et al., 2015). It has been found in high concentrations in the mushroom *Pleurotus ostreatus* (Jacq.) P. Kumm. and is also a constituent of carrots and honey (Anandhi et al., 2013; Park et al., 2018). *Oroxylum indicum* Vent. is an herb that has commonly been used in traditional medicine in the Orient and it has been stated that its activity may be due to chrysin (Anitha and Rajadurai, 2014). Chrysin has a molecular formula of $C_{15}H_{10}O_4$, a molecular weight of 254.241 g/mol and its 2D molecular structure is as shown in Figure 1.4 (National Center for Biotechnology Information, 2005c). Chrysin possesses a range of properties such as anticancer, antidiabetogenic, anti-estrogenic, antihypertensive, anxiolytic, anti-inflammatory, anti-oxidant, antiviral and antibacterial activity (Williams et al., 1997; Anitha and Rajadurai, 2014; Liu et al., 2014; Renuka et al., 2016). It has also been shown to possess potential clinical effects as an anti-aging agent, and for the treatment of depression and epilepsy (Nabavi et al., 2015). With regards to its structure-activity relationship, Anitha and Rajadurai (2014) have reported that the effects of chrysin are mainly due to the presence of hydroxyl and keto groups in its rings. Despite its many uses, research into chrysin has been limited due to its poor bioavailability (Chadha et al., 2017). However, research into formulations that will increase the bioavailability of chrysin is currently being undertaken therefore further research into this compound will be beneficial (Chadha et al., 2017). Chrysin is sub-classified as a flavone (Liu et al., 2014). Like pinocembrin and galangin it also has antibacterial activity, however, higher concentrations of chrysin are needed to produce an inhibitory effect**.** Mercan et al. (2006) point out that chrysin is capable of inhibiting Gram-negative bacteria at high concentrations. Babu et al. (2006) tested chrysin and its analogues against a number of pathogens and found that derivatives of chrysin possesses activity against *Bacillus subtilis, Bacillus sphaericus, Klebsiella aerogenes* and *S. aureus* (values ranging from 6.25 to 50 µg/ml) but has no activity against *P. aeruginosa* at 50 µg/ml. Information on the antifungal effects of chrysin is scanty although Chabot et al. (1992) have reported that chrysin significantly reduces

the percentage of spore germination in the fungus *Gigaspora margarita* Becker and Hall after discovery that hyphal elongation of this fungus was reduced by ten times when exposed to chrysin.

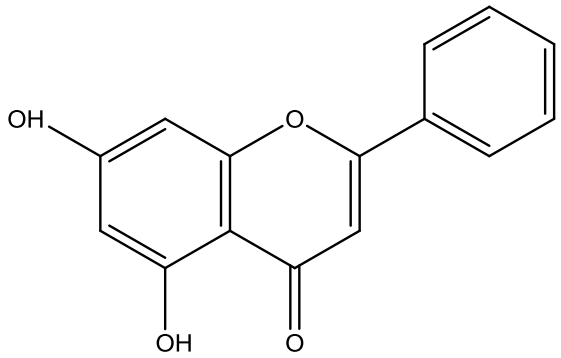


Figure 1.4: The chemical structure of chrysin.

1.4. Mechanism of action of propolis and its compounds

The mechanism of action of propolis is not completely understood, although a number of researchers have aimed to elucidate the manner in which propolis exerts its antimicrobial activity. Dziedzic et al. (2013) have reported that the mechanism of action is multidirectional, by working simultaneously in a number of ways. Possible mechanisms include disordering the cytoplasmic membrane and cell wall; bacteriolysis; and protein synthesis inhibition (Dziedzic et al., 2013). When Fokt et al. (2010) tested an ethanolic extract of propolis on *Streptococcus agalactiae*, they found that propolis worked through a number of mechanisms, which included the formation of pseudo-multicellular forms and cytoplasm disorganization, amongst others.

The antimicrobial properties of propolis have widely been attributed to its high flavonoid content, particularly that of pinocembrin and galangin (Cushnie and Lamb, 2005; Suleman et al., 2015). A few studies have been undertaken on the mechanisms of these compounds. A previous study has shown that pinocembrin works against *P. italicum* through inhibition of mycelial growth and disruption of the energy homeostasis and cell membrane disorganization (Peng et al., 2012). Rasul et al. (2013) have reported that pinocembrin exerts its effects through a metabolic engineering technique (i.e. it is able to identify and analyse the micro-organism's metabolic pathway in order to inhibit the micro-organism at a specific phase of development) which then causes cell lysis in

certain pathogens. It has been proposed that the mechanism of action of galangin is cell aggregation causing damage to the cytoplasmic membrane (Cushnie et al., 2007). Another study investigated the effects of galangin against *S. aureus* and found that this compound works by disrupting the cytoplasmic membrane, thus contributing to the loss of potassium ions from the cell (Echeverría et al. 2017). No clear mechanism of action has been discovered for the antimicrobial effects of chrysin (Liu et al., 2014).

1.4.1. Synergy between propolis compounds

It is not known specifically which flavonoids impart the antimicrobial activity of propolis, or if the mechanism of action is partially due to synergism between the components (Drago et al., 2007). Kosalec et al. (2003) have suggested that it is not the individual flavonoids that impart their antimicrobial effects to propolis, but rather synergy between the flavonoids. This conclusion was drawn after noting that propolis from both the Adriatic region and propolis from the continental regions displayed similar antimicrobial profiles despite containing differing concentrations of flavonoids.

The term additivity is used when the effects of a combination is equal to the sum of its components; while synergy (or super additivity) means that the effects of the combination is even greater than the sum of its components, or better than could be predicted (Ginsburg and Deharo, 2011; Tallarida, 2011). The efficacy of natural products often lies in the synergistic or additive interactions that occur between their components and it is thus difficult to predict that a single entity will have the same or better activities than that of the parent product (Ginsburg and Deharo, 2011). As a number of compounds are present in any natural product and thus it is of great benefit to test the interactions between the compounds to determine if synergy is apparent. Several useful outcomes could occur as a result of testing compounds for their combinatorial effects. Firstly, synergistic or additive interactions could be discovered which would enhance any antimicrobial effect of the individual compounds and validate the use of the tested combination. Secondly, antagonistic interactions could be ruled out. Thirdly, compounds are used in lower dose when they are combined, which - unless toxicity is also affected by synergism - reduces the potential of toxicity and adverse effects occurring. Lastly, testing and using compounds in combination

reduces the chances of resistance building up to the compounds (Eliopoulos and Eliopoulos, 1988; Tallarida, 2011; Ginsburg and Deharo, 2011). No previous studies have been found on the combinations of the major bioactive compounds found in South African propolis.

1.5. Aim and objectives

This study aims to investigate the antimicrobial effects of pinocembrin, galangin and chrysin, singularly and in various combinations against several different micro-organisms. This will be achieved by addressing the following objectives;

Objective one: To determine the antimicrobial efficacy of pinocembrin, galangin and chrysin using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) studies.

Objective two: To determine the antimicrobial interaction of pinocembrin, galangin and chrysin at 1:1 and at 1:1:1 concentrations (resulting in the calculation of the fractional inhibitory concentration (ΣFIC)) and at various concentration combinations (resulting in the formation of isobolograms).

Objective three: To determine the anti-quorum sensing activity of pinocembrin, galangin and chrysin and their combinations.

Objective four: To determine the antibiofilm activity of pinocembrin, galangin and chrysin and their combinations.

Objective five: To determine the toxicity of pinocembrin, galangin and chrysin and their combinations.

CHAPTER 2

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

2.1. Introduction

In vitro susceptibility tests are important in determining the effect an agent has on micro-organisms which are responsible for causing disease or infections and to detect resistance patterns (EUCAST, 2003). This aids treatment plans as it serves as a guide for clinicians in choosing the appropriate drug for a specific condition (EUCAST, 2003; Jorgensen and Ferraro, 2009). One of the most popular methods for *in vitro* susceptibility testing is the broth microdilution method, used to determine the minimum inhibitory concentration (MIC) of an agent (EUCAST, 2003; Jorgensen and Ferraro, 2009). This method allows for rapid screening of multiple samples (Fokt et al., 2010). The MIC of a substance or compound is defined as the lowest concentration of the substance that inhibits the growth of a micro-organism after incubation under defined test conditions (Andrews, 2001; Wiegand et al., 2008). The MBC of a substance is defined as the lowest concentration of that substance that will prevent the growth of an organism after sub-culture onto test free media and is complementary to the MIC (Andrews, 2001; Novais et al., 2017).

Propolis is widely known to be an antimicrobial agent and many studies on the MICs of propolis have been undertaken (Bosio et al., 2000; Fokt et al., 2010; Noori et al., 2012). A number of studies have shown that propolis displays particularly strong antimicrobial activity against Gram-positive bacteria and yeasts, and lesser activity against Gram-negative micro-organisms (Grange and

Davey, 1990; Stepanović et al., 2003; Vardar-Ünlü et al., 2008; Fokt et al., 2010). To illustrate, Stepanović et al. (2003) demonstrated that an ethanolic extract of propolis showed activity against Gram-positive bacteria at concentrations of between 0.078% and 1.25% while higher concentrations (1.25%–>5%) was needed to illicit inhibitory activity against Gram-negative bacteria. Similarly, Vardar-Ünlü et al. (2008) tested poplar propolis samples against a panel of micro-organisms and found that while the samples showed inhibitory activity against the Gram-positive bacteria at concentrations of 4 mg/ml or less, the same concentrations had no effect against their Gram-negative counterparts.

Flavonoids, commonly found in propolis, are also known to possess antibacterial properties, and are thus thought to be responsible for the antibacterial activity (Kujumgiev et al., 1999; Wagh, 2013; Afrouzan et al, 2018). This is illustrated by Darwish et al. (2010), who tested seven fractions of propolis against resistant and standard strains of *S. aureus* and *E. coli* and found that the fraction with the best antibacterial activity, also contained the most flavonoids. Supporting this theory is a study by Park et al. (1998) who tested the antibacterial activity of propolis samples from various areas in Brazil against *Streptococcus mutans* and discovered that the propolis sample with the highest concentration of pinocembrin and galangin, possessed the best anti-streptococcal activity. Very little previous research has been done on the antibacterial activity of isolated pinocembrin, galangin and chrysin, although two studies were found that proposed that the independent compounds possess better activity than does propolis itself (Quiroga et al., 2006; Buchta et al., 2011). A study undertaken by Quiroga et al. (2006), showed better antimicrobial activity for pinocembrin and galangin compared to purified propolis extracts. This is corroborated by Buchta et al. (2011), who found that the flavonoids extracted from propolis had a better antimicrobial activity than raw propolis samples.

On the other hand, several opposing studies have reported that the effects of propolis are not due to its components alone, but rather to interactions between them, particularly between the flavonoids or other phenolic compounds present (Kujumgiev et al., 1999; Drago et al., 2007; Boisard et al., 2015). A study undertaken by Kujumgiev et al. (1999) found that propolis from various geographical locations, with differing chemical compositions, displayed similar antibacterial profiles. The authors thus deduced that the activity of propolis is not ascribed to a

single compound, but rather to the combination of its components (Kujumgiev et al., 1999). However, no previous antimicrobial studies were found on the combination of propolis compounds.

Furthermore, it has not been clearly elucidated whether the compounds found in propolis are bacteriostatic or bactericidal as most studies researching these compounds have only used the MIC method as a means of determining antimicrobial activity. The lack of data in this field clearly indicated that further research needed to be done on the antimicrobial effects of the major compounds found in South African propolis and their combinations. The objective of this chapter was to investigate the antimicrobial properties of pinocembrin, galangin and chrysin. This was done by determining the antimicrobial activities of the compounds alone; by testing the antimicrobial activities of two-compound combinations at equal and varied ratios, and by testing the combination of all three compounds at an equal ratio.

2.2. Materials and methods

2.2.1. Preparation of compounds

The compounds pinocembrin, galangin and chrysin were obtained from Sigma-Aldrich. Pinocembrin appears as a white-yellow powder, lot number 20170315, with a purity standard of ≥ 98.0%; whilst chrysin, lot number STBF6934V, had a purity standard of 97% and appeared as a yellow powder. Galangin, lot number MKCC3398, had a purity percentage of ≥95% and was also identified as a yellow powder. Compounds were dissolved in acetone (Merck) to make up to a starting concentration of 5 mg/ml using Equation 2.1 and kept in small amber bottles in the refrigerator.

Equation 2.1: Volume of acetone to add to make up a compound concentration of 5 mg/ml.

$$\text{Volume of acetone to add (µl)} = \frac{\text{Weight of compound (mg) x 1000}}{\text{5 mg/ml}}$$

The purity of the compounds was also tested independently to ascertain if pinocembrin, galangin and chrysin contained traces of other substances, before any further assays were undertaken. Purity

testing of pinocembrin, galangin and chrysin was done using ultra performance liquid chromatography-mass spectrometry (UPLC-MS), which allows for fast and sensitive analyses of compounds (Taleuzzaman et al. 2015). The analysis was performed on a Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA). Separation was achieved on an Acquity UPLC BEH C18 column (150 mm × 2.1 mm, i.d., 1.7 μm particle size, Waters) maintained at 35 °C. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.45 mL/min. The gradient elution was executed as follow: 90% A: 10% B changed to 60% A: 40% B in 2.5 min, changed to 55% A: 45% B in 8.5 min, changed to 20% A: 80% B in 1.5 min, keeping for 0.5 min and back to initial ratio in 0.5 min, equilibrium the system for 1.5 min. The total running time was 15 min and the injection volume was 1.0 μL (full-loop injection).

The same column, elution gradient and flow rate were used for the UPLC-MS analysis. The mass spectrometry was operating in a negative ion electrospray mode and nitrogen (N2) was used as the desolvation gas. The following parameters were then set: capillary voltage was 3000 V; sampling cone voltage was 40 V; extraction cone was 4 V; source temperature was 100°C; desolvation temperature was 500°C; desolvation gas flow was 600 L/h. Chromatographic software Masslynx 4.1 was used to process and obtain all the chromatographic data. Data, collected over the range m/z 100 to 1200, were centroided during acquisition using independent reference lock-mass ions via the LockSprayTM interface to ensure mass accuracy and reproducibility.

2.2.2. Preparation of cultures

The Clinical Laboratory Standards Institute (CLSI, 2003), formerly known as the National Committee for Clinical Laboratory Standards (NCCLS) guidelines were adhered to in the preparation of the micro-organisms. Nine American Type Culture Collection (ATCC) strains were prepared for use in this study. These were based on the causes of infections that propolis is generally used to treat, as well as taking cognizance of the pathogens used in a propolis study by Suleman et al. (2015). The pathogens included three Gram-positive micro-organisms; *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212) and *Listeria monocytogenes* (ATCC 19111), three Gram-negative micro-organisms; *Pseudomonas aeruginosa*

(ATCC 27858), *Escherichia coli* (ATCC 25922), and *Klebsiella pneumoniae* (ATCC 13883); and three yeast strains; *Candida albicans* (ATCC 10231), *Cryptococcus neoformans* (ATCC 14116) and *Candida tropicalis* (ATCC 750). A waiver for the use of these micro-organisms was obtained from the University of the Witwatersrand Human Research Ethics Committee (reference number W-CBP-180228-01, see Appendix C).

Bacterial cultures were grown in Tryptone Soya broth (TSB) (Oxoid) at 37°C for 24 hours for bacterial strains and 37°C for 48 hours for yeast strains. Micro-organisms were kept viable by sub-culturing every two weeks. Cultures were streaked out onto Tryptone Soya agar (TSA) plates and incubated under suitable conditions in order to confirm purity of cultures.

2.2.3. Antimicrobial assays

2.2.3.1. Minimum inhibitory concentration assay (MIC) assay

MIC assays were performed as described by Eloff (1998). Compounds were dissolved in acetone to a starting concentration of 5 mg/ml. A volume of 100 µl of sterile TSB was placed into each well of a 98 well micro-titre plate. The compounds were placed into the top row of the micro-titre plate in volumes of 100 µl after which serial doubling dilutions were performed to subsequently achieve compound concentrations of 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02 and 0.01 mg/ml. A 0.5 McFarland's standard was prepared by diluting overnight growth of micro-organism into TSB at 1:100 dilutions and was thereafter added to all the wells of the micro-titre plates. Plates were sealed and incubated at 37°C for 24 hrs for bacterial species and at 37°C for 48 hrs for yeast species. After the plates were incubated, 40 µl of a 0.4% *p*-Iodonitrotetrazolium (INT) violet indicator solution was added to the inoculated wells. A change in colour of the wells from clear to pink was used as an indication of the presence of microbial growth. Plates were analysed, and MIC values interpreted as the lowest concentration at which growth was inhibited, visually noted as the first clear well (van Vuuren et al., 2010). All samples were tested in triplicate to ensure accuracy. An illustration of the microdilution MIC assay used is depicted in Figure 2.1.

2.2.3.2. Negative, positive, culture and comparative controls

In order to ensure that it was not the solvent itself that was responsible for the antimicrobial effects noted, acetone was prepared at 5 mg/ml and used as the negative control in this study. To ascertain antimicrobial susceptibility of the cultures used, ciprofloxacin and amphotericin B (both obtained from Sigma-Aldrich) were used as the positive controls for bacteria and yeasts, respectively. Ciprofloxacin was prepared using sterile water as a vehicle to make up to stock concentrations of 0.01 mg/ml, whilst amphotericin B was first dissolved in a small amount of dimethyl sulfoxide (DMSO) and then made up to 0.1 mg/ml with sterile water.

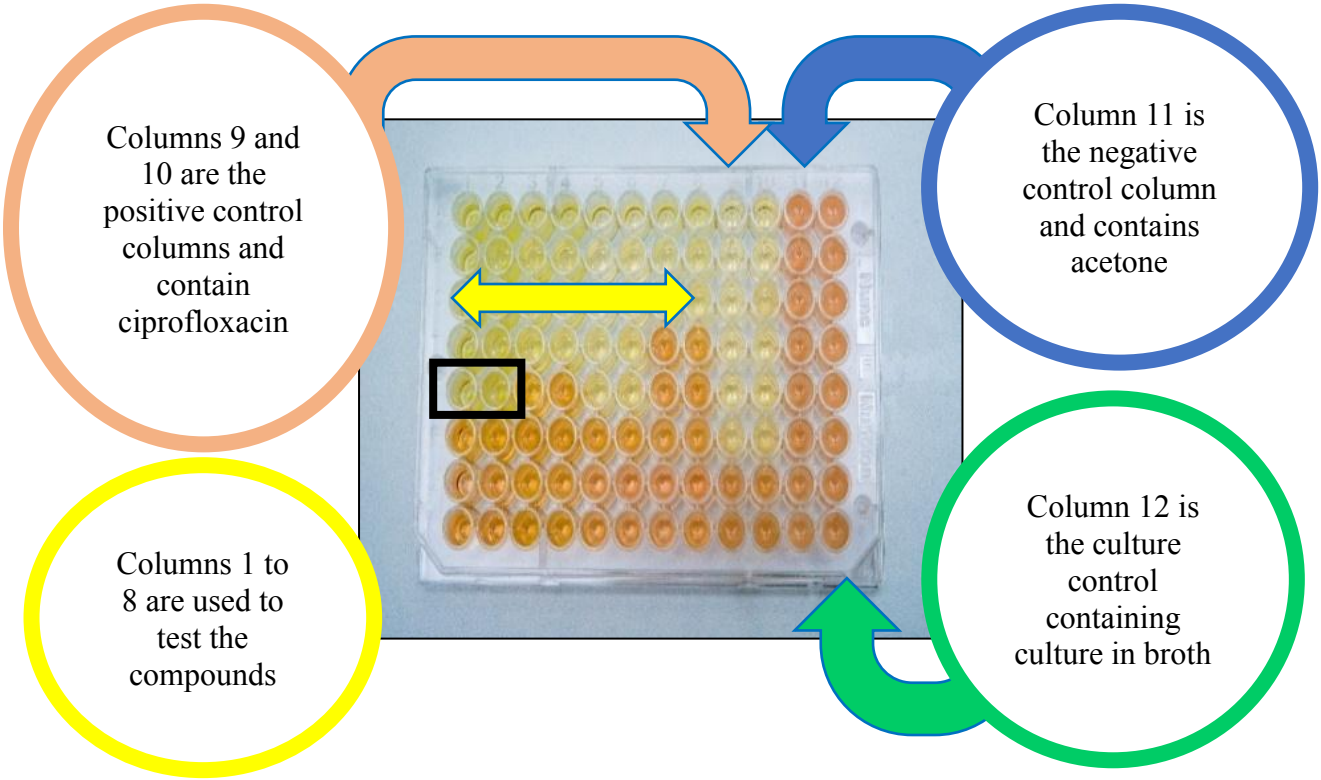


Figure 2.1: Minimum inhibitory concentration (MIC) microdilution assay.

The presence of a pink colour indicates microbial growth. The boxed off area represents the MIC of the compound tested in the first and second columns.

In order to ensure that the broth used was capable of supporting microbial growth, a culture control was included using 100 µl of TSB and 100 µl of the relevant micro-organism. A crude propolis sample, sourced from the North West province in South Africa, was used as the comparative control in this study. This was the same sample used in the study by Suleman et al. (2015) wherein it was demonstrated that propolis from the North West displayed noteworthy antimicrobial activity against a number of micro-organisms and was prepared as follows: 3 g of macerated propolis was weighed and submerged in 10 ml of absolute ethanol (MK Labs), then placed in an orbital shaker incubator (Labcon) at 37°C for seven days. Following this process, ethanol was siphoned off and the extract was left to dry at ambient room temperature. When ready, the extracts were dissolved in acetone to make up stock concentrations of 25 mg/ml which were then used to test antimicrobial activity. Both the stock solutions and the raw extracts were stored in the refrigerator away from light.

The propolis sample that was used was quantified for its alkaloid content, to ensure that it contained pinocembrin, galangin and chrysin and could thus be used as a control. For the sample collection, 2 mg of the propolis sample was weighed. Next, 2 mL methanol was added, sonicated for 10 min and filtrated through a 0.2 µm syringe filter. The filtrate was collected for UPLC-MS injection. The UPLC-MS analyses was done as described below. The experiment was performed on a Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA). Separation was achieved on an Acquity UPLC BEH C_{18} column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 35 °C. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.35 mL/min. The gradient elution was executed as follow: 90% A: 10% B changed to 60% A: 40% B in 2.5 min, changed to 55% A: 45% B in 8.5 min, changed to 20% A: 80% B in 1.5 min, keeping for 0.5 min and back to initial ratio in 0.5 min, equilibrium the system for 1.5 min. The total running time was 15 min and the injection volume was 1.0 µL (full-loop injection). The quantitative aspect of the method was validated by determining the linearity, recovery, and limit of detection (LOD) and the limit of quantification (LOQ). The same column, elution gradient and flow rate were used for the UPLC-MS analysis. The mass spectrometry was operating in a negative ion electrospray mode and nitrogen (N2) was used as the desolvation gas. The following parameters were then set: capillary voltage was 3000 V; sampling cone voltage was 40 V; extraction cone was 4 V; source

temperature was 100°C; desolvation temperature was 500 °C; desolvation gas flow was 600 L/h. Chromatographic software Masslynx 4.1 was used to process and obtain all the chromatographic data. Data, collected over the range *m/z* 100 to 1200, were centroided during acquisition using independent reference lock-mass ions via the LockSprayTM interface to ensure mass accuracy and reproducibility.

2.2.3.3. Minimum bactericidal concentration assay (MBC) assay

After MIC assays were conducted, an MBC assay was undertaken by obtaining a loop-full of the sample/culture mix from the micro-titre plate wells indicating inhibition and subsequently streaking onto a TSA plate. This was then incubated appropriately according to the micro-organisms inoculated (37°C for 24 hours for bacteria and 37°C for 48 hours for yeasts). Bactericidal activity was indicated by the complete absence of growth on the agar plate. An illustration of the MBC assay is depicted in Figure 2.2.

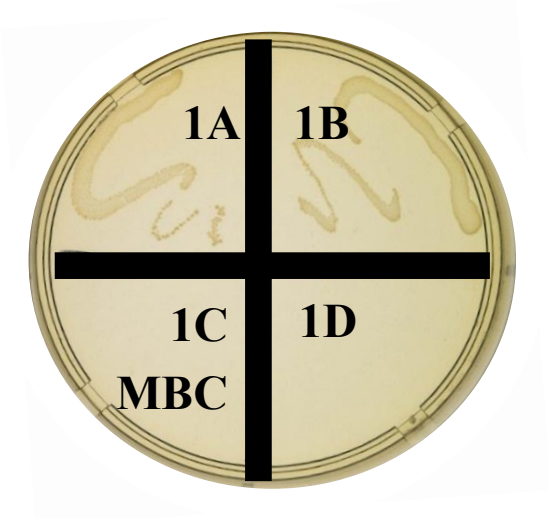


Figure 2.2: Minimum bactericidal concentration (MBC) assay.

Sections 1A–D represents decreasing concentrations of the compound concentrations inoculated from the MIC micro-titre plate. 1A = 1.25 mg/ml, 1B = 0.63/ml, 1C = 0.31 mg/ml, 1D = 0.16 mg/ml. The MBC = 0.31 mg/ml.

2.2.4. Interactive efficacy studies

2.2.4.1. Combination studies

Pinocembrin, galangin and chrysin were investigated for their interactive efficacies. These substances were introduced at starting concentrations of 5 mg/ml. A volume of 100 µl of sterile TSB was placed into each well of the 96 well micro-titre plates. A 1:1 or a 1:1:1 ratio of the compounds was then introduced into the first row of the micro-titre plate and the serial doubling dilution technique was employed. The interactions between the compounds were then analysed using the fractional inhibitory concentration (FIC) and the FIC index (ΣFIC), calculated as per Equation 2.2;

Equation 2.2: Fractional inhibitory concentration (FIC) calculations

$$FIC^{(i)} = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}} \qquad FIC^{(ii)} = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

Where (a) is the MIC of the one compound in the combination and (b) is the MIC of the other compound used in the combination. The sum of the FIC, known as the FIC index, is thus calculated as: $\Sigma FIC = FIC^{(i)} + FIC^{(ii)}$.

The ΣFIC values for each combination can be interpreted as synergistic ($\Sigma FIC \leq 0.5$), additive (ΣFIC values $> 0.5 – 1.0$), non-interactive (ΣFIC values $> 1.0 \leq 4.0$), or antagonistic (ΣFIC values > 4.0) (van Vuuren and Viljoen, 2011). When synergy was observed in the 1:1 ΣFIC analysis, further in-depth assessments of the respective combinations was undertaken. The compound combinations were tested in different ratios i.e. (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) to determine at which concentration the optimal antimicrobial interaction took place. The MIC values were plotted onto an isobologram using Graph Pad Prism® software (Version 5), which takes into account ratio values based on the MIC result and compound concentration. This allowed for a figurative representation of the interactions of the various ratios to be depicted. Generated isobolograms were interpreted as follows: all points that fell under or aligned on the 0.5:0.5 line were considered as synergistic. This is represented by the purple chequered quadrant in Figure 2.3, as per van Vuuren and Viljoen (2011). Points falling into the red lined quadrant, which depicts the

area between the 0.5:0.5 line and the 1.0:1.0 line, were interpreted as additive while those that fell into the green lined quadrant, which plots the area between the 1.0:1.0 line and the 4.0:4.0 line, as non-interactive. Antagonistic interactions were interpreted as those that were plotted above the 4.0:4.0 line, in the dotted blue quadrant (van Vuuren and Viljoen, 2011, de Rapper, 2014). All combination studies were done in duplicate or in triplicate to ensure accuracy and reproducibility of results.

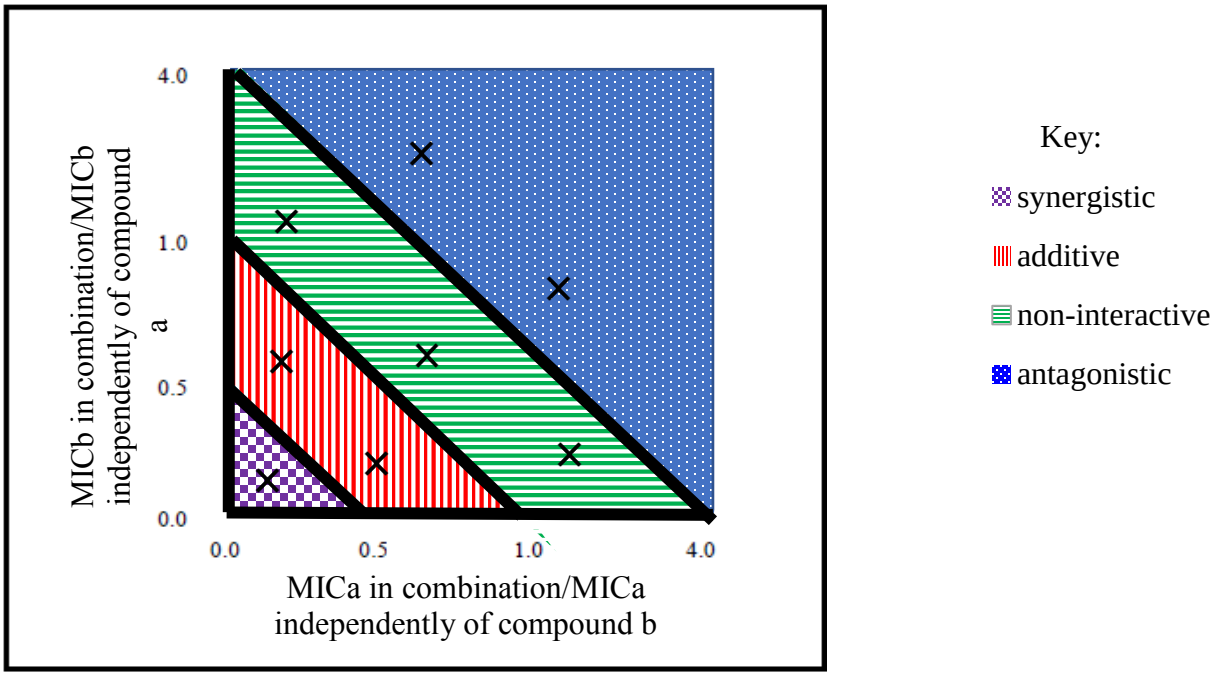


Figure 2.3: Interpretation of isobolograms.

