INVESTIGATING THE MECHANISM OF ACTION OF *ARISTEA ECKLONII'S* SUSPECTED ANTIPYRETIC PROPERTIES

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A dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, in fulfilment of the requirement for the degree of Master of Science in Medicine

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DECLARATION

I, Miles Christopher Muller, declare that this dissertation was solely composed by myself, that the work contained herein is my own and that any assistance has been acknowledged and all sources duly referenced. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Miles Muller

_____17th _____day of____July_____2024

PRESENTATIONS

Conference presentations

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Abstract

Aristea ecklonii (*A. ecklonii)* (Baker) is an indigenous, evergreen perennial medicinal plant used amongst South Africans to treat fever. This study aimed to investigate the antipyretic properties of aqueous *A. ecklonii* root extracts. Male Sprague-Dawley rats (250 – 300 g) received a subcutaneous (s.c.) injection of zymosan (300 mg/kg) or saline before receiving an intraperitoneal injection (i.p.) of paracetamol (50 mg/kg), aqueous *A. ecklonii* root extract (55 mg/kg) or saline. The i.p. injection was administered 15 h after the s.c. injection. Abdominal temperature was measured using temperature sensitive radio-transmitters. Blood, cerebrospinal fluid (CSF) and hypothalamic tissue was collected 90 min after the i.p. injection for the measurement of cytokines, prostaglandin E2 (PGE2) and enzymes involved in PGE2 synthesis. Zymosan increased abdominal temperature which peaked at 39.42 ± 0.14 °C. Compared to rats that received saline, rats receiving zymosan had increased concentrations of blood plasma IL-1β and IL-6, increased PGE2 in the CSF and increased hypothalamic expression of COX-2 and mPGES1 ($P < 0.05$). Paracetamol and aqueous *A. ecklonii* root extract reduced the zymosan-induced fevers. Rats that received saline, followed by an i.p. injection of aqueous *A. ecklonii* root extract, had a decrease in abdominal temperature and an increase in blood plasma IL-1β, IL-6, TNF-α and IL-10 concentrations compared to rats that received saline ($P < 0.05$). Thus, the antipyretic properties of the *A. ecklonii* root extract seems to be related to its ability to induce systemic inflammation and not reduce the synthesis of hypothalamic PGE2 and thus should be used with caution in ill individuals.

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LIST OF ABBREVIATIONS

AM404	N-arachidonoylaminophenol
ANOVA	analysis of variance
BC	before Christ
CBD-1	cannabinoid-1 receptor
cDNA	complementary deoxyribonucleic acid
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
COX-3	cyclooxygenase-3
CSF	cerebrospinal fluid
DAMPs	damage-associated molecular patterns
ELISA	enzyme-linked immunosorbent assay
i.p.	intraperitoneal
IL-1	interleukin 1
IL-10	interleukin 10
IL-12	interleukin 12
IL-1β	interleukin 1-β
IL-6	interleukin 6

MIP-1α	macrophage inflammatory protein 1 alpha
mPGES1	microsomal prostaglandin synthase-1
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NAPQI	N-acetyl-p-benzoquinoneimine
NSAIDs	nonsteroidal anti-inflammatory drugs
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PGE2	prostaglandin-E2
PRR	pattern recognition receptors
ROS	reactive oxygen species
RCF	relative centrifugal force
RT-PCR	real-time polymerase chain reaction
s.c.	subcutaneous
TLR	toll like receptor
TNF-α	tumour necrosis factor-α
TRPA1	transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid 1

PREFACE

Phytomedicine is a prominent source of alternative healthcare and a foundation for clinical grade pharmaceutical development globally. As a South African, I have a vested interest in comprehending and improving our local healthcare system and associated literature. Traditional medicine is a topic of great controversy as unsupported effects have resulted in numerous ailments, and it has been the source of many patients rejecting life-saving modern medicine. However, one cannot discredit traditional and alternative medicine entirely, given the large demand for alternative treatments based on cultural connections and aversions to Western medicine. Therefore, the practices and techniques of phytochemistry and pharmacology should be studied to better understand the effects of traditional medicines.