In this diagram, 'a' represents compound one and 'b' represents compound two (van Vuuren and Viljoen, 2011; de Rapper, 2014).

2.3. Results

2.3.1. Quantification of alkaloid content in propolis sample.

The linearity, recovery, and limit of detection (LOD) and the limit of quantification (LOQ) was validated. The results are tabulated in Table 2.1. below.

Table 2.1: Validation of quantified results obtained from assaying propolis for its alkaloid content.

Compound	Linear range (ug/mL)	Regression equation	r^2	LOD (ug/mL)	LOQ (ug/mL)	Recovery (100%)
Chrysin	1.0-100	Y=24744X+4313	0.999	0.149	0.452	104.8
Pinocembrin	1.0-100	Y=15316X+4031	0.998	0.311	0.944	101.9
Galangin	1.0-100	Y=21614X-15469	0.999	0.298	0.902	97.9

The final results showed that each gram of propolis contained 223.6 mg of pinocembrin; 63.3 mg of galangin; and 12,3 mg of chrysin. As this sample contained all three of the compounds focused on in this dissertation, it is used hereafter as the comparative control.

2.3.2. Purity of compounds

The purity analysis of the pinocembrin, galangin and chrysin standards are shown in Figure 2.4.

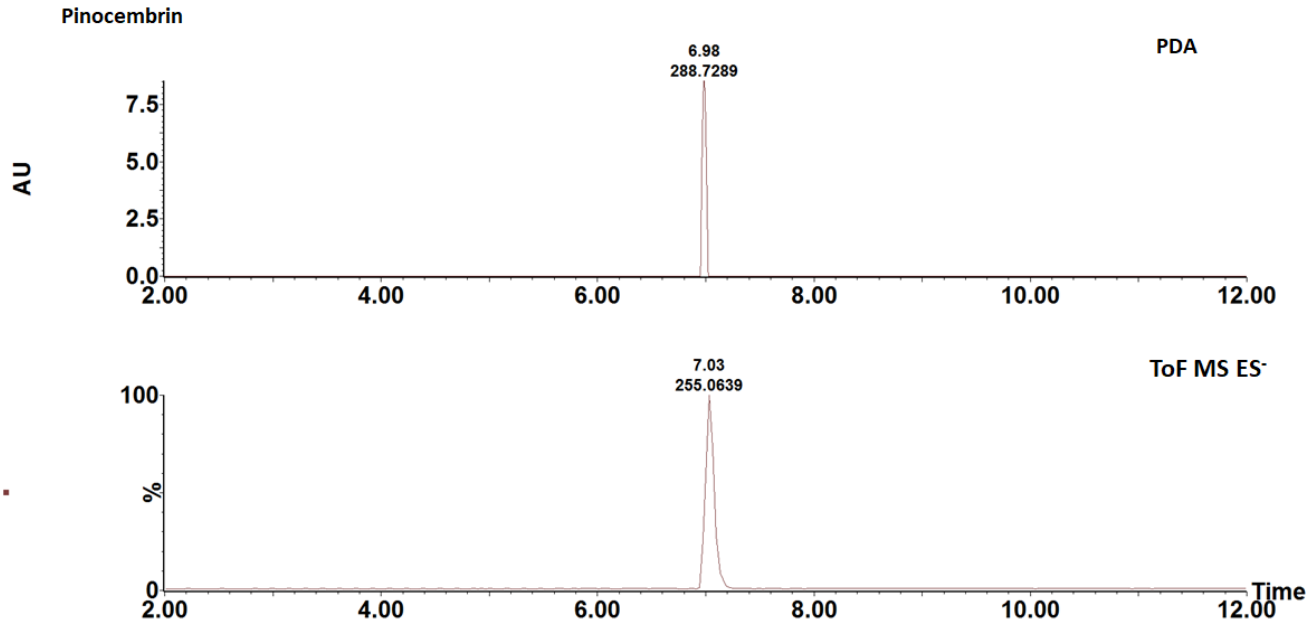


Figure 2.4A

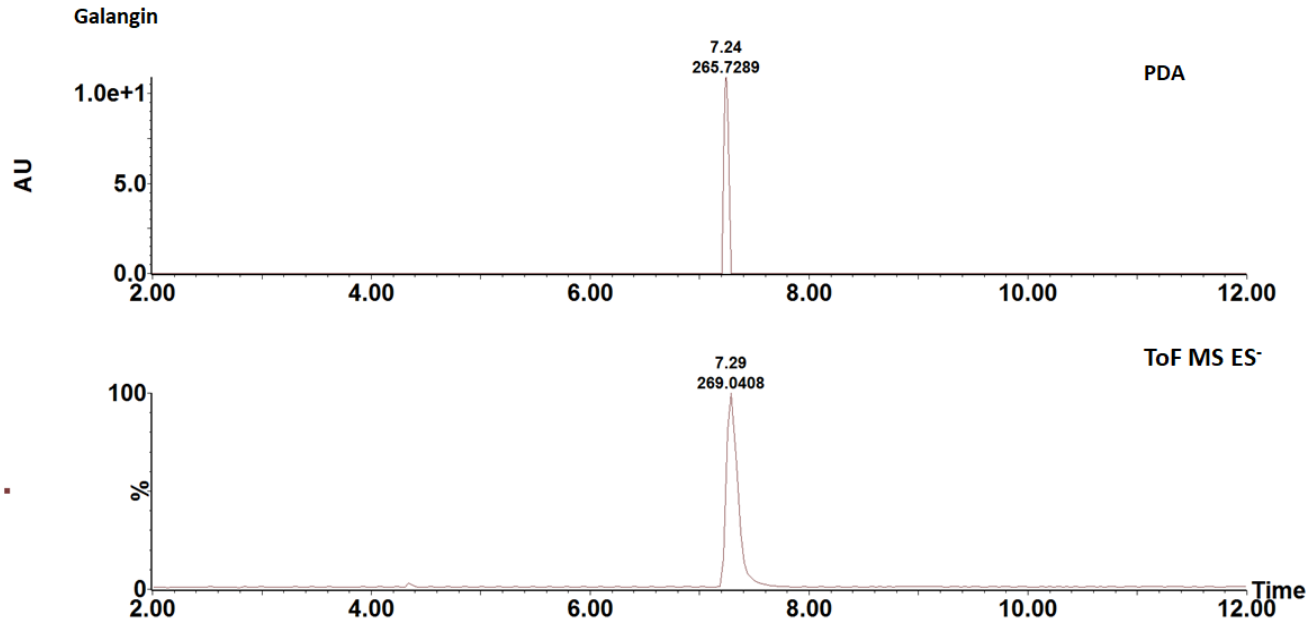


Figure 2.4B

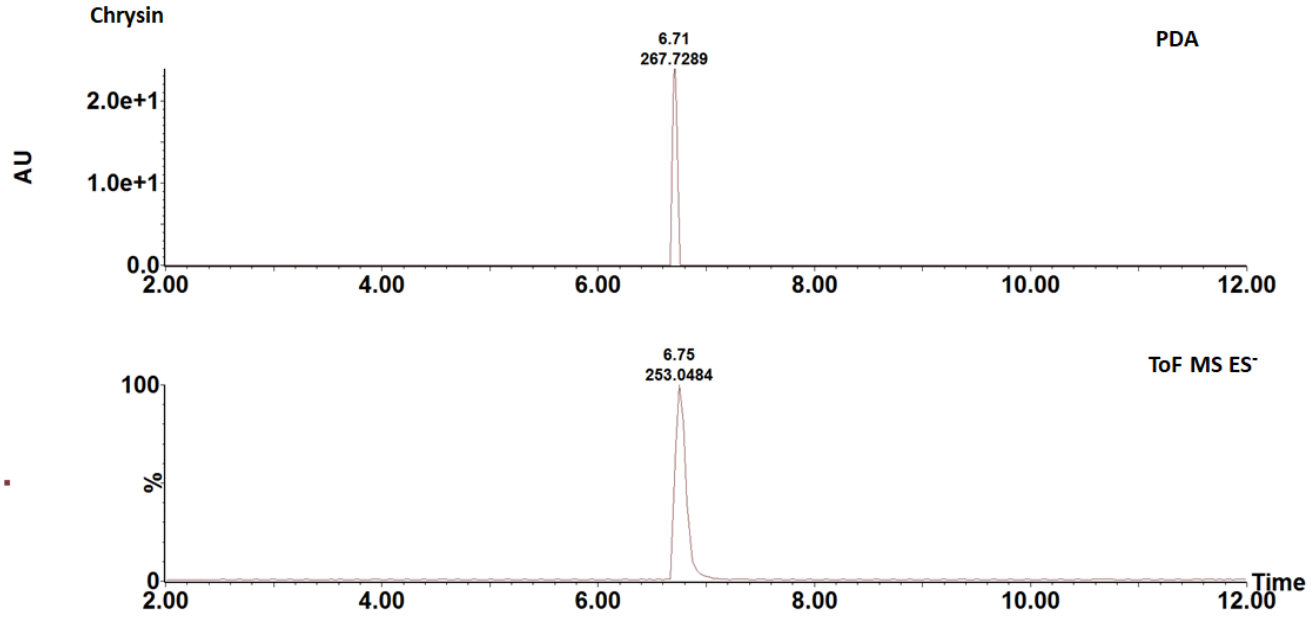


Figure 2.4C

Figure 2.4: UPLC-PDA (upper) and UPLC-MS (lower) chromatograms of the standards pinocembrin (2.4A) , galangin (2.4B) and chrysin (2.4C).

2.3.3. MIC and MBC determination of the independent compounds

Results from the MIC assay of the compounds pinocembrin, galangin and chrysin tested against Gram-positive and Gram-negative are reported in Table 2.2. Against the three Gram-positive bacteria that were tested, MIC values of the compounds ranged from 0.16 mg/ml (pinocembrin against *L. monocytogenes*) up to 0.63 mg/ml (chrysin against *S. aureus*). Against the three Gram-negative bacteria that were tested, MIC values of the compounds ranged from 0.16 mg/ml (pinocembrin against *E. coli* and *P. aeruginosa)* up to 1.00 mg/ml (chrysin against *E. coli*). From the three compounds, pinocembrin showed the best overall antibacterial activity (average MIC = 0.23 mg/ml), followed by galangin (average MIC = 0.42 mg/ml), and finally chrysin (average MIC = 0.54 mg/ml). Concerning the activity of the compounds against Gram-positive versus Gram-negative bacteria, the following results were found; pinocembrin showed similar activity against both classes (average MIC against Gram-positive bacteria = 0.24 mg/ml; against Gram-negative bacteria = 0.21 mg/ml). However, both galangin and chrysin demonstrated stronger activity against Gram-positive bacteria than against Gram-negative bacteria (galangin: 0.31 mg/ml against Gram-positive bacteria and 0.52 mg/ml against Gram-negative bacteria; chrysin: 0.42 mg/ml against Gram-positive bacteria and 0.65 mg/ml against Gram-negative bacteria). From the Gram-positive bacterial species, the strain of *L. monocytogenes* displayed the best susceptibility to the compounds (average MIC against *L. monocytogenes* = 0.26 mg/ml), whilst from the Gram-negative bacterial species, *K. pneumoniae* was the most susceptible (average MIC = 0.31 mg/ml). None of the compounds displayed bactericidal activity when tested independently.

Results from the MIC assay of the compounds pinocembrin, galangin and chrysin tested against yeasts are reported in Table 2.3. For the yeast strains tested, values ranged from 0.04 mg/ml (pinocembrin and chrysin against *C. neoformans*) up to 1.25 mg/ml (galangin against *C. tropicalis*). From the three compounds, pinocembrin showed the best antifungal activity (average MIC = 0.15 mg/ml), followed by chrysin (average MIC = 0.43 mg/ml), and finally galangin (average MIC = 0.65 mg/ml). *Cryptococcus neoformans* was the most susceptible yeast, with an average MIC of 0.05 mg/ml. None of the compounds had fungicidal activity on their own at the concentrations tested.

Propolis from the North West province of South Africa was used as a comparative control. Results are shown in Tables 2.1 and 2.2. The MIC values of the propolis samples tested varied from 0.10 mg/ml (both samples against *L. monocytogenes*) up to 0.78 mg/ml (propolis 1 against *S. aureus* and *P. aeruginosa*; propolis 1 and 2 against *K. pneumoniae*). From the propolis extracts tested, the average activity was 0.38 mg/ml for the Gram-positive bacteria; 0.59 mg/ml for the Gram-negative bacteria; and 0.27 mg/ml for the yeasts. The most susceptible pathogens from each class was *L. monocytogenes* (average MIC = 0.10 mg/ml), *E. coli* (average MIC = 0.39 mg/ml), and *C. neoformans* (average MIC = 0.20 mg/ml).

2.3.4. Interactive efficacy results

2.3.4.1 Equal ratio combinations (1:1)

Interactive efficacy studies were conducted on the combinations of the compounds, the results of which are shown in Table 2.4. Overall, from the 27 combinations tested, six combinations (22%) showed synergy, nine combinations (33%) showed additivity and 13 combinations (48%) were non-interactive (Figure 2.5). No combination was antagonistic. The combination of galangin and chrysin against *C. tropicalis* was especially interesting as the MIC value of the combination (0.13 mg/ml) was much lower than the compounds (galangin = 1.25 mg/ml and chrysin = 0.62 mg/ml) when tested individually. This combination demonstrated the lowest ΣFIC (0.16), and thus the strongest synergistic activity. It also showed the lowest MBC value of 0.16 mg/ml. The highest number of synergistic interactions were observed for the yeasts (three synergistic interactions), followed by the Gram-positive bacteria (two synergistic interactions) and then the Gram-negative bacteria (one synergistic interaction). All six of the combinations showing synergy, included galangin as one of the two compounds in the combination. The combination of galangin and pinocembrin displayed three synergistic interactions as did the combination of galangin and chrysin. The latter combination also showed three additive interactions. *Listeria monocytogenes* was the pathogen most susceptible to the compound combinations, with two sets of combinations against it synergistic and one set additive. Although no bactericidal activity was noted for any of the individual compounds, eight combinations showed cidal activity. *Candida albicans* proved to be the most susceptible pathogen to the cidal effects of the combinations. All three combinations showed MBC values of 1.25 mg/ml or less against this pathogen.

Table 2.2: Antimicrobial activity (mg/ml) of pinocembrin, galangin and chrysin against nine strains of bacteria tested.

Compound	Gram-positive bacteria			Ave MIC Gram-positive bacteria	Gram-negative bacteria			Ave MIC Gram-negative bacteria
	S. aureus (ATCC 25923)	*E. faecalis* (ATC 29212)	*L. monocytogenes* (ATCC 19111)		*E. coli* (ATCC 25922)	*P. aeruginosa* (ATCC 27853)	*K. pneumoniae* (ATCC 13883)	
Pinocembrin	0.26	0.31	0.16	0.24	0.16	0.16	0.31	0.21
Galangin	0.31	0.31	0.31	0.31	0.63	0.63	0.31	0.52
Chrysin	0.63	0.31	0.31	0.42	1.00	0.63	0.31	0.65
Propolis	0.39	0.39	0.10	0.29	0.39	0.39	0.78	0.52
Pos control*	$0.31x10^{-3}$	$0.52x10^{-3}$	$0.21x10^{-3}$		$0.21x10^{-3}$	$0.31x10^{-3}$	$0.16x10^{-3}$	

*Ciprofloxacin

Table 2.3: Antimicrobial activity (mg/ml) of pinocembrin, galangin and chrysin against three yeast strains.

Compound	Yeasts			Ave MIC against yeasts
	C. albicans (ATCC 10231)	*C. tropicalis* (ATCC 750)	*C. neoformans* (ATCC 14116)	
Pinocembrin	0.32	0.08	0.04	0.15
Galangin	0.63	1.25	0.07	0.65
Chrysin	0.63	0.62	0.04	0.43
Propolis	0.20	0.20	0.20	0.20
Pos control*	$0.63x10^{-2}$	$0.31x10^{-2}$	$0.16x10^{-2}$	$0.36x10^{-2}$

*****Amphotericin B

Table 2.4: Interactive efficacy of pinocembrin, galangin and chrysin against nine micro-organisms.

Micro-organism	Pinocembrin - Galangin				Pinocembrin - Chrysin				Galangin - Chrysin			
	MIC	**MBC**	**ΣFIC**	**Interpretation**	**MIC**	**MBC**	**ΣFIC**	**Interpretation**	**MIC**	**MBC**	**ΣFIC**	**Interpretation**
Gram-positive bacteria												
S. aureus (ATCC 25923)	0.31	–	1.10	Non-interactive	0.21	–	0.57	Additive	0.31	–	0.75	Additive
E. faecalis (ATCC 29212)	0.31	–	1.00	Non-interactive	0.31	–	1.00	Non-interactive	0.25	–	0.81	Additive
L. monocytogenes (ATCC 19111)	0.04	1.25	0.19	**Synergistic**	0.16	0.63	0.76	Additive	0.08	–	0.26	**Synergistic**
Gram-negative bacteria												
E. coli (ATCC 25922)	0.16	–	0.63	Additive	0.31	–	1.12	Non-interactive	0.50	–	0.65	Additive
P. aeruginosa (ATCC 27853)	0.13	–	0.52	Additive	0.21	1.25	0.82	Additive	0.31	–	0.49	**Synergistic**
K. pneumoniae (ATCC 13883)	0.31	–	1.00	Non-interactive	0.31	–	1.00	Non-interactive	0.31	–	1.00	Non-interactive
Yeasts												
C. albicans (ATCC 10231)	0.31	0.31	0.74	Additive	0.52	0.63	1.24	Non-interactive	0.63	1.25	1.00	Non-interactive
C. tropicalis (ATCC 750)	0.07	0.63	0.44	**Synergistic**	0.16	–	1.13	Non-interactive	0.13	0.16	0.16	**Synergistic**
C. neoformans (ATCC 14116)	0.02	–	0.40	**Synergistic**	0.04	–	1.00	Non-interactive	0.04	–	1.00	Non-interactive

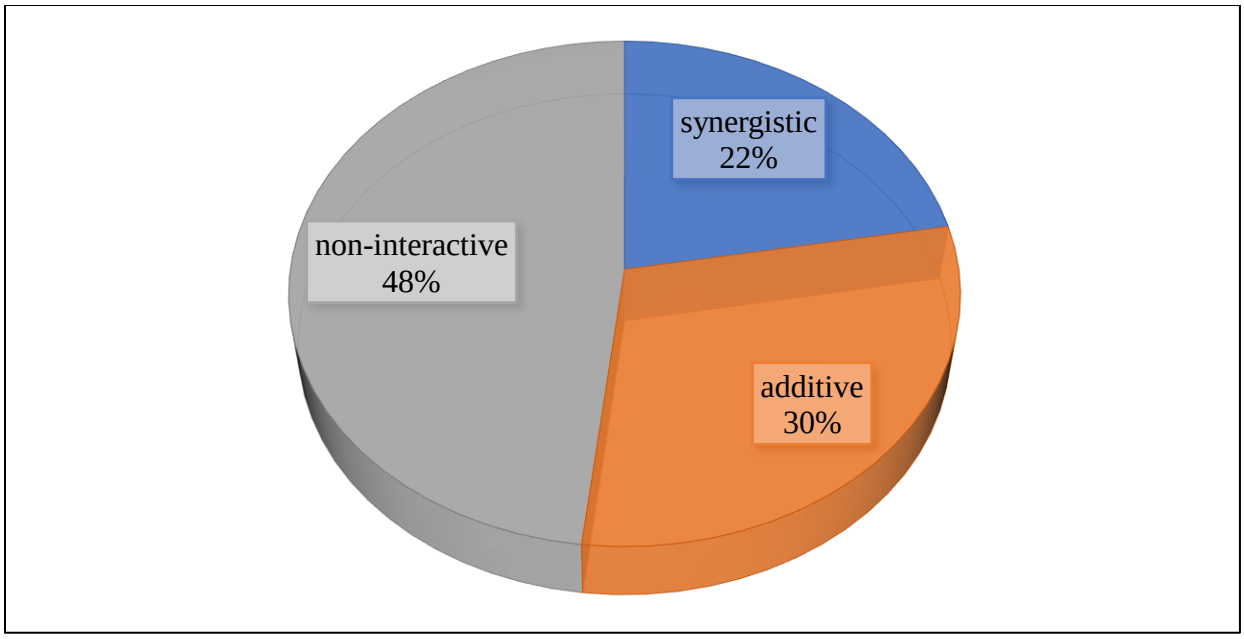


Figure 2.5: Overall interactions with 1:1 combinations.

Two combinations (pinocembrin-galangin and pinocembrin-chrysin) demonstrated bactericidal activity against *L. monocytogenes* with values of 1.25 and 0.63 mg/ml respectively.

2.3.4.2. Varied ratio combinations

2.3.4.2.1 The antimicrobial effect of propolis compounds in varied ratios against *L. monocytogenes* (ATCC 19111)

Synergy or additivity was noted for all 1:1 combinations tested against *L. monocytogenes*. When testing various ratios (Figure 2.6A), the combination of pinocembrin and galangin showed synergy for two ratios (7:3 and 6:4 – pinocembrin in majority) in addition to the 1:1 ratio. Additive interactions were observed for three ratios (9:1 and 8:2 – pinocembrin in majority and 4:6 – galangin in majority). Synergy was also present in the 9:1 combination of pinocembrin: chrysin combination, however, the majority of the ratios demonstrated additivity.

For the combination of galangin: chrysin, four ratios (8:2; 7:3; 6:4 – galangin in majority and 5:5 – equal ratio) were synergistic and the remainder of the ratios except one (9:1 – pinocembrin in majority) were additive.

2.3.4.2.2. The antimicrobial effect of propolis compounds in varied ratios against *P. aeruginosa* (ATCC 27858).

Against the micro-organism *P. aeruginosa*, seven points were noted as synergistic (Figure 2.6B). The interaction between pinocembrin and galangin was found to be synergistic at two ratios (9:1 and 8:2 – pinocembrin in majority) and additive at all other ratios except for one (1:9 – galangin in majority) which was non-interactive. Although no synergy was noted when pinocembrin was combined with chrysin, additivity was observed for all except for three ratios (3:7; 2:8; 1:9 – chrysin in majority) which were non-interactive. Galangin in combination with chrysin also shows promise: synergy was observed from this combination at five ratios (9:1; 8:2; 7:3; 6:4 – galangin in majority and 5:5 – equal ratio) against *P. aeruginosa*.

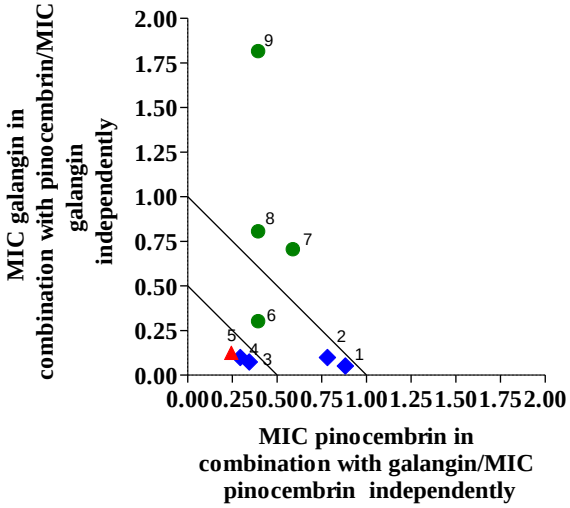
2.3.4.2.3. The antimicrobial effect of propolis compounds in varied ratios against *C. tropicalis* (ATCC 750).

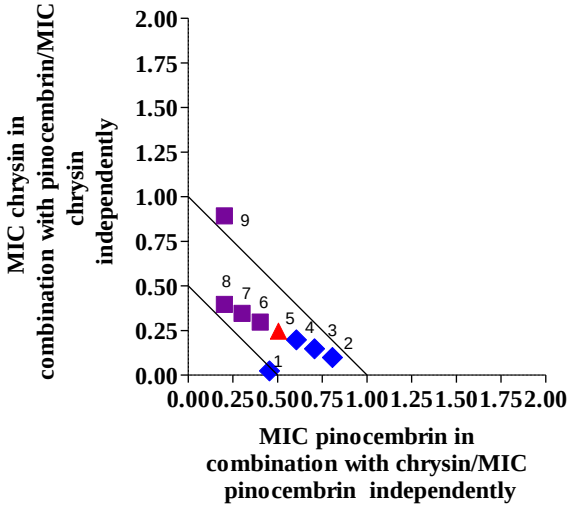
When the compounds found in South African propolis were tested against *C. tropicalis*, synergy and additivity were found for most of the ratios (Figure 2.6C). The combination of pinocembrin and galangin demonstrated five ratios that were synergistic (9:1; 8:2; 7:3; 6:4 – pinocembrin in majority; and 5:5 – equal ratio) and one which was additive (4:6 – galangin in majority). As for the combination of pinocembrin and chrysin: three ratios were found to be synergistic (9:1; 8:2; 6:4 – pinocembrin in majority) while an additional four ratios were additive (7:3 – pinocembrin in majority; and 1:9; 2:8; and 3:7 – chrysin in majority). The interactions between galangin and chrysin was possibly the most notable in this section, as these two compounds demonstrated synergy, irrespective of the ratio at which it was assayed.

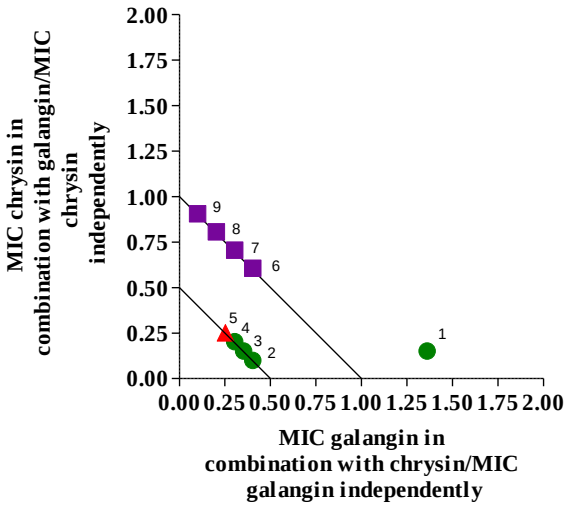
2.3.4.3. Equal ratio triple combinations (1:1:1)

Results of the triple combination studies at a ratio of 1:1:1 pinocembrin: galangin: chrysin are set out in Table 2.5. Out of the nine combinations, six combinations (67%) were synergistic and three combinations (33%) were additive (Figure 2.7). There were no non-interactive or antagonistic interactions.