As researchers and scientists, it is crucial to strive towards improving the quality of patient care given in our field. Literature can be expanded, and evidence can be uncovered to prevent harm from any treatment, be it traditional or modern. Expanding knowledge and enhancing supported practices may allow the integration of traditional medicine with modern medicine to create safe, accessible, affordable, and relevant treatments. However, motivating the specific form of treatment to be studied is essential, as the cost and time invested in a study should yield significant outcomes for the benefit of humanity.

Thus, this dissertation, focuses on *Aristea ecklonii*, a plant with a wide array of claimed effects under traditional use, some of which have been studied. The studies undertaken as part of this dissertation aim to determine the mechanism of action of *A. ecklonii*, specifically its suspected antipyretic effects, and how it affects the body and immune system. By advancing the understanding of the plant's effects, one can determine its appropriate applications and usage. Such findings would hopefully push the use of *A. ecklonii* away from traditional healer practices as an antipyretic. Our work on *A. ecklonii* not only aims to expand the understanding of phytomedicine thereby creating a safer medical environment within South Africa, but also by emphasizing the importance of animal-based studies as a screening tool for commonly used traditional medicines so as to ensure their safety and efficacy.

Chapter 1

Introduction

Pathogenic micro-organisms that breach physical barriers and invade the human body trigger an array of immune, physiological, metabolic, and behavioural responses collectively known as acute phase responses (Dantzer, 2004). One prominent aspect of these responses to infection is fever, recognized by physicians as well as patients as an elevated body temperature. In addition to fever, infected individuals commonly exhibit non-specific behavioural changes like lethargy, depression, increased sleepiness, pain, and suppressed appetite, collectively referred to as "sickness behaviour" (Hart, 1988; Dantzer, 2001; Johnson, 2002, Kelley *et al.*, 2003). These adaptive responses, including fever and sickness behaviour serve as a well-coordinated strategy employed by the host to combat infection (Hart, 1988). However, there is evidence suggesting that fever can have a detrimental effect on the hosts ability to cope with infection, by causing damage to cellular and tissue function when temperatures are at the higher end of the febrile range (Liu *et al.*, 2012, Harden *et al*., 2015). While the predominant view suggests that a modest fever can often be beneficial, as fever is observed to enhance the function of various components of the immune response, many individuals seek relief from the uncomfortable symptoms experienced while being ill. Antipyretics can enhance a person's daily functioning and overall comfort while being ill (Harden *et al*., 2015). In such cases, individuals often resort to various remedies, including traditional medicine, home remedies and phytomedicine, as alternatives to pharmaceutical antipyretics.

Around 60% of the global population, and approximately 80% in developing countries, still rely on phytomedicine and traditional practices as their primary source of healthcare (Sultana *et al*., 2015, Khan & Ahmad, 2019). Phytomedicine has several potential benefits as an alternative treatment, including increased global access, affordability, and supposedly fewer side-effects when compared to pharmaceutical medicine. As such, there has been an increase in studies investigating phytomedicines as an alternative for pharmaceuticals over the past two decades (Khan & Ahmad, 2019). The increased interest in phytomedicines as treatment options has highlighted the importance of ensuring the safety and efficacy of these plant-based medicines. Researchers need to identify, develop, certify, and advance the innovation of new treatment techniques, medications, and policies to meet the demand for green medicine (Qadri *et al.*, 2021). By introducing safe and effective novel medication, the selection of available drugs, particularly affordable and sustainable options, can be expanded. In doing so there is the provision of a wider range of choices for patients and physicians during treatment. One such example of a plant that is readily available and showing potential as an antimicrobial is *A. ecklonii* (Pretorius *et al.*, 2002; Chanda, 2014).

The root and leaf extracts from *A. ecklonii* have traditionally been used amongst South Africans as an herbal medicine for the treatment of numerous symptoms of illness, fever and coughing related ailments (Gerstner, 1939, Zondi, 2013). A substantial problem faced in utilizing phytomedicine and more specifically *A. ecklonii* is the lack of verifiable data on the plant's efficacy and safety. While there is anecdotal evidence as well as claimed traditional practices supporting *A. ecklonii's* use, there is very little in the way of scientific evidence for its medicinal benefits. For the most part *in vitro* studies have examined the potential of *A. ecklonii* as an antimicrobial agent, yet for the supported use of the plant in humans, there is a need to promote the advancement of phytomedical studies to an *in vivo* model.