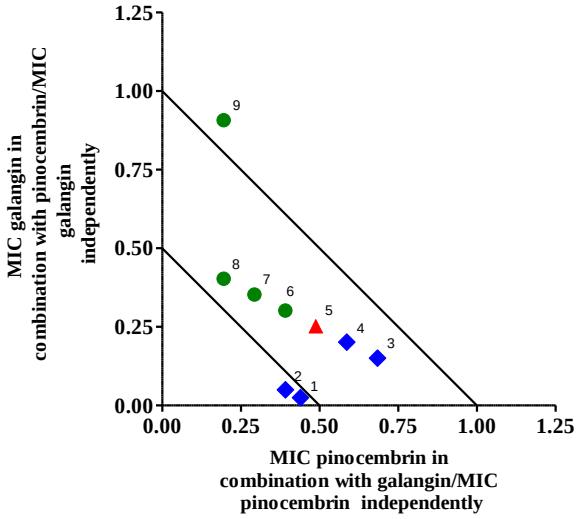
(2.6A)

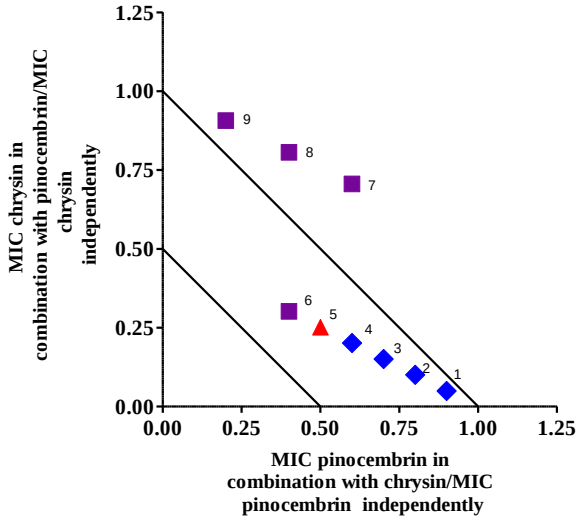

MIC galangin in combination with pinocembrin/MIC galangin independently
MIC pinocembrin in combination with galangin/MIC pinocembrin independently

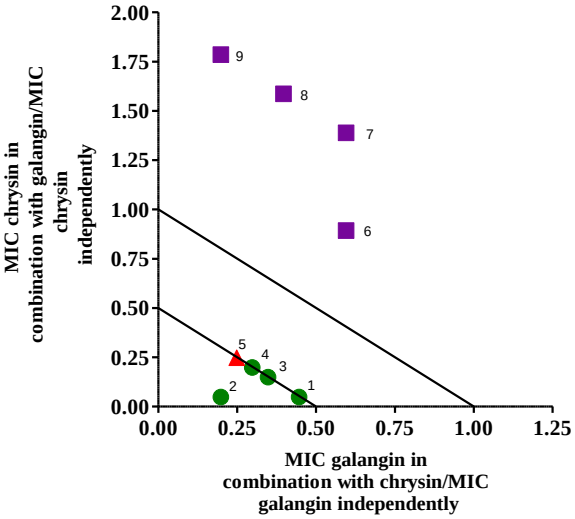

MIC chrysin in combination with pinocembrin/MIC chrysin independently
MIC pinocembrin in combination with chrysin/MIC pinocembrin independently


MIC chrysin in combination with galangin/MIC chrysin independently
MIC galangin in combination with chrysin/MIC galangin independently

(2.6B)


MIC galangin in combination with pinocembrin/MIC galangin independently
MIC pinocembrin in combination with galangin/MIC pinocembrin independently


MIC chrysin in combination with pinocembrin/MIC chrysin independently
MIC pinocembrin in combination with chrysin/MIC pinocembrin independently


MIC chrysin in combination with galangin/MIC chrysin independently
MIC galangin in combination with chrysin/MIC galangin independently

(2.6C)

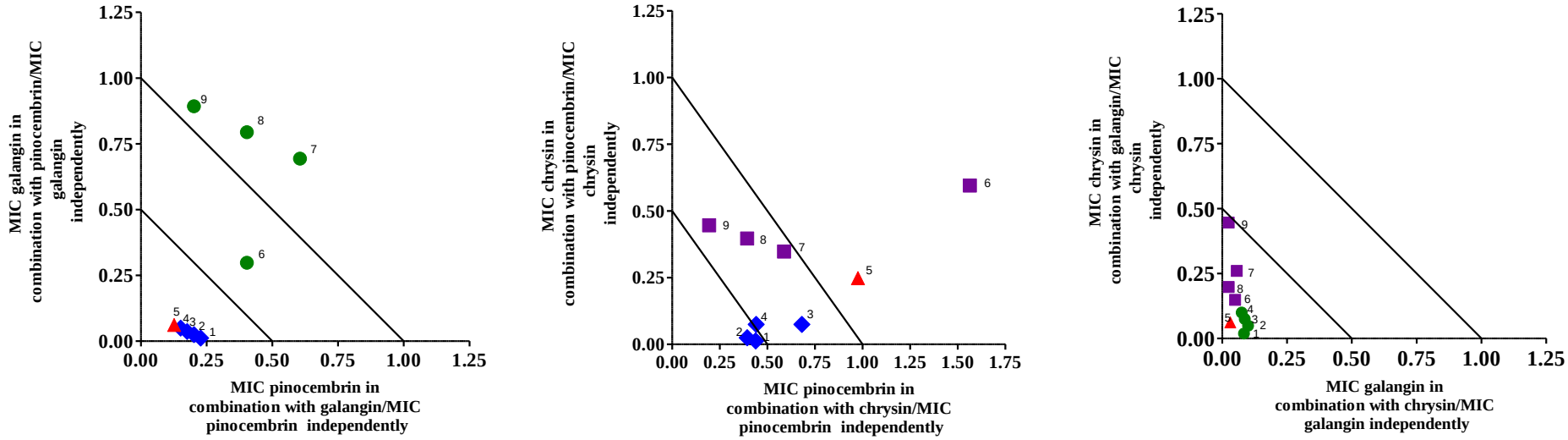


Figure 2.6: Isobologram representation of pinocembrin, galangin and chrysin in combination at various ratios against *L. monocytogenes* (ATCC 19111) (1A), *P. aeruginosa* (ATCC 27853) (1B) and *C. tropicalis* (ATCC 750) (1C).

Key: ◆ pinocembrin in majority ratio ● galangin in majority ratio ■ chrysin in majority ratio ▲ equal ratio of the compounds

Plot point 1 = 90 : 10 ratio
Plot point 2 = 80 : 20 ratio
Plot point 3 = 70 : 30 ratio
Plot point 4 = 60 : 40 ratio
Plot point 5 = 50 : 50 ratio
Plot point 6 = 40 : 60 ratio
Plot point 7 = 30 : 70 ratio
Plot point 8 = 20 : 80 ratio
Plot point 9 = 10 : 90 ratio

Table 2.5: Interactive efficacy of the triple combination of pinocembrin, galangin and chrysin.

Micro-organism	MIC	MBC	ΣFIC	Interpretation
Gram-positive bacteria				
S. aureus (ATCC 25923)	0.16	–	0.46	**Synergistic**
E. faecalis (ATCC 29212)	0.16	–	0.52	Additive
L. monocytogenes (ATCC 19111)	0.067	–	0.29	**Synergistic**
Gram-negative bacteria				
E. coli (ATCC 25922)	0.16	–	0.47	**Synergistic**
P. aeruginosa (ATCC 27853)	0.13	–	0.42	**Synergistic**
K. pneumoniae (ATCC 13883)	0.21	–	0.68	Additive
Yeasts				
C. albicans (ATCC 10231)	0.31	–	0.66	Additive
C. tropicalis (ATCC 750)	0.08	1.25	0.40	**Synergistic**
C. neoformans (ATCC 14116)	0.02	–	0.43	**Synergistic**

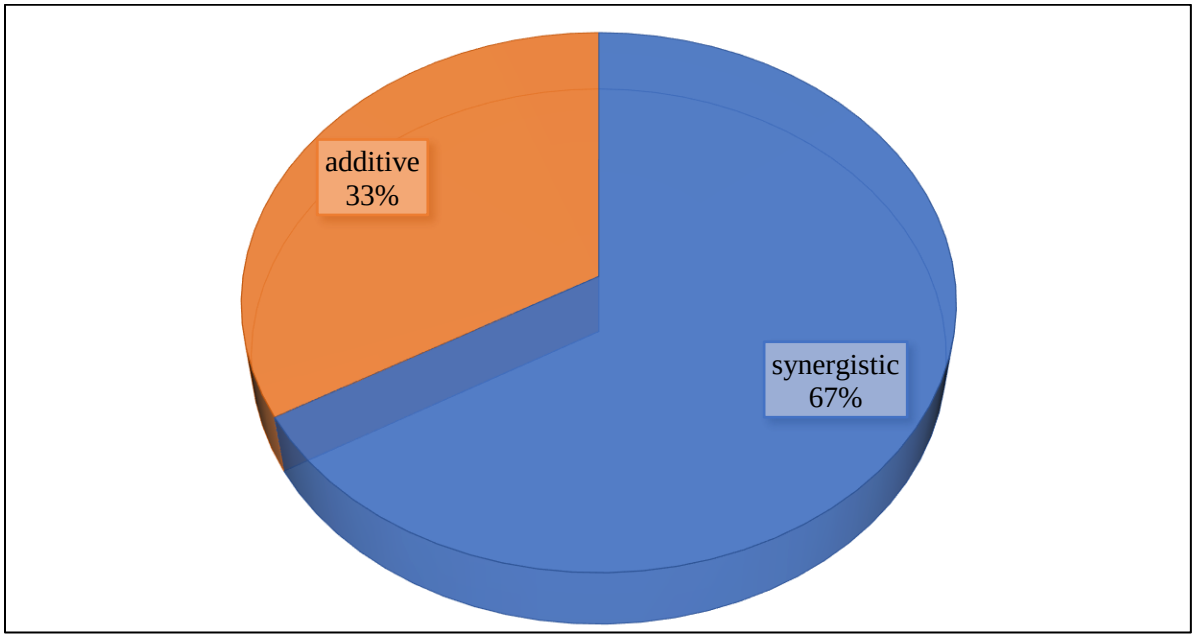


Figure 2.7: Overall interactions with 1:1:1 combinations

The effect of the triple combination against *L. monocytogenes* is especially worth mentioning, with the lowest MIC value of 0.07 mg/ml and an ΣFIC value of 0.29. This combination was bactericidal against *C. tropicalis* with an MBC of 1.25 mg/ml.

2.4. Discussion

Although propolis itself has been extensively researched, very little research has been done on the antimicrobial effects of the compounds found in propolis. Fokt et al. (2010) pointed out that although flavonoids and ester derivatives of phenolic acids are thought to be responsible for the excellent antimicrobial activity of propolis, these effects may be due to the combination and ratio of its constituent compounds.

As discussed in Section 2.1, two studies were published in which the constituents of propolis were found to possess better antimicrobial activity than pure propolis extracts (Quiroga et al., 2006; Buchta et al., 2011). However, Metzner et al. (1977), as cited in Buchta et al. (2011) found that propolis exhibited better antimicrobial activity than pinocembrin; and Boisard et al. (2015) reported better activity from an organic sample of propolis than from pinocembrin, galangin or chrysin. In this study, diverse antimicrobial activities of the propolis samples and compounds were apparent from the MIC assays conducted. These activities depended on the micro-organism and the sample tested, and no compound displayed a superior profile. This demonstrates that the properties of propolis probably cannot be attributed to any singular compound.

It should be noted that although MIC values less than 0.16 mg/ml for crude extracts are considered noteworthy, these values cannot be applied to pure compounds (van Vuuren and Holl, 2017). The following benchmarks were therefore applied: MIC values of less than 0.01 mg/ml were considered noteworthy, values of between and including 0.01 and 0.1 mg/ml considered moderate with clinical relevance, and values more than 0.1 mg/ml considered negligible (Rios and Recio, 2005; Kuete, 2010; and Kuete et al., 2011; van Vuuren and Holl, 2017). As per these criteria, it can be noted that pinocembrin, galangin and chrysin show negligible activity against all six strains of bacteria tested, with values ranging from 0.16 mg/ml up to 0.63 mg/ml.

This study showed that propolis displayed greater activity against Gram-positive bacteria than against Gram-negative bacteria, which fits in with several previous studies which have reported the same pattern (Grange and Davey, 1990; Fernandes et al., 2001; Lotfy, 2006; Yaghoubi et al., 2007). Similarly, the compounds found in propolis also showed greater activity against the yeasts and Gram-positive pathogens (average MICs of 0.32 mg/ml and 0.41 mg/ml, respectively) than against the Gram-negative pathogens (average MIC of 0.46 mg/ml). No previous could be found comparing the effects of pinocembrin, galangin and chrysin against the different microbial classes.

It should be noted that while the compounds had negligible activity against bacterial strains, when they were tested against yeast strains, 44% showed moderate activity, most of these being from the activity of the compounds against *C. neoformans*. Although no studies were found in which any of the compounds were tested against *C. neoformans*, this finding confirms research by Quiroga et al. (2006) who reported antifungal activity for pinocembrin and galangin against the fungi *Aspergillus niger, Penicillium notatum, Saccharomyces carlsbergensis, Phomopsis* spp., *Fusarium* spp., *Trichoderma* spp., and *Rhodotorula* spp., where it was reported that the compounds had MIC values equal or less than 0.05 mg/ml against all fungi. The MIC values noted against the yeasts in this study, supported by MIC values from previous studies, leads to the implication that the compounds found in South African propolis possess antifungal activity at a dose of > 0.02 mg/ml.

The average MIC of chrysin against the six strains of bacteria tested, was 0.53 mg/ml, as compared to 0.42 mg/ml for galangin and 0.22 mg/ml for pinocembrin. This indicates that the order of overall efficacy of the three compounds against bacteria is pinocembrin > galangin > chrysin. This corresponds to a study undertaken by Mercan et al. (2006) which demonstrated that higher concentrations of chrysin, when compared to pinocembrin and galangin, are needed to produce an antimicrobial effect. The order of efficacy against fungi was noted as pinocembrin (average MIC= 0.15 mg/ml) > chrysin (average MIC = 0.43 mg/ml) > galangin (average MIC = 0.65 mg/ml). This is in agreement with a study by Boisard et al. (2015) who found that pinocembrin had greater activity against *C. albicans, Candida glabrata* and *Aspergillus fumigatus* than did galangin or chrysin. It is also interesting to note that South African propolis samples contains a much higher concentration of pinocembrin than of galangin and chrysin (The South African propolis sample

tested contained 223.6 mg pinocembrin; 63.3 mg galangin; and 12.3 mg chrysin per gram). It is thus plausible to consider that pinocembrin is the sole agent responsible for the antimicrobial activity of propolis, however, this does not take interactions between the compounds into effect.

None of the three compounds exhibited bactericidal activity on their own, at the concentrations tested. This is not surprising as propolis itself has been found to possess bacteriostatic properties at lower concentrations and bactericidal effects at higher concentrations (Lu et al., 2005; Aamer et al., 2014; Fokt et al., 2010; Suleman et al., 2015). Little research has been done on the bactericidal effects of pinocembrin, galangin and chrysin, although Koo et al. (2002) found that pinocembrin had a bactericidal effect on *Streptococcus sobrinus* at a concentration of 0.12 mg/ml, and Pepeljnjak and Kosalec (2004) found galangin to possess bactericidal activity against *S. aureus*, *Enterococcus* spp. and *P. aeruginosa* at concentrations of 0.27 mg/ml, 0.44 mg/ml, and 0.23 mg/ml respectively. These differences might be due to the galangin samples used; Pepeljnjak and Kosalec (2004) used galangin extracted from propolis while this study used relatively pure commercially available galangin. No previous studies were found on the bactericidal effects of chrysin.

Twenty-two percent of the 1:1 combinations tested in this study were found to be synergistic. Synergism occurs when the effect of the compound combination is greater than the effect of the sum of the individual compounds (Breitinger, 2012). Many studies have theorized that the antibacterial activity of propolis is due to synergy between its components, although no research has been found on the combination of compounds found in propolis (Kujumgiev et al., 1999; Drago et al., 2007; Boisard et al., 2015). The most compelling argument in favour of the interactions between propolis components being responsible for its activities, is the findings that that biological activity does not necessarily diminish with a decrease in the content of flavonoids and other phenolic compounds (Kujumgiev et al., 1999; Benhanifia et al., 2013). This leads to the assumption that the compounds show stronger antimicrobial activity when used in combination, however, no studies proving this assumption have been found. Kujumgiev et al. (1999) have put forward the theory that since bees use propolis as a defence against infections, its properties depend not on any singular compound, but on propolis as a whole. The findings of synergism between certain combinations in this study supports the theory that interactions between components play a part in contributing towards the overall antimicrobial effect noted from propolis. Interestingly,

all of the combinations showing synergy included galangin as one of the two compounds. Although no previous research was found on the combination of galangin with pinocembrin or chrysin, Lee et al. (2008) found that galangin also showed synergy when combined with the antibiotic gentamycin. Furthermore, Pepeljnjak and Kosalec (2004) attributed good propolis activity to high concentrations of galangin.

In addition to the synergistic interactions that were found, additivity was discovered for a further 30% of the 1:1 combinations. This means that the effect of the combination is equal to the sum of its parts, which could also play a part in contributing to the antimicrobial activity shown by propolis. Furthermore, the remaining 48% of the 1:1 combinations were non-interactive. Therefore, when the compounds found within South African propolis are mixed, the result of their antimicrobial effects could either be enhanced or be non-interactive. However, no antagonistic effects take place, thus lessening the potential of increasing toxicity or adverse effects occurring as a result of combining compounds.

Synergy depends both on the properties of each of the drugs in combination, and on the doses of each agent (Tallarida, 2011). One pathogen from each of the three microbial classes (Gram-positive – *L. monocytogenes*, Gram-negative – *P. aeruginosa*, and the yeast – *C. tropicalis*) which displayed the highest number of synergistic interactions in the 1:1 ratio combination study was thus studied further to determine the role of varied ratios of the two constituents.

The propolis sample tested exhibited a low MIC value of 0.10 mg/ml against *L. monocytogenes*. This agrees with the findings from previous studies which have shown that *L. monocytogenes* is one of the most susceptible bacteria to the effects of propolis (Hendi et al., 2011; Ristivojević et al., 2016). *Listeria monocytogenes*, a Gram-positive foodborne pathogen, has increasingly been the cause of food contamination manifesting most commonly in gastroenteritis of which symptoms include diarrhoea, nausea, headache and abdominal pain (Wing and Gregory, 2002). Although it is found in the gastro-intestinal tract of approximately 5% of healthy adults, it can cause sepsis and meningitis in immunocompromised hosts and accounts for 25% of invasive listeriosis in pregnant patients, leading to abortion or neonatal sepsis (Wing and Gregory, 2002; Cossart and Archambaud, 2009). This pathogen, which is commonly found in the environment, has been shown

to survive even under adverse conditions including low temperature and PH levels (Cossart and Archambaud, 2009). As the propolis sample tested showed good activity against this pathogen, the next step was to investigate the activity of the compounds within propolis. From the three Gram-positive pathogens, *L. monocytogenes* proved to be the pathogen most susceptible to the antibacterial effects of the compounds pinocembrin, galangin and chrysin (average MIC = 0.26 mg/ml). When the compounds were combined, synergy or additivity was noted for all 1:1 combinations tested against *L. monocytogenes*. Varied ratio combination testing showed that the combination of galangin and chrysin produced the highest number of synergistic interactions, all with galangin either in majority or in equal parts (Figure 2.6A). This further supports the use of galangin in combination. Pinocembrin in combination with galangin also showed three synergistic interactions against *L. monocytogenes*, inferring that when used in combination, pinocembrin should be added an equal or slightly higher concentration than galangin. When pinocembrin is used together with chrysin, pinocembrin should dominate (at a ratio of 9:1, pinocembrin : chrysin) in order to achieve an optimal effect against *L. monocytogenes*.

The MIC value of the propolis sample against *P. aeruginosa* was 0.39 mg/ml. *Pseudomonas aeruginosa*, a Gram-negative pathogen, is one of the most common microbes found associated with wounds. Correspondingly, propolis has historically been used for the treatment of skin and burn wounds, and layman literature promotes the use for these ailments. Other manifestations include nosocomial pneumonia, urinary infections, respiratory infections, gastrointestinal infections, keratitis, otitis media, and bacteremia (El Solh and Alhajhusain, 2009; Morita et al., 2014). Due to its low nutritional demands, and its ability to grow in both aerobic and anaerobic environments, this pathogen is capable of surviving even under adverse host conditions (El Solh and Alhajhusain, 2009; Morita et al., 2014). Development of resistance by this pathogen to conventional antimicrobials is rapidly increasing and thus it is becoming more difficult to treat *P. aeruginosa* related infections (Lister et al., 2009). However, propolis has been shown to be effective against this pathogen, as shown by Zeighampour et al. (2014), who presented results where an MIC value of 0.75 mg/ml of propolis was observed against *P. aeruginosa*, reflecting the findings from this study. Using propolis at this concentration could be effective in the treatment of wounds. Interestingly, the individual components showed an average of 0.47 mg/ml, most of this activity imparted by pinocembrin, which exhibited an average MIC of 0.16 mg/ml. It is

possible that the antimicrobial effect of propolis against *P. aeruginosa* is due to pinocembrin, however, it is more likely due to the interaction between its compounds. When pinocembrin, galangin and chrysin were tested at a 1:1 ratio, a synergistic or additive effect was noted for all three combinations. When tested at varying ratios, the combination of pinocembrin and galangin showed synergy at two ratios, both of which had pinocembrin as the dominant compound. From this data it can be deduced that pinocembrin is important both alone and in combination with galangin, in contributing to the activity of propolis against *P. aeruginosa* and should optimally be used at a ratio of 9:1 or 8:2, pinocembrin : galangin. Similar to the results found from varied ratios against *L. monocytogenes*: when tested against *P. aeruginosa*, the combination of galangin and chrysin yielded the highest number of synergistic interactions. All ratios that included galangin as the compound in majority or at an equal ratio demonstrated synergy. This shows that the combination of galangin and chrysin can be used to treat *P. aeruginosa* infections, regardless of the ratio at which it is tested, but more that galangin is to be the predominant compound.

When tested against *C. tropicalis*, this study found an MIC value of 0.20 mg/ml from the propolis sample tested. *Candida tropicalis* is a yeast pathogen which falls under the category of *Candida*-non-*albicans* species and is a common cause of nosocomial candidemia (Kothavade et al., 2010). It is more common in tropical regions and the incidence of candidemia caused by *C. tropicalis* varies from 3-66%, depending on geographical area (Chai et al., 2010). It is found to penetrate the gastro-intestinal tract of patients, and is highly virulent (Chong et al., 2012). It was also found to be the causative agent in certain cases of pyelonephritis, lower urinary tract infections, thrombophlebitis, arthritis, bursitis, meningitis, multiple organ infection, pericarditis, and candidal vulvovaginitis (Fromtling et al., 1987). Resistance of this pathogen to azole drugs (the class of drugs most commonly used to treat *Candida* infections) has been increasing (Law et al., 1996). When pinocembrin, galangin and chrysin were tested independently against *C. tropicalis*, their activity ranged from moderate to negligible. However, when these compounds were combined at a 1:1 ratio, two synergistic interactions were discovered. Furthermore, out of the three pathogens that were tested, varying ratios of these compounds against *C. tropicalis* produced the best results (18 synergistic interactions). The combinations of pinocembrin and galangin, and pinocembrin and chrysin, proved promising with five and four synergistic interactions each. However, the true highlight of this section lies with the combination of galangin and chrysin, as these two compounds

demonstrated synergy irrespective of the ratio at which it was mixed. This proves that synergy between components is undoubtedly a major factor contributing to the antimicrobial activity of propolis.

This finding is further substantiated by the triple combination studies undertaken in this study, which showed synergy against 67% of the micro-organisms tested, and additivity against the remaining 33% of pathogens. The absence of both non-interactive and antagonistic reactions indicates that the combination between all three compounds has better activity than combinations between any two compounds, and that interactions between all three compounds are responsible for the activity of crude propolis samples.

2.5. Overview

- Pinocembrin, galangin and chrysin, when tested independently, did not display bactericidal or fungicidal activity at the highest concentration tested (1.25 mg/ml).
- Pinocembrin showed the best antimicrobial activity out of three compounds (average MIC value of 0.23 mg/ml against the six bacterial strains and 0.15 mg/ml against the three yeasts tested.
- The most susceptible pathogen to the effects of the individual compounds was *C. neoformans*.
- The compounds were then tested in combinations of 1:1 ratios. Twenty two percent of the 1:1 combinations showed synergy; 30% of the combinations were additive; and 48% were non-interactive. No combination showed antagonism.
- The lowest MIC value noted from a 1:1 combination was 0.02 mg/ml, resulting from the combination of pinocembrin and galangin against *C. neoformans*.
- The strongest synergistic interaction noted, was for the combination of galangin and chrysin against *C. tropicalis*, which had a ΣFIC value of 0.16.
- Bactericidal activity was noted for eight 1:1 combinations. The most pronounced bactericidal activity was from the combination of galangin and chrysin against *C. tropicalis*, with an MBC value of 0.16 mg/ml.

- Out of the 81 ratios generated from nine isobolograms, 32 ratios (40%) showed synergy, while 31 ratios (38%) were additive. The remaining 18 ratios (22%) were non-interactive.
- The combination of galangin and chrysin against *C. tropicalis* showed the best activity when tested at varied ratios, as all nine ratios of these two compounds in combination, showed synergy.
- From the triple combination studies, synergy was noted against six pathogens (67%) and additive interactions noted for the remaining three pathogens (33%). No antagonistic or non-interactive interactions were found for the triple combination. The triple combination also showed bactericidal activity against *C. tropicalis* at 1.25 mg/ml.

CHAPTER 3

Anti-quorum Sensing Activity

3.1. Introduction

Quorum sensing (QS) is a cell survival mechanism in which bacteria synchronize their behaviour and actions through a signalling technique, to act as a multicellular organism (Abudoleh and Mahasneh, 2017). Central to this process is the release of auto-inducers, or chemical signals, that are released from bacteria capable of QS. These auto-inducers promote communication between cells, which leads to alteration of specific genes (Miller and Bassler, 2001). They do this by gathering in the extracellular matrix (number of auto-inducers dependent on cell density population) until enough have amassed, after which they diffuse back into the cells and modify gene expression of their respective cells in unison, thus making sure cell behaviour is synchronized (Abudoleh and Mahasneh, 2017). This process depends on several factors including the synthesis and release, and later the cell uptake of, auto-inducers (Asfour, 2017). Virulence depends on the number of auto-inducers that are produced, which in turn depends on cell density (Namasivayam and Vivek, 2016). By suddenly gaining characteristics of a multi-cellular organism, without warning to the host's immune system, bacteria can resist attack from usual defence mechanisms (Namasivayam and Vivek, 2016). Auto-inducers can be classified into one of three categories: Gram-negative bacteria which produce N-acyl homoserine lactones (AHLs); Gram-positive bacteria which produce oligopeptides or autoinducing peptides; and both Gram-positive and Gram-negative bacteria which produce auto-inducer-2, allowing communication between these two classes of bacteria (Xavier and Bassler, 2003; Asfour, 2017). N-acyl homoserine lactones (AHLs) are produced by the *Luxl* protein family, while auto-inducer-2 is produced by the *LuxS* family of proteins (Asfour, 2017). The process of QS is essential to a number of consequential community physiological processes including virulence, luminescence, symbiosis, conjugation, bacterial mobility, toxin release formation of spores, and the formation of biofilms (Miller and Bassler,

2001; Mahumane, 2016; Abudoleh and Mahasneh, 2017). Although many bacteria exhibit QS properties, the most studied bacterium is *Pseudomonas aeruginosa* due to the array of common infections that it causes (Whitehead et al., 2001). *Chromobacterium violaceum*, a Gram-negative facultative anaerobe, is also often used to monitor for QS as it produces a purple colour that anti-QS agents can stop the production of, without killing the cells (Abudoleh and Mahasneh, 2017; Skogman et al., 2016). This is done through a feedback circuit, in which *C. violaceum* depends on both CviI, an auto-inducer synthase homologue, and CviR, a cognate receptor homologue (Stauff and Bassler, 2011). When cell density reaches a threshold, *C. violaceum* releases the auto-inducers N-hexanoyl-L-acylhomoserine lactone (C6-AHLand C4-AHL), which bind to the CviR receptor, after which violacein, indicated as a purple pigment, is produced (Stauff and Bassler, 2011).

The 21st century has brought about the emergence of antibiotic resistance. Older antibiotics have stopped working as effectively due to the evolution and adaptation of microbes and the development of newer antibiotics is stagnating (Fair and Tor, 2014). Although human misuse and errors in prescribing have played a large part in the development of resistance patterns, another reason is that bacteria are constantly evolving and adapting to find new ways to survive (Davies and Davies, 2010; Namasivayam and Vivek, 2016). Quorum sensing is one such mechanism that bacteria use. Apart from QS leading to biofilm formation, which make antibiotic penetration into cells very difficult, it also contributes to the working of efflux pumps which expel antibiotics out of the cells at which they are targeted (Varga et al., 2012; Amaral et al., 2014). Finding agents that stop QS signalling is thus imperative.

The term quorum quenching (QQ) refers to the inhibition of QS pathways; the agents capable of doing so, referred to as quorum sensing inhibitors (QSIs) (Kalia, 2013). Quorum sensing can be inhibited through a number of different pathways, as explained by both Kalia (2013) and Asfour (2017). The concentration of AHLs, a QS signal produced by Gram-negative bacteria, can be reduced by either inhibiting AHL synthase, the enzyme necessary for AHL production, or by lessening the activity of the AHL receptor by utilizing antagonistic agents or analogues of AHLs. It is also possible to degrade the other auto-inducers involved in QS through enzymatic techniques, or to completely inhibit the synthesis, secretion or the transport of the relevant auto-inducer. Antibodies against the auto-inducers have also been considered as a viable method of inhibiting

QS. Thus far, enzymatic degradation of auto-inducers have been the most common method of quorum quenching (Kalia, 2013).

In the search for new QSIs, natural products have grown as a source of interest. A study undertaken by Damte et al. (2013) tested the anti-QS activity of 18 indigenous Korean plant extracts and discovered that four extracts showed significant anti-QS activity against both *C. violaceum and P. aeruginosa*. Some other examples of natural QSIs are garlic against *P. aeruginosa*; clove against *Escherichia coli*, *P. aeruginosa*, and *C. violaceum*; ginseng against *P. aeruginosa* and *C. violaceum*; certain essential oils such as rosemary and tea tree against *E. coli* and *C. violaceum*; and many others as indicated in a review by Koh et al. (2013). Flavonoids have also been shown to possess QQ activity (Skogman et al., 2016; Asfour, 2017; Paczkowski et al., 2017). Vandeputte et al. (2011) tested the effects of a number of flavonoids, including the subcategories of flavones, flavanones, flavanols and chalcones. Their results showed that the flavonoids belonging to the flavanone group, namely; naringenin, eriodictyol and taxifolin, showed the best quorum quenching activity with very little effect on bacterial growth. Conversely, the flavones and flavanols tested showed no quenching activity but were shown to be either bacteriostatic or bactericidal against the bacteria tested. The authors theorize that differences in the hydroxyl group and double bond in the central C ring between these subclasses could be two possible reasons for the differences in activity. This agrees with the work of Paczkowski et al. (2017), who have reported that "novel flavonoids possessing dihydroxyl moieties in the flavone A-ring backbone" have activity against QS. However, Skogman et al. (2016) also report quorum quenching activity of kaempferide, which is a flavanol; and Paczkowski et al., 2017 also point out that baicalein (a flavone) and quercetin (a flavanol) have previously been shown to have QQ activity. Paczowski et al. (2017) have reported that these flavonoids bind to the two QS receptors (LasR and RhlR) and thus "reduce their ability to bind to DNA encoding QS regulated promoters".

With regards to bee products, both honey and propolis have been studied for anti-QS activity. Lamberte et al. (2011) studied the effect of the ethanolic extract of propolis, against QS using *C. violaceum* as a monitor strain, and found it to be an effective QSI. Similarly, Savka et al. (2015) studied the effects of ten propolis samples, each from different areas of the United States and found them all to significantly disrupt violacein production of the test strain *C. violaceum*. The study

went on to test the anti-QS activity of pinocembrin, one of the compounds found in propolis and found it to be effective, to the extent that it exhibited a similar level of inhibition to the positive control, the pure long-chain acyl-homoserine lactone 3-O-C12 . Gemiarto et al. (2015) tested the effects of manuka propolis, bee pollen and honey on QS. The authors discovered that while bee pollen had no QSI activity, honey had bactericidal activity and manuka propolis showed the strongest anti-QS activity. The major compound isoprenyl caffeate, found in manuka propolis, achieved complete QQ at a concentration of 50 µg/ml with little change to bacterial growth. Truchado et al. (2009) examined the anti-QS activity of 29 different uni-floral honey samples and while effects differed slightly (with chestnut and linden honey found to have the best activity; orange and rosemary less so), all samples inhibited the violacein production of the monitor strain at 100 mg/ml. This was done through the inhibition of AHLs produced by *C. violaceum* (Truchado et al., 2009). Although propolis and honey have been studied in detail, not much research has been found on the anti-QS activity of the compounds found in propolis, apart from the research on pinocembrin and isoprenyl caffeate mentioned earlier. The objective of this chapter was to investigate the effects of propolis compounds pinocembrin, galangin and chrysin, and their combinations, using *C. violaceum* (ATCC 12472) as a monitor strain.

Previous studies which have explored the anti-QS activity of natural products using *C. violaceum* as a monitor strain, have employed the macrodilution method (Khan et al., 2009; Ahmad et al., 2014). There are, however, disadvantages to this method: it is tedious, requires a large volume of reagents, and utilizes a lot of space and equipment (Reller et al., 2009). The broth microdilution method, which was used in Chapter 2, is more economical and easier to undertake (Reller et al., 2009). This study appears to be the first to investigate the use of the microdilution method in the determination of anti-QS activity.

3.2. Materials and methods

3.2.1. Preparation of compounds and controls

The compounds pinocembrin, galangin, and chrysin were made up to stock concentrations of 5 mg/ml, using the same methods described in Chapter 2. To determine whether it was the solvent that was responsible for anti-QS activity, acetone was used as a negative control at 5 mg/ml.

Vanillin, a known QSI, was used as a positive control to ensure that the strain of *C. violaceum* used was susceptible to an anti-QS agent (Chenia, 2013). Vanillin (Merck) was made up to a stock concentration of 5 mg/ml and tested in the same manner as the compounds. Untreated *C. violaceum* was added as a comparative control to use as a reference of violacein inhibition, and to ensure that the broth used was capable of supporting growth of *C. violaceum*.

3.2.2. Preparation of cultures

In order to monitor for QS activity, *C. violaceum* (ATCC 12472) was chosen as a biosensor strain. As mentioned earlier, this bacterium produces a purple pigment when undergoing QS, which is easily observed on a macroscopic level. The strain of *C. violaceum* used in this study was obtained from Dr. H. Chenia from the University of KwaZulu-Natal.

The growth medium chosen for the inoculation of *C. violaceum* was Luria Bertani broth (LBB) and agar (LBA), which was obtained from Oxoid and made up as per given instructions. For preparation of the broth, 25 g of the powder was added to one litre (or l) sterile water and autoclaved for 20 min at 121°C. Luria Bertani agar was made by adding 25 g of LB powder and 15 g of bacteriological agar to one litre of sterile water and autoclaved for 20 min at 121°C. To prepare the culture, *C. violaceum* was incubated, under aerobic conditions, in LBB at 30°C for 24 hr in an orbital shaker incubator (Labcon) at 140 revolutions per minute (rpm). This culture was then streaked out onto agar plate containing LBA and incubated at 30°C for 24 hr to check for purity. A single colony from this streak plate was then inoculated into a test tube containing 10 ml of LBB and incubated at 30°C for 24 hr at 140 rpm. After incubation, this culture contained approximately 5×10^6 Colony Forming Units (CFU). This suspension was then diluted by a factor of ten using LBB as a solvent to achieve a culture suspension of 5×10^5 CFU/ml which was used for the anti-QS assay.

3.2.3. Anti-quorum sensing assay

3.2.3.1. Macrodilution method

Anti-QS studies were undertaken using the methods described by Ahmad et al. (2014) and Mahumane (2016). Test tubes containing 5 ml of LBB each were prepared. For each compound, or combination of compounds, eight test tubes were required, six for the varying concentrations of the compounds and two as controls. The compound being assayed was added into the six test tubes using aseptic manipulation, at different volumes from the stock concentration of 5 mg/ml, to produce concentrations of 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04 mg/ml, respectively. Volumes needed were determined using Equation 3.1 and shown in Table 3.1.

Equation 3.1: Volume of compound to add from a stock concentration of 5 mg/ml.

$c_1v_1 = c_2v_2$

Where c_1 = concentration of the stock suspension, i.e.: 5mg/ml;

c_2 = the concentration of compound required;

v_1 = the volume of compound to add; and

v_2= the volume of LBB used

Table 3.1: Amount of compound to add to a test tube containing 5 ml LBB, in order to achieve the desired concentration.

Dilutions	Concentration required (mg/ml)	Vol. to add for individual compounds (ml)*	Vol. to add for individual compounds (µl)	Vol to add for 1:1 combinations (µl)	Vol to add for 1:1:1 combinations (µl)
1	1.25	1.25	1250.00	625.00	416.67
2	0.63	0.63	625.00	312.50	208.33
3	0.31	0.31	312.50	156.25	104.17
4	0.16	0.16	156.25	78.13	52.08
5	0.08	0.08	78.13	39.06	26.04
6	0.04	0.04	39.07	19.53	13.02

*From a stock concentration of 5 mg/ml made up in acetone.

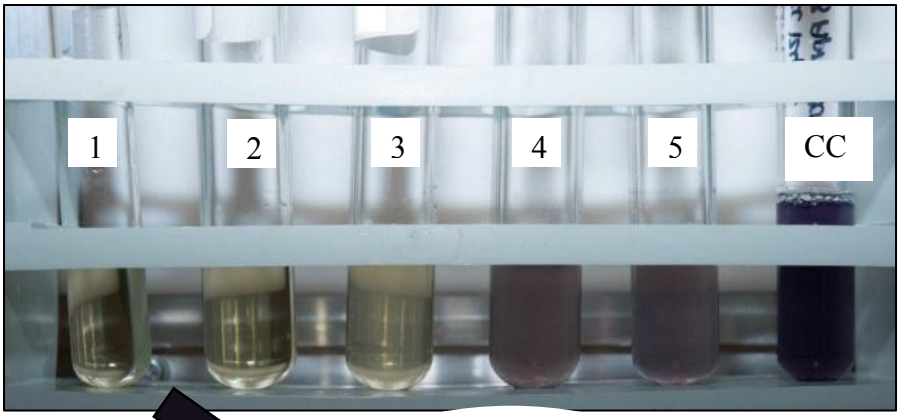
It was important to use differing volumes of the compounds, in order to determine the effect of increasing concentration on violacein production. Acetone was added into the seventh test tube, to

act as a negative control. After all the compounds and controls were prepared, 100 µl of *C. violaceum* from the stock suspension was added into all eight test tubes. The eighth test tube, containing only LBB and *C. violaceum*, was the culture control and acted as a baseline from which to compare the growth in the other test tubes. All test tubes were incubated at 30°C for 24 hr with agitation at 140 rpm in an orbital shaker incubator (Labcon). The following day, test tubes were vortexed and observed macroscopically (Figure 3.1).

Test tube 8, the culture control (CC), was expected to have produced a purple colour, the result of violacein release. Using this as a benchmark, the other test tubes were analyzed. Anti-QS activity was interpreted based on growth (turbidity) and absence of violacein, the purple pigment produced upon activation of the CviR receptor. Results were interpreted as one of two: the presence of the purple colour along with turbidity indicated that there was no anti-QS activity and that the *C. violaceum* cells were still alive, and the absence of violacein production was interpreted as the minimum quorum sensing inhibitory concentration (MQSIC). (Mahumane, 2016). Usually, the MIC would be read as the first test tube that did not produce turbidity, but because the compounds used in this study had a cloudy tint themselves when dissolved in acetone, this was difficult to ascertain. Instead, MBC studies were conducted on the effects of the compounds and their combinations on *C. violaceum*. This was done by streaking out samples from the test tubes which showed QS inhibition, onto an agar plate which was left to incubate overnight at 30°C. The MBC was read as the lowest concentration that did not produce growth (Figure 3.1).

After overnight incubation, 1 ml aliquots were taken from each test tube and transferred into a corresponding 15 ml conical bottom centrifuge tube. These tubes were centrifuged at 5000 rpm for 10 min in an Eppendorf centrifuge 5804 (Merck Millipore). This allowed for the bacteria to form pellets, which settled to the bottom and retained the colour of the *C. violaceum*. The clear supernatant was thereafter discarded. The following step was done in order to solubilize the violacein: the pellets were suspended in 1 ml of 100% dimethyl sulfoxide (DMSO) (Riedel-de-Haën) solution and each tube was vortexed for 20 seconds. Dimethyl sulfoxide (DMSO) was used as the vehicle of choice as violacein is insoluble in water and poorly soluble in acetone (Mahumane, 2016).

3.1A



3.1B

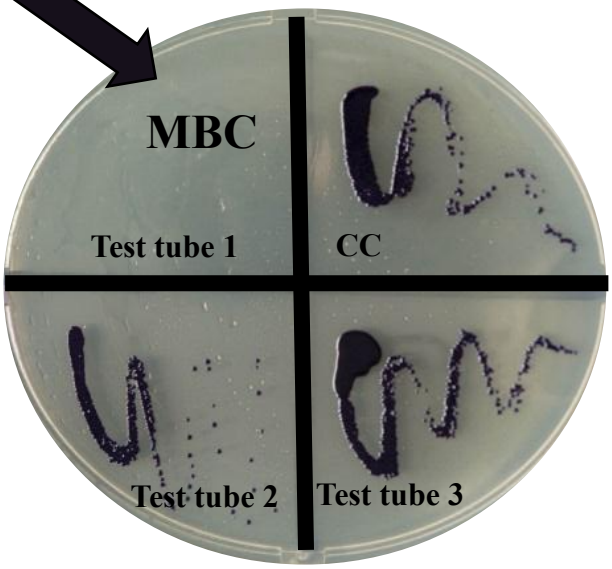


Figure 3.1: Visual representation of the anti-quorum sensing assay. The absence of a purple pigment in test tubes 1-3 (3.1A) indicates anti-quorum sensing activity, whilst the absence of growth from test tube 1 when streaked onto an agar plate (3.1B) indicates bactericidal activity.

Test tubes 1-5 represent decreasing concentrations of the compound concentrations inoculated from test tubes. Test tube 1 = 1.25 mg/ml, 2 = 0.63/ml, 3 = 0.31 mg/ml, 4 = 0.16 mg/ml, 5 = 0.08 mg/ml. CC= culture control.

Following this step test tubes were further centrifuged at 5000 rpm for 7 min to separate the bacterial cells from the solution, and 200 µl of the supernatant from each tube was transferred into a well of a 96-well micro-titre plate. This was done in duplicate. The absorbance of the supernatant in each well was then read at an optical density (O.D) of 595 nm using FilterMax F5 multi-mode

microplate reader (Molecular Devices). The percentage violacein inhibition was calculated using Equation 3.2.

Equation 3.2: Percentage violacein production using the test and control optical densities.

$$\% \text{ violacein production} = \frac{\text{Culture control @ OD 595} - \text{test compound @ OD 595 x 100}}{\text{Culture control @ OD 595}}$$

Quantitatively, the lowest concentration at which violacein production was inhibited by or more than 50% using Equation 3.2, was considered the MQSIC. This study thus used any result of ≥ 50% as an indication of anti-QS activity (Ahmad et al., 2014).

3.2.3.2. Microdilution method

A second method was also used to test the effects of pinocembrin, galangin and chrysin against QS. This method is adapted from the principle of MIC tests described by Eloff (1998) but has not previously been tested or published. A summary of the differences in methodologies between the two methods is given in Table 3.2.

Table 3.2: Differences in anti-QS methodologies between the macrodilution and microdilution method.

	Macrodilution method	**Microdilution method**
Primary equipment	Test tubes.	48-well micro-titre plate.
Step 1	Prepare a stock suspension of *C. violaceum*.	Prepare a stock suspension of *C. violaceum*.
Step 2	Make up a 5 mg/ml stock solution of compound and add 5 ml of LBB to six test tubes, labelled 1-6.	Make up a 2.5 mg/ml stock solution of compound and add 1 ml of LBB to six wells from one column of the micro-titre plate.

	Macrodilution method	Microdilution method
Step 3	Add a calculated volume (1250 µl) from the stock solution, to the first test tube of LBB, to make up to a concentration of 1.25 mg/ml.	Add 1 ml of the stock solution to the first well, to make up to a concentration of 1.25 mg/ml.
Step 4	Add half of the volume from step 4 (625 µl) from the stock solution, to the second test tube of LBB, to make up to a concentration of 0.63 mg/ml.	Using a micropipette, transfer 1 ml of the mixture from the first well down into the second well. The concentration of compound in the second well is now 0.63 mg/ml.
Step 5	Add half of the volume from step 5 (312.5 µl) from the stock solution, to the third test tube of LBB, to make up to a concentration of 0.31 mg/ml.	Using a micropipette, mix the contents of row two and then transfer 1 ml of the mixture from the second well down into the third well. The concentration of compound in the third well is now 0.31 mg/ml.
Step 6	Continue as above until you get to test tube 6. The concentration of compound in this test tube should be 0.04 mg/ml.	Continue as above until you get to row 6. The concentration of compound in this well should be 0.04 mg/ml.
Step 7	Add 100 µl from the stock suspension of *C. violaceum to* each test tube used.	Add 20 µl from the stock suspension of *C. violaceum* to each micro-titre plate well used.
Step 8	Repeat for each compound using a new set of six test tubes.	Repeat by using a new column of the micro-titre plate for each compound.
Step 9	Incubate test tubes overnight at 30°C for 24 h with agitation at 140 rpm.	Incubate micro-titre plate overnight at 30°C for 24 h with agitation at 140 rpm.
Step 10	Observe results macroscopically and record.	Observe results macroscopically and record.

	Macrodilution method	Microdilution method
Step 11	Transfer 1 ml from each test tube to a corresponding centrifuge tube and perform the anti-QS assay.	Transfer 1 ml from each well to a corresponding centrifuge tube and perform the anti-QS assay.

* Shaded areas show the same principle but in lower volumes.

Briefly, compounds were made up to a stock concentration of 2.5 mg/ml in acetone. A volume of 1 ml of sterile Luria Bertani broth (LBB) was placed into each well of a 46 well micro-titre plate. Compounds were placed into the top row of the micro-titre plate in volumes of 1 ml after which serial doubling dilutions were performed to subsequently achieve compound concentrations of 1.25, 0.63, 0.31, 0.16, 0.08 and 0.04 mg/ml. A stock culture of *C. violaceum* was prepared in the same way described in Section 3.2.2, and 20 µl was transferred to each well of the micro-titre plate. A new column of the micro-titre plate was used for each compound, and columns were also reserved for the culture control (20 µl of culture into 1 ml of LBB) and for the positive and negative controls (vanillin and acetone, respectively). A representation of the microdilution anti-QS assay is shown in Figure 3.2.

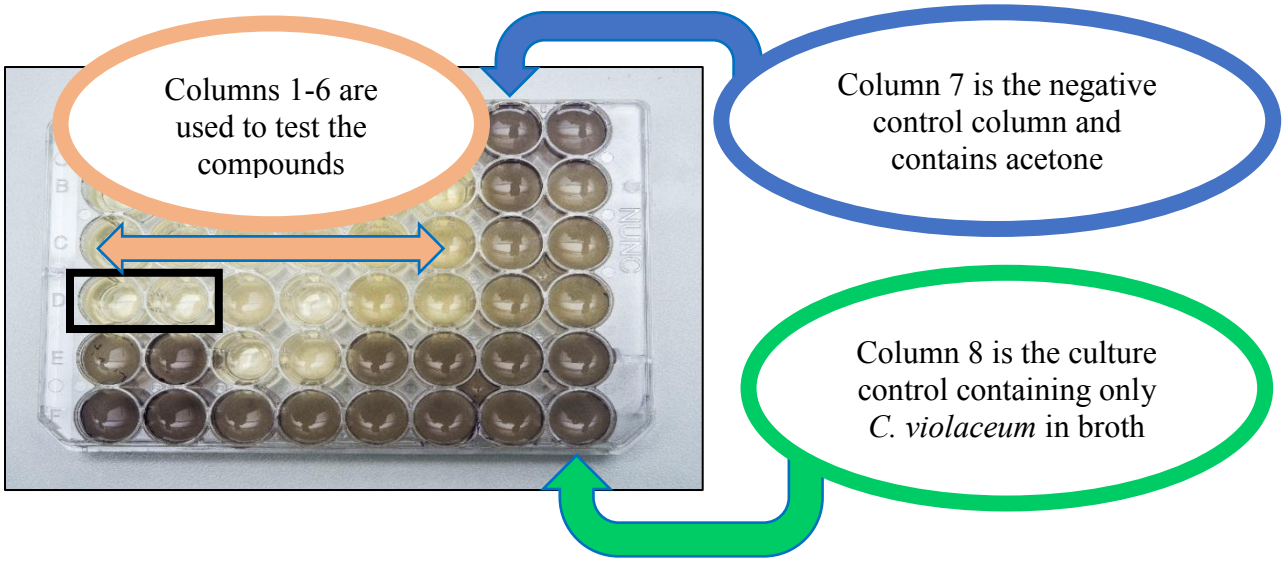


Figure 3.2: A visual representation of the microdilution anti-QS assay.

The presence of a purple pigment indicates the occurrence of quorum sensing. The boxed off area represents the MQSIC of the compound tested in the first and second columns.

3.2.3.3. Interactive efficacy studies

Synergy between compounds has been the most popular explanation for the unusually high antimicrobial activity shown by propolis (Kosalec et al., 2003). It was thus considered important to study not only the effects on QS from the three individual major compounds found in propolis, but also the effects on QS given by the combinations of the compounds in order to determine whether the anti-QS activity shown by propolis is, indeed, due to interactions between compounds. The method was followed as in Section 3.2.3.1, where a volume from each compound was added to 5 ml LBB, except that in the case of 1:1 combinations, because two compounds were used, the original volume was halved, and equal amounts of both compounds were added to the LBB. For example, 0.63 ml of pinocembrin and 0.63 ml of galangin was added to 5 ml of LBB to produce a total concentration of 1.25 mg/ml, instead of adding 1.25 ml of just pinocembrin. For 1:1:1 combinations, the original volume was divided by three and the resultant amount was added into 5 ml of LBB from each of the three compounds. The rest of the assay remained the same. All tests were done in duplicate.

To determine the types of interactions between compounds, both a fractional quorum sensing inhibitory concentration (FQSIC) and a fractional bactericidal concentration (FBC) index were used. The FQSIC index was calculated by dividing the MQSIC value of the compound in combination by the MQSIC value of each compound independently, as shown in the Equation 3.3 for 1:1 combinations. The FBC was calculated by dividing the MBC value of the compound in combination by the MBC value of each compound independently.

Equation 3.3: Fractional quorum sensing inhibitory concentration (FQSIC) calculations

$$FQSIC^{(i)} = \frac{\text{MQSIC (a) combined with (b)}}{\text{MQSIC (a) independently}} \qquad FQSIC^{(ii)} = \frac{\text{MQSIC (b) combined with (a)}}{\text{MQSIC (b) independently}}$$

Where (a) is the MQSIC of the one compound in the combination and (b) is the MQSIC of the other compound used in the combination. The sum of the FIC, known as the FQSIC index

(ΣFQSIC) is thus calculated as: ΣFQSIC = FQSIC $^{(i)}$ + FQSIC $^{(ii)}$. Triple combinations had an extra FQSIC $^{(iii)}$ value which was then plugged into the ΣFQSIC equation.

The ΣFQSIC values for each combination can be interpreted as synergistic (ΣFQSIC ≤ 0.5), additive (ΣFQSIC values > 0.5 – 1.0), non-interactive (ΣFQSIC values > 1.0 ≤ 4.0), or antagonistic (ΣFQSIC values > 4.0) as adapted from van Vuuren and Viljoen (2011).

3.3. Results

3.3.1. Anti-QS activity of individual compounds: broth macrodilution method

Table 3.3 details the percentage violacein inhibition, as well as the macroscopic appearance and the presence or absence of growth on an agar plate when pinocembrin, galangin and chrysin were tested independently using the macrodilution method. The average inhibition of the compounds over the six concentrations were as follows: pinocembrin = 52.87%; galangin = 51.28%; and chrysin = 59.97%, which indicated that chrysin had the best anti-QS activity from the three compounds tested. This is also apparent when examining varying concentrations of the compounds. At the highest concentration tested (1.25 mg/ml), the percentage violacein inhibition of chrysin was 78.82% as compared to 78.23% for pinocembrin and 77.63% for galangin. This pattern follows for every subsequent concentration going down up until the concentration of 0.08 mg/ml, where galangin showed the highest inhibition at 43.72%. However, it must be noted that only percentage inhibitions of 50% and over are considered significant in terms of quorum quenching activity. Taking this into consideration, chrysin and pinocembrin showed anti-QS activity from 0.16 mg/ml, while galangin displayed anti-QS activity at a slightly higher concentration of 0.31 mg/ml. These values are interpreted as the MQSIC, or the lowest concentration at which a compound is capable of inhibiting QS signals. A summary of the MQSIC, MBC and the percentage violacein inhibition at the highest concentration tested of pinocembrin, galangin and chrysin is given in Table 3.4. It must be noted that the qualitative data (macroscopic appearance) and the quantitative data (absorbance values and subsequent calculations) closely matched one another. MQSIC values of over 50% for every compound correlated to the first test tube in which no purple pigment was observed. Because two of the compounds used in this study have a yellow tint, it was sometimes difficult to distinguish between what was clear and turbid,

and so samples from all test tubes were streaked out. Most MBC values correlated to the first clear test tube, although some test tubes that looked clear turned out not to be the MBC, which was instead the next highest concentration of the compound. Obtaining quantitative data is thus imperative for accuracy of results.

Important also to note, is that both pinocembrin and galangin paradoxically showed an enhancement of QS signaling at 0.04 mg/ml, whilst chrysin did not. For every compound, the percentage inhibition of violacein production increased with increasing concentration. Minimum bactericidal concentration (MBC) values were also determined. Surprisingly, chrysin showed growth at all the concentrations tested. A 2.5 mg/ml concentration of chrysin was then prepared, and as it did not grow on the agar plate this value was determined to be the MBC value. Pinocembrin and galangin showed lower MIC values, at 1.25 mg/ml. All compounds showed comparable activity to vanillin, a known QSI.

While the MQSIC for vanillin was slightly lower than any of the other compounds tested (0.08 mg/ml), the MIC value (1.25 mg/ml) was the same as that of pinocembrin and galangin, and the percentage violacein inhibition produced was lower than that of pinocembrin, galangin and chrysin.

3.3.2. Anti-QS activity of individual compounds: macrodilution vs microdilution results

The results obtained from the microdilution method are shown in Table 3.5, together with the results from the macrodilution method. When observed macroscopically, the macrodilution and microdilution method yielded the same results – the purple violacein pigment was produced at the same concentrations of the compounds in both assays. Therefore, when interpreting qualitative results for the microdilution method, the MQSIC values of pinocembrin, galangin and chrysin were the same as recorded in the macrodilution method (0.16, 0.31 and 0.16 mg/ml, respectively).

Table 3.3: Percentage violacein production inhibition (%), macroscopic appearance, and the presence of growth on an agar plate for the compounds pinocembrin, galangin and chrysin.

Conc	Pinocembrin			Galangin			Chrysin			Vanillin		
	%	App	Growth	%	App	Growth	%	App	Growth	%	App	Growth
1.25	78.23	Clear	No	77.63	Clear	No	78.82	Clear	Yes	74.30	Clear	No
0.63	77.22	Clear	Yes	76.44	Turbid	Yes	78.64	Clear	Yes	74.86	Clear	Yes
0.31	74.45	Turbid	Yes	75.16	Turbid	Yes	78.38	Turbid	Yes	74.52	Turbid	Yes
0.16	67.01	Turbid	Yes	47.59	Purple	Yes	75.3	Turbid	Yes	73.07	Turbid	Yes
0.08	22.96	Purple	Yes	43.72	Purple	Yes	41.93	Purple	Yes	61.84	Turbid	Yes
0.04	–2.65	Purple	Yes	–12.88	Purple	Yes	6.73	Purple	Yes	40.88	Purple	Yes
Ave	52.87			51.28			59.97			66.58		

Table 3.4: Summary of the MQSIC, MBC and the percentage violacein inhibition of the compounds pinocembrin, galangin and chrysin.

Compound	% inhibition at 1.25 mg/ml	MQSIC (mg/ml)	MBC (mg/ml)
Pinocembrin	78.23	0.16	1.25
Galangin	77.63	0.31	1.25
Chrysin	78.82	0.16	2.50
Vanillin	74.30	0.08	1.25

For quantitative results, the percentage violacein inhibition was measured. The results of the highest three concentrations tested (1.25; 0.63; and 0.31 mg/ml) were comparable with percentage differences of less than 10% between both methods while the results from the lower concentrations (0.16; 0.08; and 0.04 mg/ml) deviated. The exceptions to this pattern are chrysin and vanillin, which showed comparable results between the macrodilution and microdilution methods for the four highest concentrations. When using the microdilution method, all the compounds showed anti-QS activity (i.e. their percentage violacein inhibition percentages were above 50%) at the concentrations tested, with the exception of chrysin at 0.04 mg/ml with a violacein inhibition of 44.54%. When interpreting the quantitative results from the microdilution method, the MQSIC for pinocembrin (< 0.4 mg/ml), galangin (< 0.04 mg/ml) and chrysin (0.04 mg/ml) differed substantially from the macrodilution method.

3.3.3. Anti-QS activity of combinations

Compounds were tested in combination for their effects at inhibiting QS. Table 3.6 details the percentage violacein inhibition, as well as the macroscopic appearance and the presence or absence of growth on an agar plate when pinocembrin, galangin and chrysin were tested in combination using the macrodilution method. Given in Table 3.7 is the summary of the MQSIC, MBC and the percentage violacein inhibition of the pinocembrin, galangin and chrysin at 1.25 mg/ml.

At first glance, it is already evident that the anti-QS activity of the combinations are better than that of the individual compounds. The percentage violacein inhibition of the combinations between pinocembrin and galangin, and between pinocembrin and chrysin, at concentrations of 1.25 mg/ml are particularly impressive at 81.66% and 85.10% respectively. None of the combinations showed negative percentage inhibition values. The average percentage violacein inhibition given from the combinations over the six concentrations tested were as follows: pinocembrin-galangin 67.11%; pinocembrin-chrysin 64.73%; and galangin-chrysin 61.38%.

Table 3.5: Comparison of the percentage violacein production inhibition (%), macroscopic appearance (app), and percentage differences of violacein inhibition between the macrodilution and microdilution methods of pinocembrin, galangin and chrysin.

Conc (mg/ml)	Pinocembrin					Galangin				
	Microdilution		Macrodilution		% difference micro vs. macrodilution	Microdilution		Macrodilution		% difference micro vs. macrodilution
	%	App	%	App		%	App	%	App	
1.25	81.80	Clear	78.23	Clear	4.36	81.78	Clear	77.63	Clear	5.07
0.63	80.99	Clear	77.22	Clear	4.65	81.20	Turbid	76.44	Turbid	5.86
0.31	80.85	Turbid	74.45	Turbid	7.92	80.38	Turbid	75.16	Turbid	6.49
0.16	79.09	Turbid	67.01	Turbid	15.27	79.67	Purple	47.59	Purple	40.27
0.08	76.03	Purple	22.96	Purple	69.80	63.29	Purple	43.72	Purple	30.92
0.04	64.00	Purple	–2.65	Purple	104.14	56.75	Purple	-12.88	Purple	122.70
Conc (mg/ml)	**Chrysin**					**Vanillin**				
	Microdilution		**Macrodilution**		**% difference micro vs. macrodilution**	**Microdilution**		**Macrodilution**		**% difference micro vs macrodilution**
	%	**App**	**%**	**App**		**%**	**App**	**%**	**App**	
1.25	81.49	Clear	78.82	Clear	3.28	76.29	Clear	74.30	Clear	2.61
0.63	80.91	Clear	78.64	Clear	2.81	77.31	Clear	74.86	Clear	3.17
0.31	80.67	Turbid	78.38	Turbid	2.84	78.94	Turbid	74.52	Turbid	5.60
0.16	80.25	Turbid	75.30	Turbid	6.17	78.77	Turbid	73.07	Turbid	7.24
0.08	56.70	Purple	41.93	Purple	26.05	76.97	Turbid	61.84	Turbid	19.66
0.04	44.54	Purple	6.73	Purple	84.89	68.28	Purple	40.88	Purple	40.13

It is thus observed that even though chrysin was the most effective anti-QS agent from amongst the individual compounds, the grouping of pinocembrin with galangin demonstrated the highest percentage of violacein inhibition. This combination, along with the galangin-chrysin combination, showed the lowest MQSIC value (0.08 mg/ml). With regards to bactericidal activity against *C. violaceum*, the combination of pinocembrin and galangin once again proved to be the most effective, with an MBC of 0.31 mg/ml.

The results of the triple combination (pinocembrin, galangin and chrysin) anti-QS activity are presented in Table 3.6. The average inhibition of the triple combination over all six concentrations tested, was 66.54%, placing it just under the pinocembrin-galangin combination in terms of effectiveness. The MQSIC of the triple combination was noted at 0.08 mg/ml and the MBC, at 0.63 mg/ml. Figure 3.3 illustrates the percentage violacein inhibition for all the compounds and their combinations, over the six concentrations tested.

3.3.4. Interactions between the compounds

Interactions between all the compounds were evaluated according to the criteria adapted from van Vuuren and Viljoen (2011). A summary of the interactions between the compounds, calculated using the FBC and FQSIC indexes, is given in Table 3.7.

The FQSIC index was calculated by using the MQSIC values of the combinations in reference to the MQSIC values of the individual components. While the combination of pinocembrin and chrysin was additive with an FQSIC index of 1.00, all other combinations proved to be synergistic. The lowest FQSIC index, or indication of the strongest anti-QS activity was shared between the pinocembrin-galangin and the galangin-chrysin combinations.

Similarly, the FBC index was calculated by using the MBC values of the combinations in reference to the MBC values of the individual components. Fractional bactericidal concentration indexes for two of the double combinations, as well as for the triple combination, were all under 0.5 thus indicating synergy. The FBC index for the third double combination, between galangin and chrysin, was 0.75 suggesting that this combination had an additive effect.

Table 3.6: Percentage violacein production inhibition (%) and macroscopic appearance of the combinations between pinocembrin, galangin and chrysin.

Conc	Pinocembrin - Galangin			Pinocembrin - Chrysin			Galangin - Chrysin			Pinocembrin - Galangin - Chrysin		
	%	App	Growth	%	App	Growth	%	App	Growth	%	App	Growth
1.25	81.66	Clear	No	85.10	Clear	No	73.03	Clear	No	76.81	Clear	No
0.63	80.96	Clear	No	84.38	Clear	No	72.90	Clear	Yes	75.40	Clear	No
0.31	79.70	Clear	No	83.19	Clear	Yes	71.86	Turbid	Yes	71.54	Turbid	Yes
0.16	78.85	Turbid	Yes	57.94	Turbid	Yes	66.48	Turbid	Yes	69.48	Turbid	Yes
0.08	55.26	Turbid	Yes	40.43	Purple	Yes	66.09	Turbid	Yes	65.88	Turbid	Yes
0.04	26.23	Purple	Yes	37.33	Purple	Yes	17.94	Purple	Yes	40.11	Purple	Yes
Average	67.11			64.73			61.38			66.54		

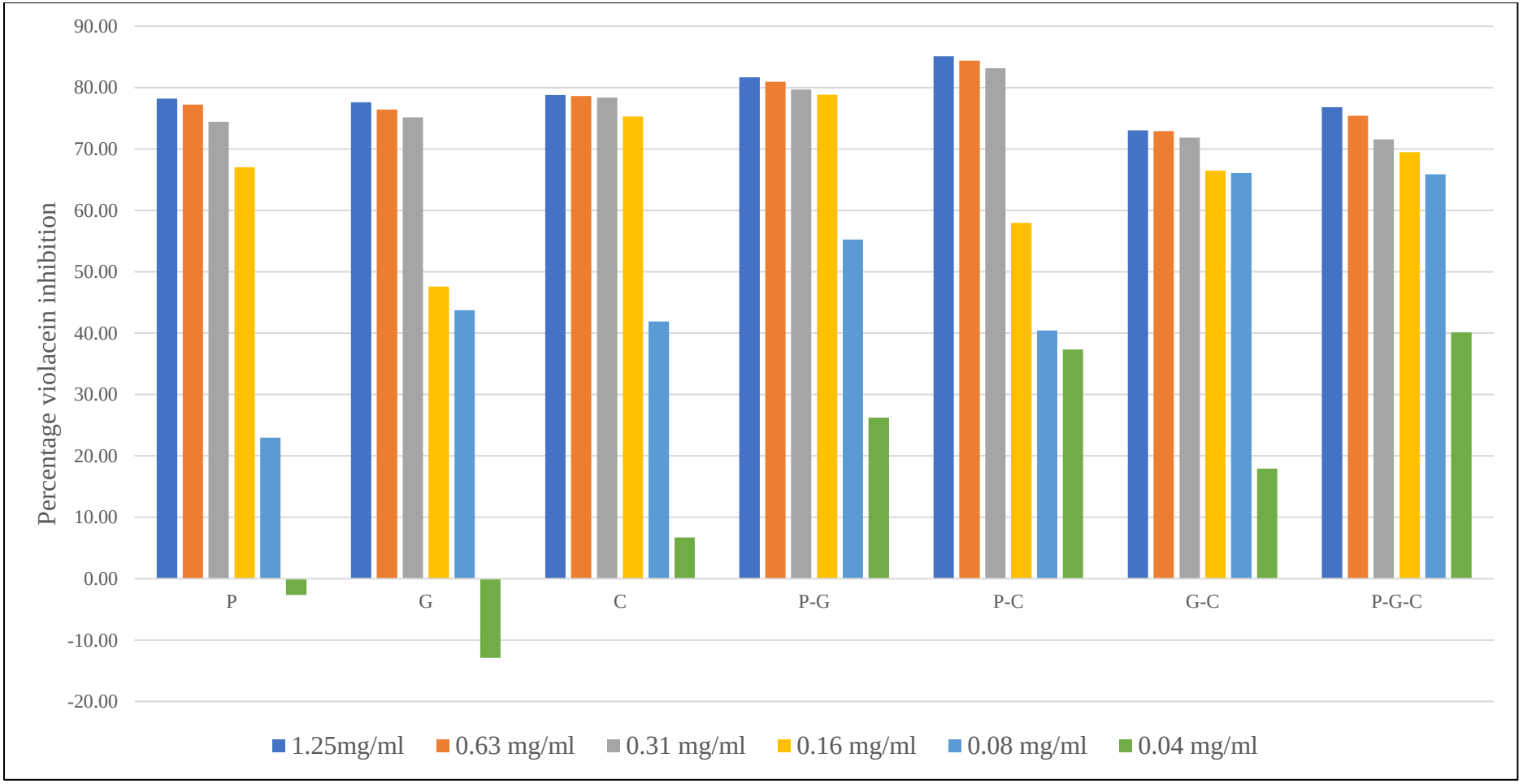


Figure 3.3: The percentage violacein inhibition of pinocembrin (P), galangin (G), chrysin (C) and their combinations (P-G = pinocembrin-galangin, P-C = pinocembrin-chrysin, G-C = galangin-chrysin).

Table 3.7: Summary of the MQSIC, MBC, percentage violacein inhibition at 1.25 mg/ml, and of the interactive efficacy of the combinations between pinocembrin, galangin and chrysin

Combination	MQSIC (mg/ml)	MBC (mg/ml)	ΣFQSIC	Interpretation	ΣFBC	Interpretation	% inhibition at 1.25 mg/ml
Pinocembrin - Galangin	0.08	0.31	0.38	**Synergistic***	0.25	**Synergistic**	81.66
Pinocembrin - Chrysin	0.16	0.63	1.00	Additive	0.38	**Synergistic**	85.10
Galangin - Chrysin	0.08	1.25	0.38	**Synergistic**	0.75	Additive	73.03
Pinocembrin - Galangin – Chrysin	0.08	0.63	0.42	**Synergistic**	0.42	**Synergistic**	76.81

*synergy indicated in bold.

The interaction between pinocembrin and galangin produced the lowest FBC index of 0.25. This combination, along with the triple combination between all three compounds, showed synergistic interactions in terms of both their anti-QS activity and their bactericidal effects.

3.4. Discussion

Although anti-QS activity has previously been reported from other flavonoids (Skogman et al., 2016; Hariri et al, 2017), this study is the first to demonstrate that pinocembrin, galangin and chrysin are capable of inhibiting violacein production from the monitor strain *C. violaceum*, and thus possess anti-QS activity.

The MQSIC is a measure of potency; the lower the MQSIC of a compound, the more potent it is (Mahumane, 2016). When testing for anti-QS activity, the optimal result would be the obtainment of a low MQSIC value together with a high percentage violacein inhibition (Mahumane, 2016). The MQSIC values noted in this study were all lower than the MBC values, agreeing with both the studies from Mahumane (2016) and Khan et al. (2009). The effects of pinocembrin, galangin and chrysin on QS are concentration dependent, as the inhibition of violacein production from these compounds correlated with an increase in compound concentration. The negative percentage inhibition noted from pinocembrin and galangin at 0.04 mg/ml means that instead of inhibition, these compounds potentiated QS. This study thus suggests that the propolis compounds should be used at higher concentrations, for the purpose of disrupting QS pathways. It must be noted that if a sufficient concentration of the required compounds is not reached at the site of the infection, a paradoxical effect may take place whereby QS may actually be potentiated. It would thus follow that to achieve a high enough concentration, a mixture of pure compounds may accord a higher bioavailability of pinocembrin, galangin and chrysin than would propolis as a natural substance.

The MQSIC can be interpreted in two ways: qualitatively, it is seen from the first test tube or the lowest compound concentration that does not produce a purple pigment when inoculated with *C. violaceum*; quantitatively, it is interpreted as the lowest compound concentration that produces a percentage violacein inhibition of over 50% (Ahmad et al, 2014; Mahumane, 2016). From the results obtained using the macrodilution method, it can easily be seen that the qualitative and

quantitative data closely match one another. While the MQSIC can thus be interpreted from the macroscopic appearance alone, it is imperative to go one step further and also procure violacein inhibition data. This will allow for quantitative analyses, which is largely considered to be more reliable (Lowhorn, 2007). Performing both qualitative and quantitative assays on QS also confirms the accuracy and reproducibility of results.

Results show that pinocembrin, galangin and chrysin all display higher violacein inhibition profiles than vanillin, the positive control and a known QS inhibitor (Chenia, 2013). This clearly demonstrates that these three compounds have great potential as anti-QS agents. However, it must also be noted the MQSIC of vanillin (0.08 mg/ml) was noted at a lower value than found for pinocembrin, galangin and chrysin. The MQSIC of vanillin in this study was lower than the results from Mahumane (2016) which showed a MQSIC of 0.39 mg/ml for vanillin as well as the findings from Tomadoni et al (2016) which reported an MQSIC value of 0.31 mg/ml for vanillin. The low MQSIC value for vanillin found in this study is nonetheless not surprising as previous studies have promoted vanillin as a promising agent to stop both QS and biofilm production (Tomadoni et al., 2016).

Apart from chrysin showing the highest percentage violacein inhibition, it also showed the lowest MQSIC value along with pinocembrin at 0.16 mg/ml. These factors combined show chrysin as a potent QSI with great potential as an anti-virulence agent. Although no other studies have tested the effects of chrysin alone on *C. violaceum*, Manner and Fallarero (2018) have reported that chrysin inhibits the QS-mediated swimming motility of *P. aeruginosa*. This is supported by Hariri et al. (2017) who have reported that a flavone mixture of apigenin, chrysin, and wogonin in combination with penicillin or streptomycin reduced planktonic growth as well as culture optical density of *P. aeruginosa*. Furthermore, Wang et al. (2011) have reported that chrysin in combination with β-lactam antibiotics is an effective agent against *Staphylococcus aureus* pneumonia, as it works by inhibiting virulence factors instead of focusing on bacterial eradication. Finally, Skogman et al. (2016) tested the effects of a number of flavonoids against QS using the monitor strain *C. violaceum* and found most of the compounds that showed good QSI activity belonged, like chrysin, to the flavone class. The authors theorize that the reason for flavones displaying potent activity against QS are due to the functional groups in their chemical structure

positively influencing QS inhibitory activity. With regards to the bactericidal activity, chrysin surprisingly displayed the highest MBC value. A high MBC value coupled with a low MQSIC value means that chrysin inhibits QS activity with little effect or selective pressure on the bacterial cells, thus making it a promising anti-virulent agent as chances of resistance to this compound is low (Ahmad et al., 2014).

Alvarez et al. (2012) have reported that propolis from Argentina decreased violacein production at 1 µl/ml and showed a bacteriostatic effects at 2 µl/ml. These are promising findings, however, the anti-quorum sensing activity of propolis varies according to the area that the propolis is sourced from due to chemical variations and varying absorption spectra (Savka et al., 2015). Although no previous studies were found on the effects of South African propolis against QS, Savka et al. (2015) tested the anti-quorum sensing activity of ten propolis samples from the United States. Their results showed that the three propolis samples which showed the best anti-QS activity, were the ones that were rich in the flavonoids pinocembrin, galangin and chrysin (Savka et al., 2015). This is a good indication that the compounds in South African propolis may also be effective quorum sensing inhibitors. LaSarre and Federle (2013) have pointed out that the separation and subsequent testing of individual compounds in natural products is necessary to understand the roles and mechanisms by which the compounds work. These authors reported that when the phenolic compound curcumin was isolated from the antimicrobial agent turmeric, the former was found to possess better QSI activity against *P. aeruginosa* than the latter. The findings that the compounds isolated from propolis are effective as quorum sensing inhibitors are therefore not surprising. Although pinocembrin and galangin have slightly lower activity against *C. violaceum* than chrysin, their potential as possible QSIs are still promising. This study showed pinocembrin to inhibit *C. violaceum* at a concentration of 0.16 mg/ml, with a percentage inhibition of 78.23% at 1.25 mg/ml. This finding is supported by Savka et al. (2015) who reported that pinocembrin isolated from Bulgarian propolis inhibited the growth of *C. violaceum* in a disk diffusion assay. No previous studies were found on the effects of galangin on QS, although other flavonoids have been studied for their anti-QS potential. Borges et al. (2014) have tested the effects of a number of flavonoids at a concentration of 1 mg/ml on violacein production and reported the percentage inhibition values as follows: epicatechin 33%; phloridzin 48%; oleuropein glucoside 51%; gallic acid 59%; ferulic acid 72%, and caffeic acid 75%. The results of the percentage violacein inhibition from the latter

two flavonoids is similar to the results from the flavonoids tested in this study. Kalia (2013) have also reviewed a number of flavonoids, and reported that naringenin, kaempferol, quercetin and apigenin all possess anti-QS activity when tested against *Vibrio harveyi*, working by inhibiting HAI-1 (*V. harveyi* auto-inducer 1) or AI-2 mediated bioluminescence. There is thus strong support for the use of flavonoids against QS.

While the results for the higher concentrations of compounds tested were comparable with the macrodilution method results, the results of the lower concentrations varied. The percentage violacein inhibition results from the lower concentrations tested in the microdilution method were much higher than those obtained from the macrodilution method. As the MQSIC is interpreted quantitatively as the lowest concentration that yields a percentage inhibition of over 50%, reporting on MQSIC values using the microdilution method would give different values and thus could not be used. A possible reason for the observed difference in results may be due to the growth pattern of *C. violaceum*: this pathogen forms a purple pigmented ring on the walls of the container it is grown in. Using the macrodilution method, test tubes could be vortexed to incorporate the purple pigment into the broth before analyzing the violacein inhibition. This could not be done with the microdilution method as the micro-titre plate could not be vortexed, leaving the purple pigmented ring attached to the walls of wells in the plate instead of including it into the broth (Figure 3.4).

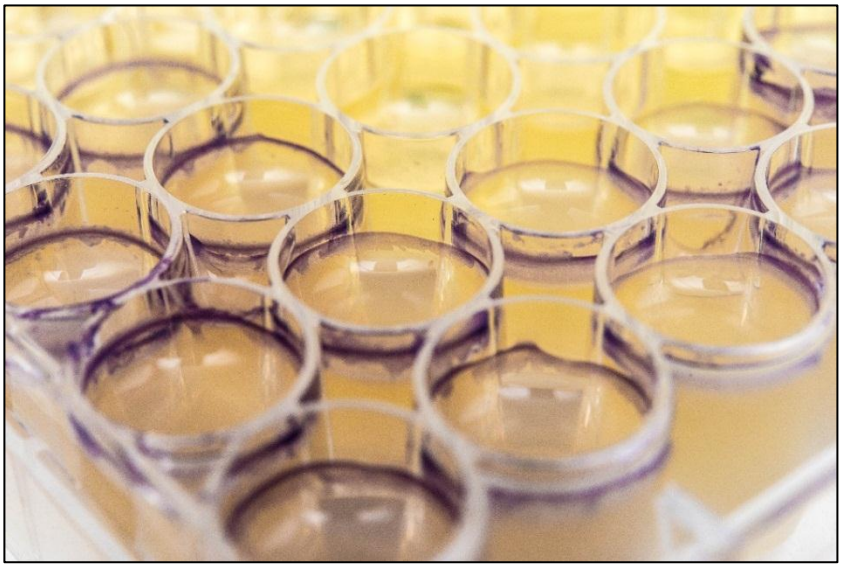


Figure 3.4: Growth of *C. violaceum* in a 48-well micro-titre plate.

This leads to the resultant solution reflecting a higher violacein inhibition value which is not accurate. This study theorizes that should a way be found to incorporate the purple pigmented ring from the wells into the broth, the microdilution method can be used as an easier and more accurate method in studying the effects of agents on QS. This could potentially be achieved by using a microplate vortex mixer. Despite the differences in the quantitative results from the macrodilution and microdilution methods, the qualitative results were consistent throughout. Therefore, the microdilution method could be used a quick and easy way to obtain qualitative data on the anti-QS activity of test compounds.

The combinatorial effects between other natural sources on QS have been studied previously, such as in the case of Mahumane (2016) who reported on the additive effect between *Eucalyptus radiata* and *Melaleuca alternifolia* essential oils in inhibiting QS. This lends credence to the testing of compound combinations against QS, especially since the antimicrobial effects of propolis have long been attributed to interactions between its constituent flavonoids (Fokt et al., 2010). This study researched the effects of interactions between pinocembrin, galangin and chrysin at 1:1 and 1:1:1 combinations on QS using the MQSIC and MBC of the combinations against *C. violaceum* as an indicator.

When the anti-QS interactions between the compounds were investigated, it was found that three combinations (75%) were synergistic, and one combination (25%) was additive. The most promising combinations were the double combination between pinocembrin and galangin at a 1:1 ratio, and the triple combination between all the compounds at a 1:1:1 ratio. Unlike the independent compounds that were tested, there were no negative violacein inhibition values noted for the combinations, even at very low concentrations. This means that the chances of the compounds enhancing quorum sensing is noticeably reduced when they are used in combination. No previous studies were found on any of these combinations against QS, although the review by Kalia (2013) discusses synergy between QSIs and antibiotics. The flavonoid baicalcein, when combined with ampicillin, showed synergy against *P. aeruginosa*, while other QSIs such as Furanone C30, patulin and garlic also displayed a synergistic effect when combined with tobramycin against *P. aeruginosa* (Kalia, 2013). This demonstrates the potential of using QSIs in combination.

It is interesting to note that whilst chrysin was the best QSI from the three compounds overall, the strongest 1:1 combination was the one that did not include chrysin as one of its components. The impact of synergy between compounds is clearly highlighted here. The final point to note is that three out of the four combinations showed synergy in exerting a bactericidal effect on *C. violaceum* cells. This indicates that the activity of the compounds is enhanced through more than just one mechanism.

3.5. Overview

- A broth microdilution method used to test the effects of agents against QS was tested for the first time in this study.
- Chrysin showed the strongest anti-QS activity with a violacein inhibition of 78.82% and a MQSIC value of 0.16 mg/ml.
- The combination of pinocembrin and galangin displayed the strongest anti-QS activity from the combinations with a MQSIC value of 0.08 mg/ml, violacein inhibition at 81.66% and the lowest MIC value of 0.31 mg/ml.
- Two out of the three 1:1 combinations (pinocembrin-galangin and galangin-chrysin) displayed synergy when tested for their ability to inhibit quorum sensing.
- Two out of the three 1:1 combinations (pinocembrin-chrysin and galangin-chrysin) displayed synergy when tested for their ability to exert a bactericidal effect on *C. violaceum* cells.
- The triple combination of pinocembrin, galangin and chrysin showed a synergy both in inhibiting quorum sensing, and in killing off *C. violaceum* cells.

CHAPTER 4

Antibiofilm Activity

4.1. Introduction

Biofilm formation is a mechanism that micro-organisms use to adapt and survive in a constantly changing environment (Malheiro and Simões, 2016). It is a complex process that involves the microbial cells adhering to a surface, multiplying, maturing and producing an extra polysaccharide (EPS) matrix (Drago and Toscano, 2016; Malheiro and Simões, 2016). The bacterial cells are then embedded within the matrix and become resistant to attack from the body's immune system due to the limited rate of antibiotic diffusion (Oliveira and de Lourdes, 2010; Drago and Toscano, 2016). These cells are then capable of detaching from the original surface and colonizing new surfaces (Drago and Toscano, 2016). The adherent population of micro-organisms are referred to as sessile bacteria, differentiated from planktonic bacteria which are single, free-floating cells (Olson et al., 2002). However, bacteria can switch between the sessile and planktonic forms, depending on the environmental factors (Garrett et al., 2008; Landini et al., 2010). Biofilm development and its subsequent structure and chemistry depends on several factors including the surrounding environment and the characteristics of microbes forming the biofilm (Jefferson, 2004). It should be noted that the growth of biofilms occurs more slowly than the growth of planktonic organisms, due to limited oxygen and nutrient availability (Donlan, 2001). Whilst planktonic micro-organisms have free access to nutrients, and are capable of multiplying at a rapid rate, sessile bacteria are limited in terms of both motility and access to nutrients (Olson et al., 2002). The growth rate is thus slowed down significantly (Olson et al., 2002). Possible causes for resistance of sessile cells to antibiotics include: slow growth of biofilm cells, the ability of the cells to form aggregates, their capability to resist clearance by the host system supporting its growth, and their ability to alter the physiology of the constituent micro-organisms (Mah and O'Toole, 2001; Olson et al., 2002). It has been postulated that bacteria in the sessile form are at an advantage

as they are capable of benefitting from what has been called "group behaviour" which includes gene transfer and the division of microbial labour (Jefferson, 2004). Studies have shown that the susceptibly of bacteria to the antibiotics, ciprofloxacin and tobramycin, decrease with decreasing growth rate, thus highlighting reduced susceptibility of biofilms to antimicrobial agents (Mah and O'Toole, 2001). Jefferson (2004) reports that the concentration of antibiotics required to kill planktonic bacteria needs to be between ten to 1000 times higher to have an effect on biofilms. Furthermore, it has been reported that the age of the biofilm affects antibiotic susceptibility, with older biofilms showing more resistance to antibiotics than newly formed biofilms, thus making it more difficult to treat conditions in which biofilms have been allowed to grow over an extended period of time (Donlan, 2001). In addition, the thickness of the biofilm layer determines the degree of antimicrobial diffusion: Mosquera-Fernández et al. (2016) report that compared to a single layer of cells, antibiotics take 100 times longer to diffuse through biofilms which contain around ten layers of cells.

Biofilms are implicated in a number of medical conditions, including native valve endocarditis, middle ear infection, osteomyelitis, and dental caries (Jefferson, 2004). In fact, any type of implanted medical device is susceptible to biofilm development, thus increasing the potential for causing infection (Bryers, 2008). This leads to an increased incidence of nosocomial infection, as corroborated by Roy et al. (2018) who reports that approximately 50% of nosocomial infections occur due to the presence of an inserted medical device. Jefferson (2004) reports that when bacteria are released from a biofilm, they are released in a large quantity and rapidly become virulent, whilst Cady et al. (2012) estimate that around 80% of microbial infections are associated with biofilm development. It is thus imperative to study biofilms and investigate compounds that are capable of acting as antibiofilm agents.

Stoodley et al. (2002) outlined the four stages of biofilm formation, using *P. aeruginosa* as a model organism. Stage one involves the initial attachment of cells to a surface, which is a reversible process. Stage two is an irreversible process that includes production of the EPS matrix and the further adhesion of the cells in the matrix to the relevant surface. Stage three comprises of the biofilm maturation process and formation of a biofilm architecture which includes the formation of channels and pores that allow for the transport of water, nutrients and waste, and the formation

of cavities which provide protection for planktonic bacteria. Stage four, the final stage, is labelled as the detachment stage in which planktonic bacteria are released from the biofilm, after which they are capable of moving elsewhere to form new biofilms (Stoodley et al., 2002; Zacchino et al., 2017; Roy et al., 2018).

Although bacteria in the laboratory are often grown in the planktonic form, in their natural environment they are often deprived of nutrients thus causing them to morph into sessile bacteria and form biofilms (Jefferson, 2004). It is thus imperative to test the effect of antimicrobial agents not only on planktonic, or free, bacteria, but also their effects on biofilm prevention and destruction. Three micro-organisms were selected to examine this further; *Listeria monocytogenes* (representing the Gram-positive pathogens), *Escherichia coli*, (Gram-negative pathogens) and *Candida albicans* (representing yeasts).

Listeria monocytogenes is a Gram-positive ubiquitous pathogen found in the soil, water and sewage and is associated with high incidences of foodborne diseases (Farber and Peterkin, 1991; Todd and Notermans, 2011). In South Africa, 748 cases of listeriosis were reported from the beginning of 2017 to the beginning of 2018, and 42% of patients with a known outcome of listeriosis, died (Frean et al., 2018). The high incidence rate of listeriosis in this case is from contaminated food processing plants (Todd and Notermans, 2011). Despite attempts to eradicate *Listeria* spp. from these food processing plants, "persister" cells have been found. These persister cells are thought to be the result of biofilm formation, which is difficult to completely eradicate (Harvey et al. 2007; Todd and Notermans, 2011). It has been discovered that *L. monocytogenes* adsorbs easily onto surfaces (Kalmokoff et al., 2001). Doijad et al. (2015) report that this pathogen can form a mature biofilm within 12 to 24 hr, and that build-up is two dimensional with no defined structure. In contrast, Marsh et al. (2003) describe a honeycomb-like architecture formed by *L. monocytogenes* biofilms. No matter the structure, it is important to find agents that reduce biofilm formation of *L. monocytogenes*, especially in the South African context, as diseases caused by this pathogen are often fatal especially in high risk groups (Marsh et al., 2003).

Escherichia coli is a facultative anaerobic Gram-negative pathogen commonly found in the gastrointestinal tract and is transmitted through the consumption of contaminated food (Beloin et

al., 2008: Chekabab et al., 2013). It is a prevalent cause of nosocomial infections in hospitalized patients (Gaynes and Edwards, 2005). Eradication of this pathogen is sometimes difficult due to the ability to form biofilms (Bazargani and Rohloff, 2016). Soto et al. (2007) have reported that this ability is what allows *E. coli* to remain in the genito-urinary tract for extended periods of time, causing urinary tract infections with alarmingly high frequency. Despite this, very few studies have been undertaken regarding the nature of *E. coli* biofilms. O'Toole et al. (2000) have reported that *E. coli* biofilms share many properties with *P. aeruginosa* biofilms, including thickness and the formation of microcolonies and water channels. The authors also point out that biofilms formed from different *E. coli* strains vary slightly. For example, the strains of *E. coli* K-12 does not form biofilms in a minimally supplemented environment, whereas, the *E. coli* O517:H7 strain only forms biofilms in a nutrient-poor environment (O'Toole et al., 2000). Unlike many other bacteria, *E. coli* does not directly use AHLs to form biofilms, but instead makes use of the auto-inducer AI-2, a quorum sensing molecule (Reisner et al., 2003; van Houdt and Michiels, 2005). Due to the increasing prevalence of infections caused by *E. coli*, it is imperative to find an agent that acts against biofilms caused by this bacterium.

Candida albicans is an opportunistic fungal pathogen often found in the gastrointestinal and reproductive tracts, as well as in the oral cavity and on the skin of resident hosts (Nobile and Johnson, 2015). Whilst *C. albicans* is usually not pathogenic, a weakened immune system allows it to attack and cause infections in humans (Nobile and Johnson, 2015). Although these infections are sometimes superficial and easily treatable, at other times they are more serious and can become life-threatening (Seneviratne et al., 2008). Mortality rates of patients infected with candidiasis have been reported at numbers as high as 60% (Seneviratne et al., 2008). Candidiasis often occurs due to *Candida* biofilm formation on indwelling medical devices (Chandra et al., 2001). In particular, *C. albicans* has commonly been implicated in disseminated bloodstream infections from biofilm formation on central venous catheters (Nobile and Johnson, 2015). In fact, *C. albicans* is the pathogen most often responsible for fungal biofilm formation (Bonhomme and d'Enfert, 2013). Due to a high level of resistance from *C. albicans* to conventional antibiotics, implant removal of any medical device is necessitated if the biofilm itself cannot be eradicated, thus leading to high hospitalization and economical costs (Seneviratne et al., 2008). Thus, the search for new agents that could eradicate *C. albicans* biofilms is imperative. The structure of *C. albicans* biofilms is

complex, involving yeasts, hyphae, pseudo hyphae, and extracellular matrix within a three-dimensional network (Nobile and Mitchell, 2006; Uppuluri et al., 2009).

Agents that inhibit biofilms act by either killing the bacteria incorporated in the biofilm; by dispersing preformed biofilms; by inhibiting the development and maturation of the biofilm; or through inhibiting the adhesion or early formation of biofilms (Oliveira and de Lourdes, 2010; Silva et al., 2016). It is widely acknowledged that inhibiting the formation of biofilms is the favoured strategy as bacteria in the planktonic form are more vulnerable to antibiotic activity (Silva et al., 2016).

The methods of inhibiting biofilms can be classified as either preventative (inhibition of bacterial adhesion) or disruptive (acting on preformed biofilms) (Alves et al., 2013). Although the application of mechanical and physical stresses to biofilms is an effective method of removing them, most surfaces are not easily accessible, thus, agents that disrupt biofilms through biochemical means need to be investigated (Silva et al., 2016). Natural products have grown as a source of interest as antibiofilm agents, as they disrupt the biofilm structure without killing off the constituent bacterial cells thus containing a low risk of bacterial resistance (Rabin et al., 2015). Amongst the natural products tested, flavonoids have shown promising activity against biofilms. A review by Silva et al. (2016) reported that flavonoids are effective against the formation of, and in the dispersal of, biofilms formed by *Staphylococcus aureus*. A popular theory in literature is that agents that are capable of inhibiting quorum sensing (QS), are also effective as antibiofilm agents (Landini et al., 2010; Silva et al., 2016).

Propolis extracts from Italy, which also contains high levels of pinocembrin, galangin and chrysin, were capable of inhibiting *P. aeruginosa* biofilms at sub-MIC concentrations and in a concentration dependent manner (De Marco et al., 2017). This is validated by Lamberte et al. (2011) who found that the ethanolic extract of propolis from the Philippines was capable of inhibiting biofilms caused by *P. aeruginosa* at lower concentrations. In contrast, Dogan et al. (2014) tested the effects of propolis from Turkey against a number of bacterial biofilms and reported that the higher concentration of propolis samples showed better biofilm inhibition percentages. However, Turkish propolis does not contain pinocembrin, galangin or chrysin but is

instead made up of different flavonoids (Dogan et al, 2014). Looking at biofilms caused by fungal pathogens, Capoci et al. (2015) report that propolis reduces the formation of biofilms caused by *Candida albicans*. Despite the adequate amount of research done on the effects of propolis on biofilms, no studies investigating the effects of the propolis compounds on biofilms were found. Many natural products have an effect on inhibiting biofilm formation and development (Silva et al., 2016). Similarly, many antibiotics are capable of reducing biofilms, but complete elimination of the biofilm structure is difficult to achieve (Roy et al., 2018). The use of antimicrobial or polyherbal combinations to achieve the complete eradication of biofilms has thus gained interest in the scientific community. Roy et al. (2018) point out that combinations are advantageous as the agents in the combination can inhibit the biofilm through different mechanisms. The example the authors give is that while one agent may inhibit the growth of dormant cells, the other agent could work against growing cells. A practical example found in literature is the combination of low molecular weight natural products with conventional antibiotics, which have been found to be effective in the eradication of biofilms (Zacchino et al., 2017).

This is the first study to investigate the effects of pinocembrin, galangin and chrysin, as well as the combinations between these compounds, on biofilm prevention and destruction using selected pathogens.

4.2. Materials and methods

4.2.1. Preparation of compounds

Pinocembrin, galangin and chrysin were found to be insoluble in water, thus a cyclodextrin inclusion complex was prepared for each compound. This was done by using the co-precipitation method of complexation as explained by Hedges (1998). The method is as follows: 50 mg of compound dissolved in 1 ml of acetone was added to a solution of 2 g of 2-hydroxypropyl-β-cyclodextrin (Aldrich) and 20 ml of water. The resultant solution was mixed overnight using a magnetic stirrer on low speed and low heat (i.e.: 25°C) and then left under a fume hood for 48 hr to allow the liquid to evaporate. The resultant crystals that were formed were dissolved using 41 mg of the complex to 1 ml of water to give a concentration of 1 mg/ml of the test compound (as

40 mg of 2-hydroxypropyl-β-cyclodextrin was used for every 1 mg of compound when making up the complex).

4.2.2. Preparation of cultures

Three American Type Culture Collection (ATCC) strains were prepared for use in this study, based on their biofilm-forming abilities and clinical relevance. The pathogens included *L. monocytogenes (*ATCC 19111), *E. coli* (ATCC 25922) and *C. albicans* (ATCC 10231). These three were inclusive of Gram-positive, Gram-negative, and fungal pathogens, respectively.

4.2.3. Preparation of controls

Conventional antibiotics that are known to prevent biofilm formation and development were used as positive controls. Ciprofloxacin was prepared to a stock concentration of 2.5 μg/ml and used as the positive control for the bacteria, while amphotericin B was prepared to a stock concentration of 5.0 μg/ml and used as the positive control for the yeast species. A negative control of 2-hydroxypropyl-β-cyclodextrin (which was used to complex the compounds), was also prepared at 40 mg/ml. To make up the cyclodextrin complex, 40 mg of 2-hydroxypropyl-β-cyclodextrin was used for every 1 mg of compound, and compounds were tested at 1 mg/ml.

4.2.4. Preparation of crystal violet stain

To make the 1% crystal violet solution needed for the biofilm assay, the following steps were carried out: 2 g of the crystal violet powder (Sigma) was dissolved in 95% ethanol. Separately, 0.8 g of ammonium oxalate monohydrate was dissolved in 80 ml sterile distilled water. Finally, the crystal violet and ammonium oxalate monohydrate solutions were mixed together to make the crystal violet stain.

4.2.5. Biofilm assay

The effects of pinocembrin, galangin and chrysin were tested using the method described by Sandasi et al. (2008). Briefly, the method of testing was performed as follows; a single colony of each culture was transferred to a corresponding test tube containing sterile Tryptone Soya broth (TSB) and incubated overnight according to optimal incubation conditions. The following day, 100 µl of each culture was transferred into separate wells of a 96-well micro-titre plate and the absorbance read at 590 nm using a FilterMax F5 multi-mode microplate reader (Molecular Devices). Acceptable absorbance is approximated around 0.02 at 590 nm, as this absorbance indicates that the culture contains about $1x10^6$ CFU/ml, approximately equivalent to a 0.5 McFarlands standard as used for the MIC assay (Chapter 2). If absorbance of the liquid cultures was found to be more than this, the cultures were diluted in sterile TSB until the desired absorbance was reached. Once the desired absorbance was reached, the culture could be used as the stock culture in the assay. A volume of 100 µl of the prepared stock culture was then added to each well of a new 96-well micro-titre plate.

To test the effect of the compounds on planktonic cells, 100 µl of each compound (or for 1:1 combinations, 50 µl of each compound; or for 1:1:1 combinations, 33.3 µl of each compound) was added to the well of the micro-titre plates immediately after the cultures were added. A blank column containing 200 µl of sterile TSB; a culture control column, containing 100 µl of sterile TSB and 100 µl of the culture used; and a positive control column, containing 100 µl of the culture being tested and 100 µl of antibiotic (ciprofloxacin for bacteria or amphotericin B for yeasts), were also included. All tests were repeated in quadruple to ensure accuracy of results. A diagram of a plate testing the effects of the three compounds and their combinations against *E. coli* is shown in Figure 4.1. The micro-titre plate was then sealed using an adhesive sealing film and incubated at 30°C overnight, after which the crystal violet staining assay was performed.

To test the effects of agents on preformed biofilms, the same method was used, with the exception that the compounds were not added immediately to the plates. Preformed biofilm assays were performed on 4 hr, 24-hr, 48 hr and 72 hr preformed biofilms. After adding the cultures to the micro-titre plates, including all the control columns, plates were incubated at 30°C according to

the required times (4 hr, 24 hr, 48 hr, or 72 hr), after which the compounds were added. Plates were sealed and incubated at 30°C overnight after which the crystal violet (CV) assay was performed. Biofilm biomass was then determined, and percentage inhibition was calculated using Equation 4.1.

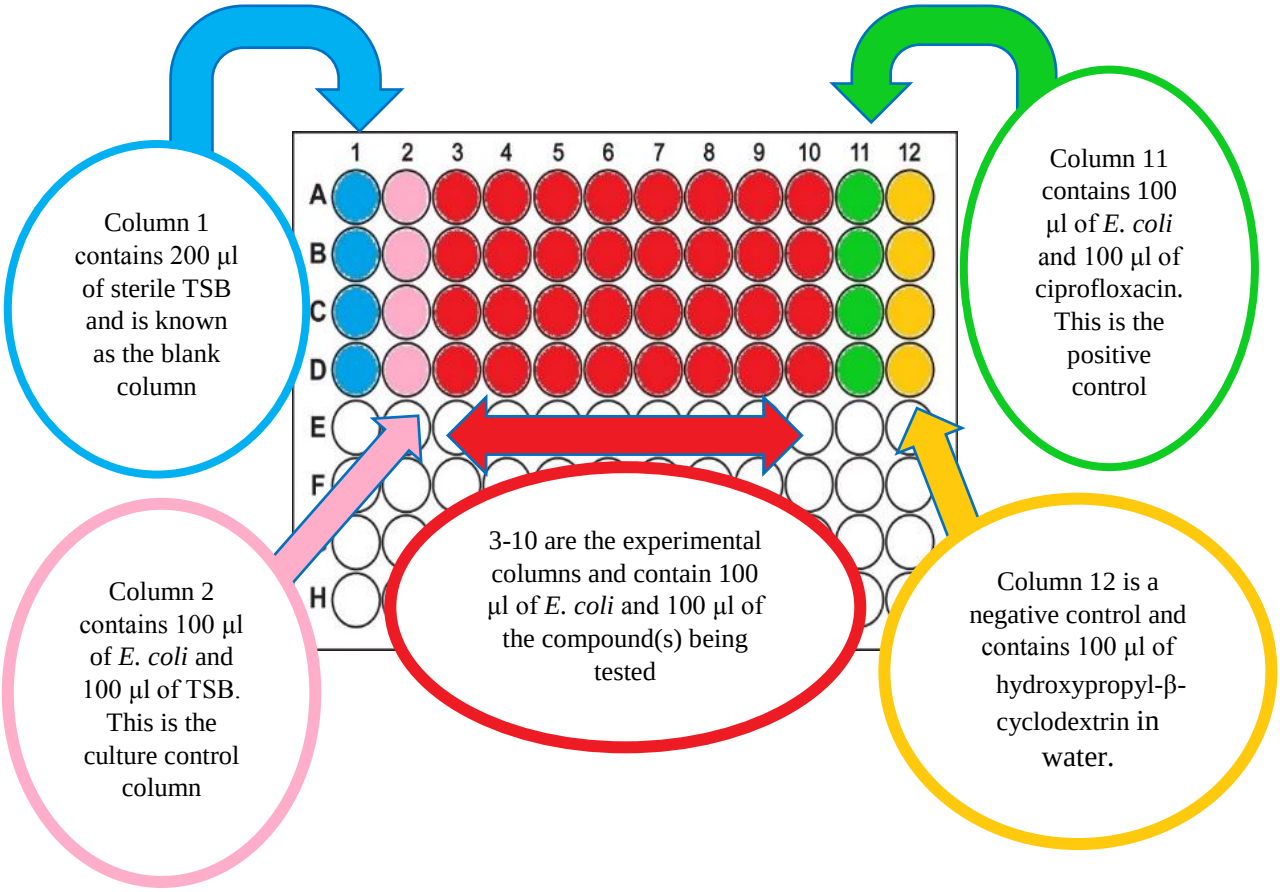


Figure 4.1: Representation of a plate testing the effects on biofilms of the three compounds and their combinations against *E. coli*.

Equation 4.1: Percentage inhibition of biofilm biomass.

$$\% \text{ inhibition} = \frac{\text{Optical density (OD) Culture control} - \text{OD Experimental} \times 100}{\text{OD Culture control}}$$

4.2.5. Crystal violet (CV) staining assay

The CV assay, described by Sandasi et al. (2011), was performed in order to assay for biofilm biomass. The CV assay was used as it allows for a quick analysis of effects of agents on biofilms, without having to undertake labour-intensive microscopic screening (Alves et al., 2013). This assay is also the most popular test done for the investigation of agents against biofilm formation and dispersal (Alves et al., 2013). The method is as follows: incubated plates were rinsed out thrice with sterile distilled water, air-dried for 15 min, and oven-dried at 60°C for 45 min. Following this, all wells were stained with 200 µl of 1% crystal violet and incubated at room temperature for 15 min. This step allowed the crystal violet to adsorb to any biofilms present in the wells. To remove the unabsorbed stain, plates were again rinsed with sterile distilled water three times. Next, 200 µl of ethanol was added to all wells to release or destain the retained colour from the sessile cells. A volume of 100 µl of the destaining solution was then transferred to a new plate (Figure 4.2) and the absorbance was determined at 590 nm using a FilterMax F5 multi-mode microplate reader (Molecular Devices). The mean absorbance of the compounds was determined, and percentage inhibition calculated as per Equation 4.1.

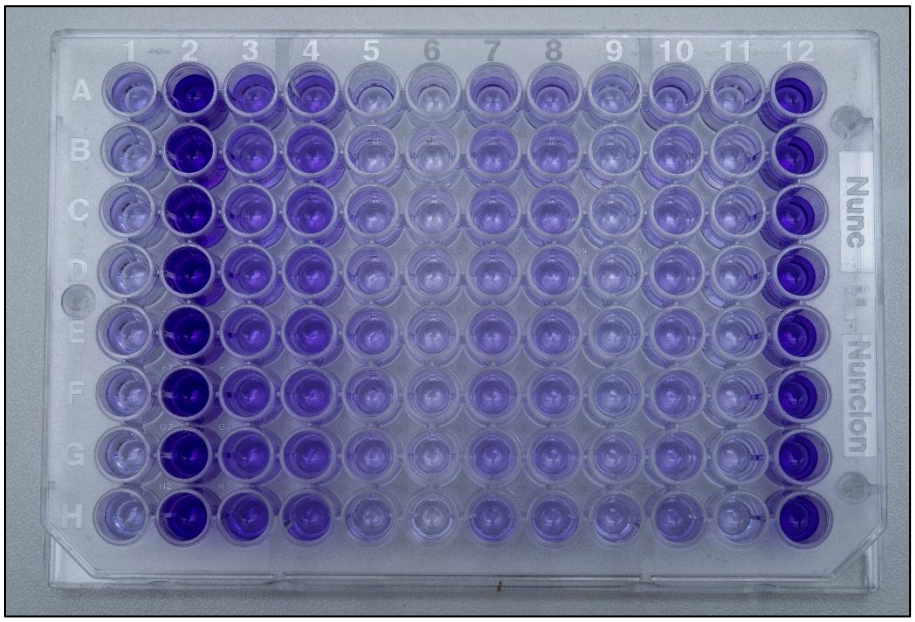


Figure 4.2: Crystal violet (CV) biofilm assay.

The compounds and controls added match those shown in Figure 4.1. Column 2, the culture control, stained the darkest as it had the highest rate of biofilm growth. Columns 3-10 contain the

compounds which all show some degree of biofilm inhibition, as indicated by the colour which is a lighter shade of purple than the culture control column.

4.3. Results

This chapter aimed to determine the antibiofilm activity of the compounds found in South African propolis (pinocembrin, galangin and chrysin) and their combinations against *L. monocytogenes*, *E. coli* and *C. albicans* using the 96-well microplate crystal violet assay at different time frames (0, 4, 24,48 and 72 hr) of biofilm development.

The results of the antibiofilm activity of all compounds and combinations are presented in Tables 4.1 – 4.3. The following criteria were set down for the interpretation of results, as determined by Sandasi et al. (2008) and Landini et al. (2010); values between >0 and 100% were interpreted as inhibitory activity, further broken down into either weak activity (>0 – 49%) or good activity (50 – 100%). Negative percentage inhibition results denoted enhancement, rather than inhibition, of biofilms. Results are categorized into five phases corresponding to biofilm developmental stages. Firstly, planktonic growth is represented at 0 hr; initial attachment of biofilms at 4 hr; biofilm formation at 24 hr; and development of a mature biofilm at 48 hr and 72 hr.

Against *L. monocytogenes*, the only compound that showed good activity at 0 hr was chrysin at 68.52%. All other compounds or combinations showed weak inhibitory activity or biofilm enhancement. The activity of ciprofloxacin, the positive control at 0 hr was excellent at an almost complete inhibition (99.16%). At 4 hr, the percentage inhibition of ciprofloxacin decreases slightly to 72.84%, but activity is still considered good. After this period, there is no enhancement of biofilm, and all the compounds and their combinations display weak anti-attachment activity. The combination of pinocembrin and galangin (percentage inhibition of 40.96%) is the only one that shows better activity than its individual components (percentage inhibition of pinocembrin and galangin 31.17% and 1.54% respectively). At 24 hr, the compounds possess optimal activity against the formation of *L. monocytogenes* biofilms. All compounds and combinations exhibit values over 50%, meaning that they all possess good activity against biofilm formation. In fact, galangin, chrysin and all four combinations actually have better activity than against biofilm

formation than did the positive control ciprofloxacin (61.94%). Interactions between the compounds seemed to be non-interactive, as combinations showed similar activity to individual compounds.

At 48 hr, activity given from the compounds decrease slightly, with only galangin showing good inhibitory activity (50.23%) against the development of a mature biofilm at this hour. At this stage too, compounds are non-interactive in combination. Interesting to note is that all compounds and 1:1 combinations show better activity than ciprofloxacin with an inhibitory effect of 16.83%. Activity of all compounds and combinations decreases at 72 hr with the best activity noted for pinocembrin at 29.64%. At 72 hr, the independent compounds have better activity than any of the combinations and ciprofloxacin.

The results obtained from the biofilm studies on *E. coli*, a Gram-negative pathogen, were vastly different from the Gram-positive *L. monocytogenes*. For *E. coli*, ciprofloxacin showed weak inhibitory activity with 23.38% inhibition at 0 hr, while all other compounds and combinations at 0 hr had lower activity than the positive control or enhancement of growth. At 4 hr, all of the compounds, as well as the galangin-chrysin combination, showed enhancement of biofilm formation while the other three combinations showed weak anti-attachment activity, possibly indicating that interactions between the compounds are at play. At 24 hr the activity of ciprofloxacin is 46.52%, which is still considered weak activity against biofilm formation. Pinocembrin showed weak activity (22.09%) while the other two compounds both have strong activity (57.84% for galangin and 79.73% for chrysin) against *E. coli* biofilm formation at 24 hr. As with *L. monocytogenes*, the inhibitory effect of the triple combination peaked at 24 hr. This combination percentage inhibition of 100% was higher than any other noted in this study. At 48 hr pinocembrin showed strong activity (77.01%) while galangin and chrysin showed weak inhibitory activity (21.18% and 16.72% respectively) against the development of a mature *E. coli* biofilm. The combinations at 48 hr showed either enhancement of the biofilm or displayed weak inhibitory activity against the developed biofilm, as did ciprofloxacin (3.56%). Apart from the combination of galangin and chrysin, all compounds and combinations showed better activity than ciprofloxacin at 48 hr.

Table 4.1: Percentage biofilm inhibition of pinocembrin, galangin, chrysin and their combinations against *L. monocytogenes* at different time intervals.

Compound	% Inhibition				
	0-hr	**4-hr**	**24-hr**	**48-hr**	**72-hr**
Pinocembrin	–92.26	31.17	55.69	48.46	29.64
Galangin	–93.31	1.54	71.43	50.23	20.4
Chrysin	68.52	12.74	62.05	36.46	18.42
Pinocembrin - Galangin	25.72	40.96	65.76	31.3	4.52
Pinocembrin - Chrysin	–63.02	36.87	75.01	42.6	5.72
Galangin - Chrysin	15.26	8.24	70.19	27.12	8.53
Pinocembrin - Galangin – Chrysin	6.55	39.01	75.84	7.1	–13.28
Ciprofloxacin	99.16	72.84	61.94	16.83	13.41
Cyclodextrin	–6.77	0.39	14.51	4.5	–3.43

Table 4.2: Percentage biofilm inhibition of pinocembrin, galangin, chrysin and their combinations against *E. coli* at different time intervals.

Compound	% Inhibition				
	0-hr	**4-hr**	**24-hr**	**48-hr**	**72-hr**
Pinocembrin	–24.47	–12.03	22.09	77.01	36.82
Galangin	–5.99	–15.63	57.84	21.18	27.17
Chrysin	0.56	–1.38	79.73	16.72	3.42
Pinocembrin - Galangin	20.12	3.11	–39.66	28.3	10.55
Pinocembrin - Chrysin	–0.24	2.3	–31.68	12.21	4.24
Galangin - Chrysin	5.06	–1.65	6.56	–2.03	26.31
Pinocembrin - Galangin – Chrysin	–65.67	11.78	100.00	3.88	5.44
Ciprofloxacin	23.38	41.72	46.52	3.56	37.72
Cyclodextrin	1.74	10.05	10.77	2.85	–1.38

Table 4.3: Percentage biofilm inhibition of pinocembrin, galangin, chrysin and their combinations against *C. albicans* at different time intervals.

Compound	% Inhibition				
	0-hr biofilm	**4-hr biofilm**	**24-hr biofilm**	**48-hr biofilm**	**72-hr biofilm**
Pinocembrin	48.06	52.11	54.42	55.55	8.83
Galangin	54.88	72.64	56.09	60.67	30.57
Chrysin	51.89	72.16	43.14	58.65	16.2
Pinocembrin - Galangin	36.95	47.06	47.15	75.31	–14.79
Pinocembrin - Chrysin	45.96	–0.05	40.72	63.63	–0.66
Galangin - Chrysin	36.7	69.14	28.85	66.32	24.99
Pinocembrin - Galangin – Chrysin	26.92	26.48	35.49	37.68	21.32
Ciprofloxacin	82.81	76.85	74.3	80.02	69.18
Cyclodextrin	5.03	18.61	16.03	12.31	–7.74

At 72 hr, all compounds and combinations possessed weak inhibitory activity against the mature biofilm, similar to the trend noted for *L. monocytogenes*. At this interval, pinocembrin was the only compound which showed similar activity to ciprofloxacin (36.82 vs 37.72%).

Activity against the fungal strains also varies from the bacterial strains. Amphotericin B was used as the positive control and showed good activity at all time intervals. At 0 hr, galangin (54.88%) and chrysin (51.89%) show good activity against the planktonic yeasts whilst pinocembrin and all of the combinations showed weak activity. Activity of all compounds when tested independently increased at 4 hr, at which time they all have good antibiofilm attachment activity. Galangin and chrysin, in particular, show excellent activity comparable to amphotericin B (72.64% and 72.16% vs 76.85% for amphotericin B). Activity of the pinocembrin-galangin and of the galangin-chrysin combinations also increased from 0 hr to 4 hr, although only the anti-attachment activity of the latter combination was considered good. Unlike with the bacterial strains, activity against *C. albicans* did not peak at 24 hr: rather, activity dropped from 4 hr for most compounds/combinations, including for amphotericin B. Pinocembrin and galangin were the only two compounds that displayed good activity against biofilm formation at this timeframe. The optimal interval in the case of *C. albicans* is at 48 hr, where all compounds and 1:1 combinations showed good inhibitory activity against the developed biofilm. The combination of pinocembrin and galangin (75.31%) at 48 hr is comparable to amphotericin B (80.02%). At 72 hr, activity of all the test compounds and their combinations against the mature *C. albicans* biofilm decreases again, mostly showing weak inhibitory activity and two of the 1:1 combinations (pinocembrin-galangin and pinocembrin-chrysin) displaying biofilm enhancement.

4.4. Discussion

Biofilm formation or development has been cited as one of the factors allowing for micro-organisms to develop a resistance to antibiotics (Høiby et al., 2010). In turn, a reduced susceptibility of bacteria and fungi to the conventional antibiotics used have led to recurring infections, as complete eradication of the biofilm is unlikely (Stewart, 2002). The severity of this problem is highlighted by a statement that an estimated 80% of chronic infections are due to the

presence of biofilms (Mishra and Wang, 2017). The results of this study show a few positive effects from the compounds and combinations studied, which could be used in the inhibition of biofilms.

Due to the large number of mechanisms given for biofilm antibiotic resistance, it is difficult to predict biofilm behavioural patterns (Romero et al., 2016). Biofilm structures are complex and heterogenous, thus, clear patterns on their susceptibility to antimicrobial agents are difficult to elucidate (Wimpenny et al., 2000). As each micro-organism shows a different biofilm structure, one micro-organism from each microbial class (Gram-positive, Gram-negative, and fungal populations) was chosen to examine for biofilm susceptibility.

Although there is a lack of studies on the compounds, particularly on their effects on biofilms, other flavonoids have been found to possess antibiofilm activity. Ramadan et al. (2017) have attributed the antibiofilm activity of a number of phytochemicals to the presence of their constituent flavonoids, including quercetin, which is also a component of propolis. A number of flavonoids have been found to possess antibiofilm activity against *E. coli* (Silva et al., 2016). The presence of hydroxyl groups on the flavonoid backbone has been reported to enhance activity against biofilms, with activity depending on the number and positions of the hydroxyl groups (Silva et al., 2016). Propolis itself has also been shown to possess antibiofilm activity (De Marco et al., 2017). It has been proposed that the mechanism of action of propolis against biofilms is structural, with the propolis ethanolic extract causing damage to the bacterial cell walls, which eventually leads to cell lysis (Bryan et al., 2015). A previous study has theorized that propolis contains both anti-bacterial and antibiofilm compounds: the antibiofilm compounds cause structural damage to the EPS matrix, reducing the thickness of the biofilm and allowing entry of the antibacterial compounds into the bacterial cells (Bryan et al., 2015).

Ciprofloxacin is effective against the *L. monocytogenes* planktonic cells at 0 hr, which is not surprising as antibiotics display better activity against bacteria in the fast-growing and dividing phase than against bacteria in the slow-growing, stationary phase (Olson et al., 2002; Sandasi et al., 2010). Interestingly, whilst the activity of ciprofloxacin decreased with each time interval, activity of the compounds increased up to the 24-hr mark, at which point all of the compounds and their combinations had good activity and most samples tested showed better activity than

ciprofloxacin. From this it can be deduced that ciprofloxacin works better on the planktonic cells and initial formation stage, while pinocembrin, galangin and chrysin work better against the formation of biofilms. One possible reason for this is that while ciprofloxacin exhibits a bactericidal effect on *L. monocytogenes* cells, the compounds all show a bacteriostatic effect at the concentrations tested (Chapter 2), possibly meaning that they take a longer time to exert their effects on biofilm cells. However, the combination of pinocembrin and chrysin, which was the only combination that showed a bactericidal effect against *L. monocytogenes* at a concentration of less than 1 mg/ml in Chapter 2, was also the combination that showed the best activity against the *L. monocytogenes* biofilm at 24 hr. In their study, Andriani et al. (2017) categorize their results into three groups: (1) samples that possess both antibacterial and antibiofilm activities; (2) samples that possess antibiofilm activity but lack antibacterial properties; and (3) samples that possess antibacterial properties but do not have antibiofilm activities. The authors explain that generally, there exists a direct correlation between the ability to inhibit biofilms and the ability to stop bacterial growth. In this study, the best antibiofilm activity was noted for *L. monocytogenes* at 24 hr. Correspondingly, when inhibitory and cidal activity against planktonic cells was tested at this same time interval, all compounds and combinations displayed bacteriostatic effects of less than 1 mg/ml. Most samples tested displayed antibacterial activity at less than the concentration used for biofilm studies, however, 1 mg/ml was used as the concentration of choice for the biofilm assay as previous research has shown that higher concentrations of agents are needed for the eradication of biofilms (Barbieri et al., 2017). Romero et al. (2016) have noted that in some cases, antibiofilm activity is dose dependent. It may, therefore, be possible that the antibiofilm activities of pinocembrin, galangin, chrysin and their combinations are more effective at higher concentrations. At 48 hr, only galangin showed good inhibitory activity against the developed *L. monocytogenes* biofilms while weak activity/enhancement was noted for all samples at 72 hr.

Against *E. coli*, chrysin again showed the best activity from all the samples tested at 0 hr. In contrast, when antibacterial studies were done (Chapter 2), chrysin showed the weakest activity from the individual compounds against *E. coli*, with it being the only compound with an MIC of above 1 mg/ml. A possible reason for this is given by Andriani et al. (2017) who suggest that agents with antibiofilm activity, but no antibacterial activity may work through other mechanisms, such as disrupting the matrix, or by eliminating the nutrients needed for biofilm formation. Another

possible explanation is that the agents may possess anti-QS activity which then lends activity towards biofilm disruption (Andriani et al., 2017). As in the case of *L. monocytogenes*, the independent compounds galangin and chrysin showed optimal activity at 24 hr, with chrysin possessing the best biofilm inhibitory activity noted against *E. coli* overall. On the other hand, pinocembrin showed better activity at 48 hr with an inhibitory effect of 77.01% against the developed biofilm, much higher than ciprofloxacin at 3.56%. Interestingly, pinocembrin showed the lowest MIC value out of all the compounds when tested against *E. coli*, meaning that it has both strong antibacterial and antibiofilm activity. The antibiofilm activity for all compounds peaked at 24 hr against *L. monocytogenes* i.e. worked well at the biofilm formation stage. Comparatively, against *E. coli* the two compounds (galangin and chrysin) also worked well at the formation phase. Furthermore, pinocembrin stopped the development of a mature biofilm. The same is true of the 1:1 combinations, with both combinations containing pinocembrin showing better activity at the 48-hr interval, and the galangin-chrysin combination working better at 24 hr. However, the true highlight of this section lies in the triple combination's results at 24 hr. At this stage, the effectiveness of this combination was 100%. This was the highest percentage inhibition noted from all the samples tested, although the same combination also showed good activity against the formation of *L. monocytogenes* biofilm at 24 hr (inhibition of 75.84%). Activity of ciprofloxacin against *E. coli* biofilms was weak at all time frames. This may be because *E. coli* possesses genes that encode high-level resistance to biofilms formed by this pathogen (Stewart, 2002). Overall, *L. monocytogenes* (which had nine compounds/combinations with good inhibitory activity) showed better susceptibility to the antibiofilm effects of the compounds than did *E. coli* (which had three compounds/combinations with good inhibitory activity). These results match the activity of the compounds against the planktonic bacteria tested in Chapter two, where it was found that Gram-positive bacteria were more susceptible than Gram-negative bacteria. These findings are also supported by Bryan et al. (2015) who reported that *S. aureus* biofilms were more susceptible than *E. coli* biofilms to the effects of propolis.

Against *C. albicans*, activity of the positive control was stronger than any of the samples tested, at all stages. This is not surprising as amphotericin B has been reported as the antifungal with the best activity against biofilms, while fluconazole, a common antifungal drug, has very weak activity (Nett, 2014). Unlike with the susceptibility of the bacterial biofilms, which showed positive results

at more at the 24-hr timeframe, the compounds seemed to attack *C. albicans* biofilms better at 4 hr and 48 hr, meaning that they work at the initial attachment stage and by stopping the development of a mature biofilm. At 48 hr, all the compounds and 1:1 combinations showed good inhibitory activity against the developed biofilm. However, the triple combination which showed exceptional activity against the formation of both bacterial biofilms tested at 24 hr, showed weak activity against the *C. albicans* biofilm at all time intervals tested. This demonstrates that susceptibility of fungal and bacterial biofilms varies greatly. Notwithstanding whether against a bacterial or fungal biofilms, it is surprising that compounds showed better activity at the 24-hr or 48-hr intervals, as previous studies have shown that inhibiting initial cell attachment is easier than inhibiting preformed biofilms (Sandasi et al., 2010).

Some compounds and combinations enhanced biofilm growth rather than inhibiting them. A possible reason for this is explained by Sandasi et al. (2010) who describe what is known as a conditioning film, which promotes microbial adhesion to a surface. In the same study, the authors report that certain natural products enhance the growth of biofilms *in vitro*. This is corroborated by Sandasi et al. (2008), who report that individual compounds of essential oils promoted biofilm growth. The authors suggest that better results would be obtained if the components were used together, as in a whole essential oil for example – or in this case propolis – instead of just the individual compounds.

Concerning the effects of compounds versus their combinations, no clear pattern was observed. Whilst the effects of certain combinations – such as the pinocembrin-galangin combination against *L. monocytogenes* at 0 hr and 4 hr – were better than either of their individual components, the opposite was true of other combinations. Searching through previous literature, conflicting reports were found on combination efficacy. Slobodníková et al. (2016) report that although ellagic acid and tannic acid both inhibit *E. coli* biofilm formation independently, they do not work synergistically when combined. Similarly, Nyila et al. (2012) discovered that while an extract of the leaves of the *Acacia karroo* plant showed weak antibiofilm activity, it's components β-sitosterol and epigallocatechin had stronger activity, concluding that the compounds work better when used independently. However, other studies have discovered the effectiveness of combining newfound antibiofilm agents with conventional antibiotics, which allows for the eradication of

biofilms (Khan and Ahmad, 2011; Rabin et al., 2015; Grassi et al., 2017; Zacchino et al., 2017). This may be because the heterogenous nature of the biofilm structure requires inhibition through different mechanisms (Grassi et al., 2017). Thus, even though the compound combinations tested in this study did not have as good activity as was expected, better results may be obtained by combining these compounds with an antibiotic drug or with any other agent that targets the biofilm cells in a different manner.

4.5. Overview

- Although no compound eradicated biofilms completely, studies demonstrated that out of the 45 biofilm studies, 18 (40%) showed good inhibitory activity (> 50%) across all of the micro-organisms and time frames tested.
- In contrast, only nine out of 60 (15%) combinations showed good inhibitory activity.
- The best activities were noted at the 24 hr timeframe for the bacterial biofilms, and at the 4 hr and 48 hr intervals for the fungal biofilm.
- Generally, the compounds worked better as individual compounds than when combined, with a few exceptions.
- The most notable exception to this is the triple combination which showed excellent activity (100%) against the formation of an *E. coli* biofilm. This combination should be considered in the treatment of biofilm-related infections caused by this pathogen.

CHAPTER 5

Toxicity Testing

5.1. Introduction

The toxicity of a compound is defined as its ability to exert harmful effects on a living organism (Mendonça et al. 2013). Toxicity is often cited as the reason for the failure of potential new drugs in the drug discovery process (Martin et al., 2014). Screening potential drugs or new compounds for toxic effects early on is thus crucial (Parasuraman, 2011). Although a few components of propolis, such as benzyl benzoate, benzoic acid and phenols, have been identified as potentially toxic, propolis itself has been found to be non-toxic at approximately 70 mg/day (Mendonça et al. 2013; Wagh, 2013). Flavonoids, which are the major constituents of propolis, have been reported to have a low level of toxicity (Burdock, 1998; Kesarkar et al., 2009). Although no *in vitro* studies were found that investigated the toxicity of the compounds in South African propolis, research has shown that pinocembrin exhibits no toxicity in rats at a maximum dose of 50 mg/kg body weight (Punvittayagul et al., 2012). Similarly, galangin has also been found to be non-toxic in mice, while chrysin has been shown to have protective effects against toxic agents in animal tissues (Huh et al., 2013; Samarghandian et al., 2017). No studies could be found on the toxicity of these compounds in combination. It must be noted that toxicity of compounds may also be affected by synergism and thus their toxicity profiles need to be evaluated not just singularly, but also as mixtures.

The brine shrimp lethality test is favoured as a screening assay as it is a simple, effective and reproducible means of testing for toxicity (Sarah et al., 2017). However, care must be taken when choosing the vehicle used to dissolve the test compounds in, as some organic solvents are highly toxic *in vitro* themselves (Geethaa et al., 2013; Wu, 2014). If an organic solvent is to be used, it should be kept at a concentration of not more than 1% to avoid false positive toxicity results

(Ghisalberti, 1993; Nguta et al., 2013). Pinocembrin, galangin and chrysin have poor solubility in water (Kismet et al., 2006; Iljazović et al., 2006; Vlaia et al., 2016). The poor solubility of pinocembrin, galangin and chrysin in water have previously been overcome through the formation of cyclodextrin inclusion complexes using 2-hydroxypropyl-β-cyclodextrin (Kim et al., 2008; Kim et al., 2009; Zhou et al., 2014). Cyclodextrins are cyclic oligosaccharides, which possess a hydrophobic cavity and a hydrophilic surface; and exist in the alpha, beta, delta and gamma forms (Attama et al., 2004; Zhou et al., 2014; Cheirsilp and Rakmai, 2016). Cyclodextrins are widely used in the pharmaceutical industry to improve the stability, solubility and bioavailability of their parent drugs (Jullian et al., 2010). This study aimed to test the toxicity effects of pinocembrin, galangin and chrysin, individually and in combination, through complexing the compounds with 2-hydroxypropyl-β-cyclodextrin for use in the brine shrimp lethality assay (BSLA).

5.2. Materials and methods

5.2.1. Preparation of compounds and controls

Cyclodextrin inclusion complexes were prepared with the compounds to improve their solubility in water. This was done using the same method described under Chapter 4, Section 4.2.1. The resultant crystals that were formed were dissolved using 82 mg of the complex to 1 ml of water. This gave a concentration of 2 mg/ml of the test compound, as 40 mg of 2-hydroxypropyl-β-cyclodextrin was used for every 1 mg of compound when making up the complex. A South African propolis sample obtained the North West province from the study conducted by Suleman (2016) was used as the comparative controls and prepared as described in Chapter 2, Section 2.2.3.2. The propolis extracts were then dissolved in sterile water to yield a concentration of 2 mg/ml. Potassium dichromate (Sigma) was prepared at a concentration of 1.6 mg/ml to use as a positive control as it is highly toxic to brine shrimp. Artificial sea water, which mimics the brine shrimp's natural environment, was prepared at 32 g/l using NaCl and distilled water to use as a negative control. A second negative control of 2-hydroxypropyl-β-cyclodextrin, which was used to complex the compounds, was also prepared at 80 mg/ml. This concentration was used as 40 mg of 2-hydroxypropyl-β-cyclodextrin was used for every 1 mg of compound, and compounds were tested at 2 mg/ml.

5.2.2. Brine shrimp lethality assay (BSLA)

The Brine-shrimp lethality assay, first described by Michael et al. (1956) and subsequently adapted (Bussmann et al., 2011), was performed. Artificial seawater was prepared by dissolving 32 g of Tropic Marine® Sea Salt in 1 l of distilled water. Dried brine-shrimp (*Artemia franciscana*) eggs (Ocean Nutrition™) (0.5 g) was then added to the saltwater. The eggs were left for 48 hr to hatch under a constant light source. A rotatory pump (Kiho) was added to the mixture for aeration of saltwater. Thereafter, 400 µl saltwater (containing 40-60 live brine-shrimp) was added to each well of the 48 well micro-titre plate along with 400 µl of the compound being tested. Compounds were studied at a final concentration of 1 mg/ml in triplicate per plate, with each plate duplicated. A negative control of artificial sea water and a positive control of potassium dichromate (Sigma) were used. A visual representation of the brine shrimp assay is given in Figure 5.1.

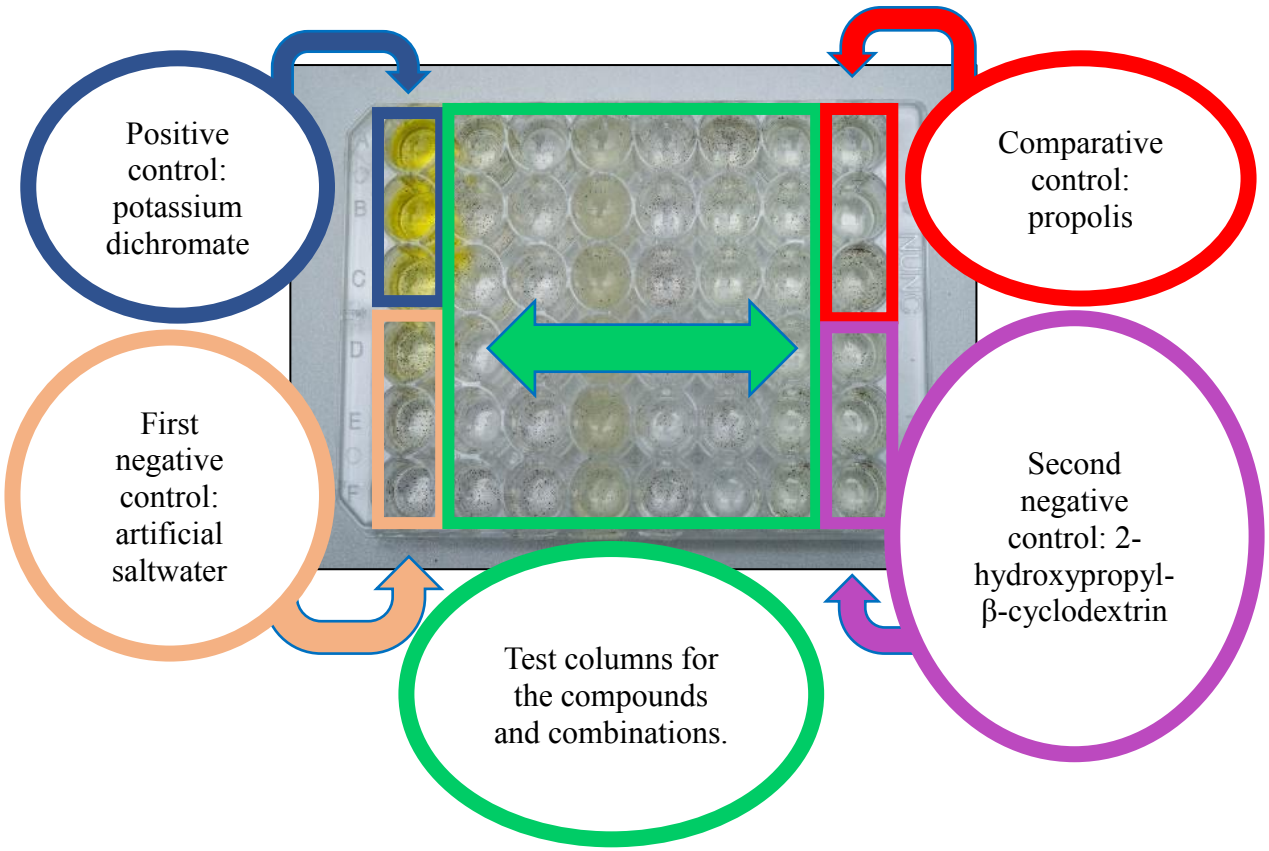


Figure 5.1: Brine shrimp lethality assay (BSLA).

The brown dots seen in the well are the brine shrimp, which are clearer under magnification.

Brine shrimp were examined under a light microscope (Olympus, 40X magnification) immediately before and after adding the compounds to determine viability. Any dead brine shrimp at the initial count was excluded from the percentage mortality calculation. Dead brine-shrimp were counted again after 24 hr by viewing plates under a light microscope. Finally, a lethal dose of acetic acid (100% (v/v); 50 µl) was added to each well in order to take a final count and thus calculate the percentage mortality. A percentage mortality of 50% or greater is considered biologically toxic (Bussman et al., 2011).

5.2.3. Interactive studies

Pinocembrin, galangin and chrysin were investigated for their interactive efficacies with regards to toxicity. The brine shrimp lethality assay was performed as described in Section 5.2.2., with 200 µl of each compound used in the 1:1 ratio combination tests, and 133.3 µl of each compound used when testing the triple combination. The interactions between the compounds were then analysed using the fractional percentage mortality (FPM) and the fractional percentage mortality index (ΣFPM), as adapted from van Vuuren and Viljoen (2011) and Ramulondi (2017) and calculated as per Equation 5.1.

Equation 5.1: Fractional percentage mortality (FPM) calculations

$$FPM^{(i)} = \frac{\text{PM (a) in combination with (b)}}{\text{PM (a) independently}} \qquad FPM^{(ii)} = \frac{\text{PM (b) in combination with (a)}}{\text{PM (b) independently}}$$

Where (a) is the percentage mortality of the one compound in the combination and (b) is the percentage mortality of the other compound used in the combination. The sum of the FPM, known as the FPM index, is thus calculated as: $\Sigma FPM = FPM^{(i)} + FPM^{(ii)}$

The ΣFPM was interpreted as follows; any value ≤ 0.5 was interpreted as the combination being synergistic and possessing a marked reduction in toxicity than any of the compounds tested independently; a ΣFPM of 0.5 – 1.0 meant that the combination was additive and had less toxicity than its components individually; a ΣFPM of 1.0 – 4.0 was interpreted as the combination being

non-interactive, with no changes to the toxicity when in combination and a ΣFPM ≥ 4.0 interpreted as antagonistic with an increase in toxicity of the combination when compared to its individual components (van Vuuren and Viljoen, 2011; Ramulondi, 2017).

5.3. Results

The results of the BSLA are shown in Table 5.1. The South African propolis sample tested was shown to possess a low level of toxicity at 2 mg/ml (percentage mortality of 14.78%). The negative control 2-hydroxypropyl-β-cyclodextrin, widely accepted to be non-toxic, used to complex pinocembrin, galangin and chrysin, led to a 7.31% mortality. This is probably a result of the culture conditions used for the brine shrimp.

The compounds identified in South African propolis had a low toxicity at a dose of 2 mg/ml compared to the negative control. The highest percentage mortality noted from the three compounds when tested individually, was for galangin at 23.53%, while chrysin showed the least toxicity at 4.15%. When assayed in combination, three of the combinations showed additive interactions and one combination was non-interactive. All the combinations had a lower toxicity than the negative control. With the exception of galangin all the compounds and mixtures had a lower toxicity than the negative control cyclodextrin at a dose of 2 mg/ml.

Table 5.1: Average percentage mortality of pinocembrin, galangin, chrysin and their combinations when tested against brine shrimp.

Compound	Ave % mortality	Combination	Ave % mortality	ΣFPM	Interpretation
Pinocembrin	4.62	Pinocembrin - Galangin	4.68	0.71	Additive
Galangin	23.53	Pinocembrin - Chrysin	4.38	1.00	Non-interactive
Chrysin	4.15	Galangin - Chrysin	6.17	0.87	Additive
		Pinocembrin - Galangin - Chrysin	5.30	0.88	Additive
Propolis	14.78				
Cyclodextrin	7.31				
Potassium dichromate	82.00				

5.4. Discussion

This study has demonstrated that the South African propolis samples have a low toxicity. The propolis sample tested showed low toxicity results, agreeing with previous studies which have reported the same trend. No toxicity studies have been found on South African propolis, however, a study by Mohammadzadeh et al. (2007) investigated the toxicological effects of Iranian propolis samples which also contained high levels of pinocembrin, galangin and chrysin. The authors studied the propolis samples at 10 mg/ml on 20 male Wistar rats and it was found that no toxicological changes occurred in the test group compared to the control group after 48 hr, thus indicating a low level of propolis cytotoxicity. Furthermore, Brazilian green propolis was found to cause no side effects in mice, rats and humans after administration to each of the groups (Wagh, 2013). It has been reported that propolis in low dosages (under 15 g/day) is considered safe but may cause allergic reactions when used in higher dosages (Castaldo and Capasso, 2002). Despite the antibacterial effects of propolis varying according to location, a review by Miguel and Antunes (2011) noted that that there is no correlation between the toxicity of propolis and its geographical origin. This may be because flavonoids, which is the major constituent of all propolis samples, are largely thought to have a low level of toxicity (Burdock, 1998). However, research into the toxic profiles of flavonoids have often been neglected as they are found in a multitude of dietary products, which do not need to be regulated by the Food and Drug Administration (Galati and O'Brien, 2004). A review by Galati and O'Brien (2004) concluded that flavonoids have the potential to be toxic if consumed in large amounts, and that further research into the toxicity of these compounds is necessitated if they are to be used as therapeutic agents.

Concerning the toxicity of pinocembrin, galangin and chrysin, very little prior research has been found. Pinocembrin showed a mortality of 4.62% after 24 hours, meaning that it has a very low potential for toxicity. This finding is supported by Zhang et al. (2016) who have pointed out that since pinocembrin is also found in honey, which is consumed by humans, its use should be considered safe. Metzner et al. (1977) as reported in Burdock (1998) found that pinocembrin showed no toxicity when administered to mice at dosages of 1000 mg/kg. Galangin, the second major flavonoid found in South African propolis, had higher toxicity against brine shrimp than the negative control with a percentage inhibition of 23.53%. This finding is supported by Kumar and

Alagawadi (2013), who tested galangin on five female Wistar rats in dosages of 2000 mg/kg orally and found no toxic symptoms in the test group after 14 days. A study by Huh et al. (2013), which reports that galangin showed no toxicity to the mice in their study, also corroborates with this finding. Chrysin, the third major compound found in South African propolis, showed the lowest toxicity with a percentage mortality of 4.15%. This is not surprising as Samarghandian et al. (2017) have reported on the protective effects of chrysin on animal tissue. In contrast, a study done by Tsuji and Walle (2008) showed that chrysin exhibited toxic effects on the liver of trout. The authors attribute this toxicity to peroxidase-like activity in the cells of the trout, which oxidise chrysin to a toxic by-product (Tsuji and Walle, 2008). However, this may not extend to human subjects.

When the compounds were tested in combination, their toxic interactions proved to be either additive or non-interactive. No antagonistic interactions were found, meaning that the toxicity profiles of pinocembrin, galangin and chrysin do not increase when used in combination. While the compounds were not toxic when they were tested on their own, their toxicity profiles decreased even further when used together. It can thus be deduced that using pinocembrin, galangin and chrysin in combination poses no risk of increased toxicity. This in turn reduces the potential for adverse effects occurring as a result of using the compounds concomitantly.

5.5. Overview

- Pinocembrin, galangin and chrysin were shown to possess a low level of toxicity when tested using the BSLA.
- Galangin had higher toxicity against brine shrimp than the negative control with a percentage inhibition of 23.53%, however, its toxicity was markedly reduced when used in combination with pinocembrin and/or chrysin.
- Three of the four combinations showed additive interactions, meaning that the toxicity of the compounds was reduced even further when they were assayed in combination.
- The compounds found in South African propolis can safely be used in combination, as their toxicity profiles are reduced even further when they are used together.

CHAPTER 6
Summary and Conclusion

6.1. General discussion

Propolis has been extensively used and researched for its therapeutic and medicinal applications, however, it has not been accepted into conventional medicine due to its varying composition and lack of standardization (Silva-Carvalho et al., 2015). Moreover, there is a surprising lack of research into the antimicrobial efficacy of the compounds found within propolis. This study investigated the antimicrobial activity of pinocembrin, galangin and chrysin, singularly and in combination, on planktonic and sessile bacteria and yeasts, and examined their toxicity effects.

6.2. Dissertation summary

The antimicrobial and toxic interactions of pinocembrin, galangin and chrysin were examined using the objectives outlined below.

Objective one: To determine the antimicrobial efficacy of pinocembrin, galangin and chrysin using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays.

Pinocembrin, galangin and chrysin were tested for their antimicrobial effects on planktonic bacteria using the MIC and MBC assays. The inhibitory activity of these compounds against the six strains of bacteria tested varied between 0.16 mg/ml and 0.63 mg/ml. Against the three yeast strains tested, inhibitory activity was noted between 0.04 mg/ml to 0.16 mg/ml, which classifies the antifungal activity of these compounds as moderate to negligible (44% moderate, 66% negligible). The most susceptible pathogen tested was *Cryptococcus neoformans*, against which all three compounds displayed moderate activity. On average, the compounds exhibited greater

antimicrobial activity against fungal and Gram-positive pathogens than against Gram-negative strains. Out of the three compounds, pinocembrin showed the best activity against both bacterial (average MIC = 0.22 mg/ml) and fungal (average MIC = 0.15 mg/ml) strains. With subsequent MBC studies, none of the compounds showed any bactericidal activity on their own at the concentrations tested, indicating that these compounds have inhibitory activity against the growth of bacterial cells rather than bactericidal activity.

Objective two: To determine the antimicrobial interaction of the compounds at equal ratio and varied ratio concentration combinations.

Pinocembrin, galangin and chrysin were tested for their interactive efficacies in combination. Firstly, combinations comprising of two compounds were tested at an equal ratio. For the 1:1 ratio study, results showed that synergy occurred in six of the 27 combinations (22%) whilst nine combinations (30%) were additive, and the remaining 12 combinations (44%) were non-interactive. No combination showed antagonism. The combination of galangin and chrysin appeared to be the most effective, with 33% of its combinations synergistic and 33% additive. *Listeria monocytogenes* was the pathogen most susceptible to the compound combinations, with two sets of combinations (pinocembrin-galangin and galangin-chrysin) demonstrating synergy and pinocembrin in combination with chrysin being additive. Bactericidal activity was noted for eight combinations. The strongest bactericidal activity was observed for the combination of galangin with chrysin against *Candida tropicalis*, having an MBC value of 0.16 mg/ml.

One pathogen from each microbial class having the highest number of synergistic interactions was then chosen for varied ratio combination studies. These pathogens were *L. monocytogenes*, *C. tropicalis* and *Pseudomonas aeruginosa*. Out of the 81 ratios generated from nine isobolograms, 32 ratios (40%) showed synergy, while 31 ratios (38%) were additive. The remaining 18 ratios (22%) were non-interactive. *Candida tropicalis* proved to be the most susceptible pathogen to the antifungal effects of the combined compounds, having 17 synergistic interactions. The combination of galangin and chrysin against *C. tropicalis* showed the best activity, as all nine ratios of these two compounds in combination, showed synergy.

Thereafter, triple combination studies were undertaken at a 1:1:1 ratio. Synergy was noted from this combination against six pathogens (67%) and additive interactions noted against the remaining three pathogens (33%). No antagonistic or non-interactive interactions were found for the triple combination. The triple combination also showed bactericidal activity against *C. tropicalis*.

The synergistic and additive interactions between the compounds found in this study supports the theory put forward by several studies suggesting that the antimicrobial effects of propolis are due to interactions between the compounds (Kujumgiev et al., 1999; Drago et al., 2007; Boisard et al., 2015).

Objective three: To determine the anti-quorum sensing (QS) activity of the compounds and their combinations.

The anti-QS activity of pinocembrin, galangin and chrysin, and the combinations, were tested using *Chromobacterium violaceum* as a monitor strain. All of the compounds and combinations studied inhibited violacein production produced by *C. violaceum*. It could therefore be deduced that the compounds are effective at inhibiting quorum sensing. When the compounds were tested independently, pinocembrin and chrysin shared the lowest MQSIC value of 0.16 mg/ml; pinocembrin and galangin had the lowest MBC value of 1.25 mg/ml; and chrysin showed the highest violacein percentage inhibition at 78.82%. When the compounds were tested together, three out of four combinations (75%) had a MQSIC of 0.08 mg/ml, which was lower than any noted for the independent compounds. These three combinations were synergistic, while the remaining one combination was additive.

For the combinations, the strongest bactericidal activity was observed for the pinocembrin and galangin combination, where an MBC of 0.31 mg/ml was obtained. The highest violacein percentage inhibition observed was from the combination of pinocembrin and chrysin at 85.10%. Pinocembrin, galangin and chrysin thus have great potential as anti-quorum sensing agents, particularly when used in combination.

Objective four: To determine the antibiofilm activity of the compounds and their combinations.

The antibiofilm effects of pinocembrin, galangin and chrysin were tested using the crystal violet staining assay at five different time intervals. Three pathogens (*L. monocytogenes, Escherichia coli* and *Candida albicans*) were tested, based on their biofilm-forming abilities. Good biofilm inhibitory activity was noted for 18 out of 45 (40%) of the compounds when they were tested independently against the three micro-organisms at various timeframes. The best activity from a single compound was observed for chrysin against *E. coli* at 24 hr, where it showed a 79.73% inhibition.

When the compounds were tested together at a 1:1 ratio, only nine out of 60 (15%) combinations showed good inhibitory activity. However, the triple combination showed an inhibitory activity of 100% when tested against *E. coli* at 24 hr, which was the best activity noted overall. Concerning the activity at the different time intervals: the best activity was noted at the 24-hr timeframe for the bacterial biofilms, and at the 48 hr intervals for the fungal biofilm formation. This has positive therapeutic implications, as the dosage intervals for the compounds, if used medicinally, would be less than that of conventional antibiotics thus reducing adverse effects and increasing patient compliance.

Objective five: To determine the toxicity of the compounds and combinations.

The toxicity effects of pinocembrin, galangin and chrysin were tested using the brine shrimp lethality assay (BSLA). All of the compounds or combinations showed low toxicity levels after 24 hr. When tested independently, the highest percentage mortality was noted for galangin, at 23.53%.

When the compounds were tested together, the highest percentage mortality was observed for the combination of galangin and chrysin, at 6.17%. Three of the four combinations showed additive interactions, meaning that the toxicity of the compounds was reduced even further when they were used together.

6.3. Observation trends between the antimicrobial assays.

Micro-organisms are capable of acting in what is now known as “group behavior” which allows them to act as a multi-cellular organisms (Jefferson, 2004; Li and Tian, 2012). Up until recently, it was believed that bacterial cells acted independently as uni-cellular organisms, and that any infections that they caused was a result of the sum of their individual effects (Greenberg, 2003; Li and Tian, 2012). It is now known that quorum sensing and the formation of biofilms allow for micro-organisms to communicate with each other and act in a synchronized manner, thus contributing to antimicrobial resistance (Greenberg, 2003; Li and Tian, 2012). This study tested the effects of pinocembrin, galangin and chrysin and their combinations on both individual and group bacteria and yeasts.

Due to the emergence of resistance that has occurred as a result of micro-organisms acting in synchronization, compounds that work against planktonic micro-organisms might not work as well against sessile micro-organisms. This is clear in the case of pinocembrin and galangin, which when tested at a low concentration (0.04 mg/ml), showed enhancement of quorum sensing rather than inhibition. Similarly, a number of compounds and combinations, which showed MIC values of less than 1 mg/ml when tested against planktonic bacteria, contrastingly showed enhancement of biofilm growth at the same concentration. It can thus be deduced that compounds may require higher concentrations to exert an antimicrobial effect against sessile micro-organisms than against planktonic ones. This is corroborated by Olson et al. (2002) and Jefferson (2004), who report that biofilms are much less susceptible to the effects of antimicrobial agents than are planktonic cells.

Another example to support the differences between individual and colony micro-organism behaviour, is the antimicrobial effects of chrysin. When tested against the six bacterial strains studied using the MIC assay, chrysin showed the weakest activity out of all three compounds. Contrastingly, when the compounds were studied for their anti-quorum sensing effects, chrysin was shown to be the strongest quorum sensing inhibitor. This proves that the mechanism of action of certain compounds might be through disrupting communication between bacterial cells rather than inhibiting individual micro-organisms. When studying the antimicrobial effects of an agent, care should be taken to research all possible targets and modes of action.

On the other hand, certain results indicated good activity against both planktonic and sessile micro-organisms. A case in point is the combination of pinocembrin and galangin. Under the MIC assay, this combination was shown to be antimicrobially synergistic against *L. monocytogenes*, with a low FIC index of 0.19. It was also synergistic against *C. neoformans* and *C. tropicalis* and additive against *E. coli*, *P. aeruginosa* and *C. albicans*. This was also the combination which showed the most promising anti-QS activity as shown in Chapter 3. When tested against a 4-hr *L. monocytogenes* biofilm in Chapter 4, this combination was the only one that displayed better activity than its individual components. Under the biofilm assay it was seen that the combination of pinocembrin and galangin was the only one out of all the compounds and combinations which showed similar activity to the positive control against a 48-hr *C. albicans* biofilm. The many positive interactions given by this combination marks it as a promising candidate for future research.

A popular theory in literature is that agents that are capable of inhibiting quorum sensing (QS), are also effective as antibiofilm agents (Landini et al., 2010; Silva et al., 2016). Landini et al. (2010) point out that since bacteria are capable of undergoing QS processes only under high bacterial cell concentrations, it is very probable that biofilms, which contain a high cell population, make use of QS communication strategies. In this study, chrysin demonstrated the strongest anti-QS activity out of the three compounds tested (Chapter 3). Chrysin also showed good antibiofilm properties against six of the 15 biofilms (40%) (Chapter 4). This suggests that there is a link between anti-QS and antibiofilm activity. Silva et al. (2016) elaborates on this idea, proposing that agents that impede QS do so by interrupting cell-to-cell communication, which in turn suppresses biofilm formation. This finding is also supported by Rabin et al. (2015) and Roy et al. (2018) who both give examples supporting this theory. The former study proves that products that inhibited QS through acting as auto-inducer-2 analogues, such as isobutyl-DPD, also caused biofilms to be thinner and less ordered when tested against *E. coli* and *P. aeruginosa*. The latter study demonstrated that the flavonoid quercetin, which inhibits QS, also acts as an antibiofilm agent against *S. aureus*.

The triple combination of pinocembrin, galangin and chrysin at a 1:1:1 ratio also demonstrated good activity against both planktonic and sessile bacteria. When tested for antibacterial properties under the MIC assay in Chapter 2, this combination showed a synergistic effect against six micro-organisms including *E. coli*. When tested against QS in Chapter 3, it was shown to have a remarkably low minimum quorum sensing inhibitory concentration (MQSIC) of 0.08 mg/ml. When tested for antibiofilm activity in Chapter 4, it showed the highest biofilm inhibition (100% against the 24-hr *E. coli* biofilm). It can thus be concluded that combining the compounds found in South African propolis enhances their antimicrobial activity and extends their spectrum allowing the triple combination to be effective against both individual and grouped micro-organisms.

6.4. Future recommendations

This study has investigated the combined antimicrobial and toxicity effects of the compounds found within South African propolis. However, certain areas were not within the scope of this study and are thus included below for future consideration.

6.4.1. Cell culture studies

Although herbal or natural products and their resulting compounds are thought to be non-toxic, this premise is often unfounded (Stickel et al., 2000). Compounds are capable of causing various toxic effects in the human body, and as such, toxicity needs to be constantly evaluated (van Tonder, 2011). Several factors need to be considered when evaluating the toxicity of an agent, including hepatotoxicity and the effects on the drugs on populations such as the elderly or immunocompromised (Bateman et al., 1998). Toxicology is evaluated throughout the drug development process (van Tonder, 2011). This is done in order to determine any risks that the compound or agent may pose, and to eliminate failure of potential drugs early on (Fielden and Kolaja, 2008; van Tonder, 2011). Toxicity can either be tested *in vivo*, using whole organisms, or *in vitro* by using molecules or cells. The BSLA which was undertaken in this study is one measure of *in vitro* toxicity, however, it is recommended that further toxicity assays on pinocembrin, galangin and chrysin be undertaken. Animal testing has previously been used as the yardstick in

the determination of safety profiles, as reactions in animal models are thought to be useful in predicting human responses to an agent (Buckberry, 1999; Bhattacharya et al., 2011). However, the use of animals as test subjects is contentious due to ethical, financial and moral implications (Buckberry, 1999). Cell line cultures are a viable alternative to animal testing and allows for the accurate evaluation of tissue responses to the test agent (Allen et al., 2005). In this method, tissue cells from various organs are removed from an animal and are kept viable by sub-culturing in a suitable growth medium. They are then exposed to the test agent and alterations in their integrity measured (Ekwall et al., 1990). This method is advantageous as it is quick, easy to carry out, and more cost-effective than animal studies (Doke and Dhawale, 2015). In addition to providing specific data on the toxicity of compounds by evaluating finite endpoints, such as growth and morphology, cell line assays also provide important pharmacokinetic information such as the mechanisms of drug metabolization (McKim, 2015; Liu et al., 2018). It is for these reasons that cell lines are recommended as the next step to further evaluate the toxicity and pharmacokinetic properties of the compounds pinocembrin, galangin and chrysin and their combinations.

6.4.2. Structure-activity relationships (SARs)

Natural products are an important source for producing novel drugs (Cheuka et al., 2016). This is due to the vast diversity that natural products offer, as well as their target affinity and specificity (Hong, 2011). With many conventional drugs failing, or resistance to conventional drugs increasing, natural products are once again being explored as a source of new pharmaceutical agents (Lahlou, 2013). One such class of naturally derived products is the flavonoids. Flavonoids are classified under polyphenols and are structured from a C6-C3-C6 skeleton which consists of two aromatic rings (A and B) linked through a three-carbon chain (Alzand and Mohamed, 2012; Xie et al., 2015). Over 8000 flavonoids have been identified (Xie et al., 2015). Flavonoids are well known to be beneficial to health, possess a large variety of biological activities, and are often used in the pharmaceutical industry (Panche et al., 2016). Pinocembrin, galangin and chrysin are all flavonoids, and this study has shown that they have great potential as novel antimicrobial agents especially when used in combination. However, before considering developing these compounds into pharmaceutical or therapeutic agents, attention must first be given to the structure-activity relationships (SARs). The study of SARs involves relating the chemical structures of a group of

compounds to their biological or toxicological activities (McKinney et al., 2000). This is done to gain an understanding of the mechanisms through which compounds work, and how their properties can be altered by modifications to their chemical structures (McKinney et al., 2000). Structure-activity relationships can also be used to optimize or increase the activity of compounds thus contributing to the development of new drugs (Lahlou, 2013; Tu et al., 2015). The SARs of flavonoids have previously been investigated, relating their chemical structures to their antibacterial activities, pro- and anti-oxidant effects, anti-diabetic properties, and their vascular functions, amongst others (Cos et al., 2000; Xu et al., 2007; Xie et al., 2015; Sarian et al., 2017). In the present study, the SARs of the flavonoids pinocembrin, galangin and chrysin found from previous literature were highlighted throughout the chapters. In Chapter 2 it was reported that pinocembrin's activity against *Penicillium italicum* is due to the hydroxyl groups present in its structure; that the the biological effects of chrysin are mainly due to the presence of hydroxyl and keto groups in its rings; and that a planar A/C ring in flavanols and the presence of O-H groups in flavonoids are essential for their antimicrobial activity (Nishino et al., 1987; Yang et al., 2011; Anitha and Rajadurai, 2014). In Chapter 3, it was mentioned that specific moieties in the chemical structure of flavones such as chrysin is associated with quorum sensing inhibition (Skogman et al., 2016). Chapter 4 stated that the presence of hydroxyl groups on the flavonoid backbone has been reported to enhance activity against biofilms, with antibiofilm activity depending on the number and positions of the hydroxyl groups (Silva et al., 2016). However, these few points extracted from previous papers are hardly sufficient in providing a full overview of the SARs of pinocembrin, galangin and chrysin with regards to their antibacterial, anti-quorum sensing and antibiofilm activities. It is thus recommended that more comprehensive studies be undertaken on the SARs of these compounds in order to comprehend how their structures relate to their activities, which will subsequently allow for the compounds to be optimized for enhanced therapeutic effects.

6.4.3. Varied ratio triple combination

This study researched the antimicrobial interactions between the three major bioactive compounds found within South African propolis, based on the theory that interactions between said compounds contributes towards the antimicrobial activity of propolis. Compounds were tested at 1:1 and varied ratios for the two-compound combinations, and at 1:1:1 ratios for the triple

combination. However, compounds within propolis are naturally not present in an equal ratio and it is therefore possible that different results would be obtained if the combination of pinocembrin, galangin and chrysin was tested at varying ratios. This would also be reliant on finding the ratio of the components in South African propolis, obtained through further chemical studies. Triple combination data can be analysed using the design systems MODDE 9.1®, which simplifies the number of variables given from combinations of more than two compounds (Eriksson et al., 2008). It is thus suggested that the triple combination be tested at varying ratios.

6.4.4. Stereoisomerism

Stereoisomers are "compounds which differ only in the spatial arrangement of their constituent atoms or groups" and are classified as either enantiomers or diastereoisomers (Hutt and O'Grady, 1996). Enantiomers, which are often formed when a carbon atom consists of four different substituents, are "stereoisomers which are non-superimposable mirror images of each other" (Hutt and O'Grady, 1996; Nguyen et al., 2006). Enantiomers are labelled as either R (rectus or right), or S (sinister or left), depending on whether their atoms are counted in a clockwise or anti-clockwise direction. They are also depicted with a (+) or (–) notation, depending on the direction in which they rotate plane-polarized light (Nguyen et al., 2006). A racemate, or racemic mixture, is a mixture of the two enantiomers and is designated as R,S (Nguyen et al., 2006; Peepliwal et al., 2010). Enantiomers cause divergent biological activities to take place within the body, and as such, can generate conflicting therapeutic effects: a compound may produce a therapeutic effect whilst the enantiomer may produce adverse effects or even no effect at all (Peepliwal et al., 2010). For example, Silva et al (2017) tested the S-(+)- and R-(–)- enantiomers of linalool against *Aeromonas hydrophila* and found that only S-(+)-linalool possessed antibacterial activity against this pathogen. On the other hand, enantiomers can also both possess therapeutic effects – for example, the enantiomers quinine (which is an antimalarial) and quinidine (an antiarrhythmic) possess very different effects (Peepliwal et al., 2010). Stereoisomerism also affects the interactions of compounds in combination, as demonstrated by van Vuuren and Viljoen (2007). In their study, the (+)-limonene and (−)-limonene enantiomers were tested singularly and in combination with the essential oil constituent 1.8-cineole, and it was found that while (−)-limonene possessed better activity when tested singularly, synergy was only present when (+)-limonene was combined with

1.8-cineole (van Vuuren and Viljoen, 2007). Surprisingly, when (−)-limonene was combined with 1.8-cineole, antagonism was noted at all ratios (van Vuuren and Viljoen, 2007). In the present study, the (S)-enantiomer of pinocembrin was tested. No previous studies were found on the (R)-enantiomer, although Du et al. (2011) have published a patent which states that when a racemate of pinocembrin is used to treat stroke, its duration of action lasts for longer than does (S)-pinocembrin. It is thus possible that (R)-pinocembrin could possess antimicrobial activities, or that it could enhance the effects of other compounds when in combination, and it is recommended that future studies be undertaken on the effects of this compound.

6.5. Limitations

There were two main limitations faced in this study. The first was the insolubility of pinocembrin, galangin and chrysin when submerged in water, which was overcome through the formation of cyclodextrin inclusion complexes. The second was the cost of the compounds, particularly for pinocembrin and galangin. Pinocembrin costs R3900 for 50 mg, and galangin costs R5950 for 100 mg. Whilst the antimicrobial activities of these compounds (particularly when used in combination) were found to be promising, their therapeutic applications may be limited due to their economic implications. Before considering future formulations incorporating these compounds, thought should be given to cost and feasibility. One should keep in mind that South African propolis is a natural source of pinocembrin, galangin, and chrysin as well as containing other compounds which may have other pharmacological activities. Not only does South African propolis works well as a natural antimicrobial but it is also more economically viable and may have other health benefits.

6.6. Final conclusion

This study examined the individual compounds found in South African propolis and concludes with a number of points: firstly, that the antimicrobial activities of pinocembrin, galangin and chrysin are enhanced when used together against planktonic bacteria. Secondly, that the mechanism of antimicrobial action alternates from bacteriostatic when the compounds are used independently, to bactericidal in the case of selected combinations. Thirdly, that the ability of the

compounds to stop quorum sensing improves substantially when they are used in combination. Fourthly, while no clear pattern was elucidated for biofilm reactivity to the compounds, good inhibitory activity was noted for certain compounds and combinations. Lastly, that the toxicity of the compounds were reduced even further when examined in certain combinations. All in all, the study firmly demonstrates that the combination of the compounds pinocembrin, galangin and chrysin found in South African propolis synergistically enhances antimicrobial activity of propolis on a number of levels whilst simultaneously further reducing the toxicity of the compounds. Furthermore, the study provides a classical example of the need to examine compound interactions and not always follow the reductionist approach of searching for a single active compound in natural product research.

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APPENDIX A

Presentations

A1. The 2018 Indigenous Plant Use Forum (IPUF) conference – Oudtshoorn, South Africa, 1-4 July 2018.

KHARSANY, K.[1], VAN VUUREN, S.F.[1], VILJOEN, A.M.[2,3]

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The new buzz: proving synergy between propolis chemical compounds

Propolis is a resinous substance produced by the *Apis mellifera* bee. The chemical composition of propolis depends, among other factors, largely upon its botanical origin. The antimicrobial activity of propolis thus mirrors that of the plant it was sourced from. The main compounds found in South African propolis are the flavonoids; pinocembrin, galangin, and chrysin. The aim of this study was to obtain an understanding of the antimicrobial activity of these compounds, both individually and in combination, to ascertain whether synergy is responsible for the exceptional activity shown by South African propolis. Antimicrobial studies include minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) assays; time kill assays; anti quorum-sensing assays; and toxicity studies.

MIC testing results showed that combinations of compounds showed better inhibitory activity than single compounds. When the combinations were tested, synergy was noted for 22% of the 1:1 combinations and for 67% of the triple 1:1:1 combinations. The effect of the triple combination against *L. monocytogenes* is especially worth mentioning, with an ΣFIC value of 0.02.

Similarly, bactericidal activity was noted for combinations rather than for individual compounds. The lowest MBC value that was noted, was of the combination between galangin and chrysin, with an MBC value of 0.16 mg/ml.

The minimum quorum sensing inhibitory concentration (MQSIC) for pinocembrin and chrysin was noted at 0.16 mg/ml and for galangin at 0.31 mg/ml. Combinations showed even better quorum sensing inhibition, in particular the double combination of galangin and chrysin and the triple combination of pinocembrin, galangin and chrysin, which both showed MQSIC values of 0.08 mg/ml.

Toxicity studies were undertaken using the brine shrimp assay, testing the compounds at 2 mg/ml. None of the compounds were found to be toxic to the shrimp after a 24-hour exposure. However, it should be noted that the percentage mortality of galangin alone (23.53%) was higher than any of the combinations containing galangin.

These results demonstrate that synergistic interactions amongst compounds within an extract (as demonstrated in South African propolis) play a bigger role than independent compounds.

A2. **School of Therapeutic Sciences Biennial Research Day – University of the Witwatersrand (Faculty of Health Sciences), Johannesburg, South Africa, 6 September 2018.**

Investigating the interactions between compounds found in South African propolis

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INTRODUCTION

Propolis is a resinous substance produced by the *Apis mellifera* bee, used to block holes and cracks in the hive(1). It has historically been used as an embalming agent, as an antipyretic, as a mouth disinfectant, and for wound healing(2). Today it is used for the treatment of burns, wound healing, skin conditions, herpes, in dental care, and for rheumatic disorders and sprains(1). Propolis possesses antibacterial, antifungal, cytostatic, wound healing, antitumor and anti-inflammatory properties, all of which have been attributed to its **high flavonoid content**(3). However, it is not known specifically which flavonoids impart the antimicrobial activity of propolis, or if the effects are due to **interactions** between the components(4). South African propolis is rich in flavonoids, particularly **pinocembrin, galangin** and **chrysin** (Figure 1), but no studies have been done on the antimicrobial activity of these compounds in combination(2).

AIM & OBJECTIVES

To investigate the antimicrobial interactions amongst compounds within propolis using the following objectives:

1.) To determine the **antimicrobial activity** of pinocembrin, galangin and chrysin, individually and in combination, against **planktonic bacteria** using the minimum inhibitory concentration (MIC) assay.

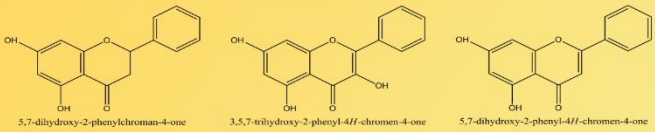
2.) To determine the **antimicrobial activity** of pinocembrin, galangin and chrysin and their combinations against **bacteria capable of group synchronization** through anti-quorum sensing testing.

METHODS

MIC: The micro titre plate dilution assay was used to determine the minimum inhibitory concentrations (MICs) of the compounds and their combinations(5). Analysis was performed on both bacterial and fungal pathogens. The compounds investigated were diluted to a starting concentration of 5 mg/ml. When testing the compounds independently, 100 µl of each compound was placed in the first row of the micro titre plate and serially diluted. When testing 1:1 combinations, 50 µl of each compound was used, and 33.3 µl used for triple 1:1:1 combinations. All assays were undertaken in triplicate. The interactions of the combinations were assessed by determining the sum of the fractional inhibitory concentration (ΣFIC). Values for each combination were interpreted as synergistic (ΣFIC ≤ 0.5), additive (ΣFIC values > 0.5 - 1.0), non-interactive (ΣFIC values > 1.0 ≤ 4.0), or antagonistic (ΣFIC values > 4.0)(5).

Quorum sensing (QS): Anti-QS studies were undertaken using the macrodilution method(6). Varying concentrations of the compounds were prepared and added to 5 ml of Luria Bertani broth with 100 µl of *Chromobacterium violaceum*. After incubation, the test solutions were vortexed and observed macroscopically. Anti-QS activity was interpreted based on growth (turbidity) and absence of violacein, a purple pigment produced by *C. violaceum*. For quantitative results, 1 ml sample aliquots were centrifuged. This allowed for the bacteria to form pellets, which retained the colour of the *C. violaceum*. The supernatant was thereafter discarded, and the violacein solubilized. After further centrifugation, supernatant from each tube was transferred into a micro titre plate and the absorbance was read. Any result of ≥ 50% violacein inhibition is an indication of anti-QS activity.

THE COMPOUNDS



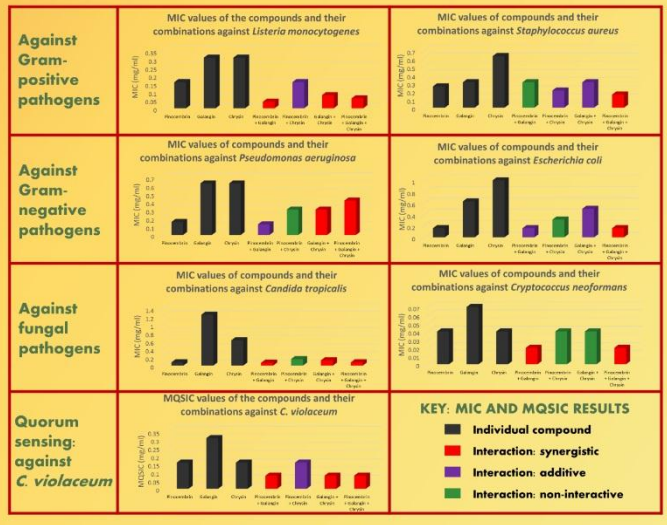
5,7-dihydroxy-2-phenylchroman-4-one — **Pinocembrin**

3,5,7-trihydroxy-2-phenyl-4*H*-chromen-4-one — **Galangin**

5,7-dihydroxy-2-phenyl-4*H*-chromen-4-one — **Chrysin**

Figure 1: 2D chemical structures of the compounds found in South African propolis

RESULTS – MIC AND MQSIC



SUMMARY

It was found that synergy occurred in 22% of the 1:1 combinations tested and 66% of the triple combinations. Synergy was also present in 75% of the combinations tested against quorum sensing. The remaining interactions were either additive or non-interactive, and no combination showed antagonism.

CONCLUSION

Although it has often been theorized that interactions between the compounds are responsible for the remarkable antimicrobial activity shown by propolis, no previous studies investigating these interactions have been undertaken. From this study, it can be concluded that synergy between the pinocembrin, galangin, and chrysin play an important role in contributing towards the antimicrobial activity of propolis, against bacteria found both in the planktonic and group forms. When considering product development, these three compounds should be used together to achieve an optimal antimicrobial effect.

ACKNOWLEDGEMENTS

University of the Witwatersrand for facilities, equipment, and funding; National Research Foundation (NRF) for funding; Mrs. Phumzile Madondo for Lab and technical assistance.

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A3. Postgraduate Botany Symposium – University of Johannesburg (Department of Botany and Plant Biotechnology) Johannesburg, South Africa, 5 November 2018.

Exploring the role of synergy and additivity amongst the compounds found in South African propolis.

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Propolis is a resinous substance produced by the *Apis mellifera* bee. The chemical composition of propolis depends, among other factors, largely upon its botanical origin. The antimicrobial activity of propolis thus mirrors that of the plant it was sourced from. The main bioactive compounds found in South African propolis are the flavonoids; pinocembrin, galangin, and chrysin. This study aimed to explore the antimicrobial activity of these compounds, both individually and in combination, and to investigate the role of interactions between the compounds. Studies undertaken include minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) assays, time kill assays, anti-quorum sensing assays, and cytotoxicity studies. Minimum inhibitory concentration assay results showed that combinations of compounds exhibit superior activity to single compounds. When the combinations were tested, synergy was noted for 22% of the 1:1 combinations and for 67% of the triple 1:1:1 combinations. Varied ratio combinations illustrated that synergy is present in many other ratios of the compounds – the most notable example is that of galangin and chrysin which showed synergy at all nine ratios tested. Similarly, MBC results showed bactericidal activity from a few combinations while the compounds on their own were unable to exhibit bactericidal activity. In line with these results, quorum sensing studies showed that compound combinations are more effective at inhibiting bacterial communication than are individual compounds. Synergy was noted for 75% of the combinations tested. Cytotoxicity studies were undertaken using the brine shrimp assay, testing the compounds at 1 mg/ml. None of the compounds were found to be cytotoxic to the shrimp after a 24-hour exposure. However, it should be noted that the percentage mortality of galangin alone (23.53%) was higher than any of the combinations containing galangin. The interactive effects of three of the combinations were additive, meaning that the compounds showed reduced cytotoxicity when used together. These results demonstrate that the interactions among compounds within an extract (as demonstrated in South African propolis) play a bigger role than independent compounds.

APPENDIX B

Publication

Original article (Accepted in Journal of Ethnopharmacology, May 2019)

The new buzz: Investigating the interactions between bioactive compounds found in South African propolis.

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ABSTRACT

Propolis is a resinous substance produced by the *Apis mellifera* bee. The main compounds predominantly found in South African propolis are the flavonoids pinocembrin, galangin, and chrysin. This study aimed to obtain an understanding of the antimicrobial activity of these compounds, both individually and in combination, and to investigate the role of interactions between the compounds. Antimicrobial assays included minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), anti-quorum sensing, biofilms studies, and toxicity studies. Minimum inhibitory concentration testing results showed that combinations of compounds showed better inhibitory activity than single compounds. When the combinations were tested in the MIC assay, synergy was noted for 22% of the 1:1 combinations and for 66% of the triple 1:1:1 combinations. Similarly, MBC results showed bactericidal activity from selected combinations, while the compounds on their own demonstrated no cidal activity. Quorum sensing studies showed that compound combinations are more effective at inhibiting bacterial communication than the individual compounds. Biofilm assays showed that the highest percentage inhibition was given from the triple combination against *E. coli* at 24 h. Finally, brine shrimp lethality studies revealed that combinations of the three compounds had reduced toxicity when compared to their individual components. These results demonstrate that the compounds found in South African propolis work synergistically to achieve an optimal antimicrobial effect whilst simultaneously minimizing toxicity.

KEY WORDS: propolis, pinocembrin, galangin, chrysin.

APPENDIX C

Ethics waiver

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms KE Kharsany
School: Therapeutic Sciences
Department: Pharmacy and Pharmacology
Medical School
University
E-mail: khadija.karsany@gmail.com

CC: Supervisor: Professor S van Vuuren <Sandy.vanVuuren@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 28/02/2018

REF: *R14/49*

PROTOCOL NO: W-CBP-180228-01 *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *The antimicrobial properties of the major compounds found in South African Propolis*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.



MSWorks2000/Iain0007/ClearScanWaiver.wps

APPENDIX D

Turnitin report

The Antimicrobial Properties of The Major Compounds Found in South African Propolis.

ORIGINALITY REPORT

11%	6%	9%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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2	Mahumane, Gillian D., Sandy F. van Vuuren, Guy Kamatou, Maxleene Sandasi, and Alvaro M. Viljoen. "Chemical composition and antimicrobial activity of Eucalyptus radiata leaf essential oil, sampled over a year", Journal of Essential Oil Research, 2016. Publication	1%
3	Z. Hübsch, R.L. Van Zyl, I.E. Cock, S.F. Van Vuuren. "Interactive antimicrobial and toxicity profiles of conventional antimicrobials with Southern African medicinal plants", South African Journal of Botany, 2014 Publication	<1%
4	Suleman, T., S. van Vuuren, M. Sandasi, and A. M. Viljoen. "Antimicrobial activity and chemometric modelling of South African propolis", Journal of Applied Microbiology, 2015. Publication	<1%
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