In this introductory chapter I have condensed the ramifications of fever and the need to treat fever while also expanding how phytomedicine is used to enhance treatment options. Within this literature review and research project, I aim to provide a comprehensive understanding of *A. ecklonii*, including its history and potential antipyretic properties. Additionally, I will explore the current understanding of our immune system and the fever and hypothermia associated with infection and inflammation, as well as the mechanisms by which current antipyretic drugs function. Finally, I will review the experimental model that I will use to induce fever in this study. By doing so, I hope to shed light on the possible antipyretic effects of *A. ecklonii* and its mechanism of action in attenuating fever.

1.1 *Aristea ecklonii*

Iridaceae otherwise known as the iris family is a diverse family of flowering plants belonging to the order Asparagales, with around 2,200 species and 66 genera (Britannica, 2020). *A. ecklonii* is a plant species in the Iridaceae family and has several stiff and upright leaves with bright blue and purple star-like flowers, giving meaning to its English names such as the African blue star iris and blue stars lily to name a few (Random Harvest, n.d.). The plant *A. ecklonii* is an indigenous, evergreen perennial that belongs to the Iridaceae family (Pooley, 2005). The plant *A. ecklonii* has mauve-blue star-like flowers that produce seeds distributed by natural vectors such as birds and environmental conditions, and is found within Southern Africa, specifically KwaZulu Natal, Eastern Cape, Limpopo and Mpumalanga (Pooley, 2005). Flowering of *A. ecklonii* can be seen in Figure 1.1A with the purple and blue flowers affiliated with the English names of the plant. *A. ecklonii* is known to be able to grow up to 70 cm in height in well-drained soils by streams and evergreen forests (Hyde *et al.*, 2020). The variety

of *A. ecklonii's* possible habitats gives the plant a hardy trait meaning it can be sourced sustainability in numerous areas at any altitude found in Southern Africa (Manning, 2008, Manning, 2012, Maroyi, 2020, Hyde *et al.*, 2020). The hardiness of *A. ecklonii* gives it the ability to easily grow and spread in most environments, with thick branching roots that form a condensed mat in the upper layer of soil (Figure 1.1B), preventing invasion by competitive species (Maroyi, 2020). Flowering occurs between late spring and early summer, often being used as an ornamental plant as well as a protective charm by traditional healers within southern and central Africa (Manning, 2008, Mabona *et al.*, 2013, Maroyi, 2020). *A. ecklonii* was originally imported into foreign countries from the plant's native country South Africa as an ornamental plant and has now become an invasive weed due to its hardiness and favourable survivability regardless of the environment (Csuches *et al.*, 1998, Marambe *et al.*, 2001, Randall, 2001, Froude, 2002, Csuches, 2008, Maroyi, 2020). The possibility of farming and extracting portions of *A. ecklonii* is not only likely but seemingly easy if replanted during harvest. The likely ability to easily farm *A. ecklonii* and produce the plant in large amounts eliminates the two major concerns of ecologists, those being the large-scale development of industrial phytomedical treatments endangering plants and the adulteration of medicine. These ecological issues can be amended by sustainable harvesting of phytomedicine (Chanda, 2014).

Figure 1.1 *A. ecklonii* in its natural habitat and its appearance when harvested.

The natural appearance of *A. ecklonii* as a shrub is shown (A); the leaf and root sections of *A. ecklonii* when unearthed (B). (Zondi, 2013, Contemplari, 2023).

1.1.1 Traditional and clinical application

The plant *A. ecklonii* has been and is used for the treatment of syphilis, shingles, sexually transmitted infections, toothache, internal sores, general pain, coughing and most pointedly

with regards to our research, general infection and fever attenuation (Hutchings *et al.*, 1996, Hutchings, 1996, Ngwenya *et al.*, 2003, Long, 2005, Zondi, 2013, Sagbo *et al.*, 2018). The extracts of *A. ecklonii* leaf and root sections as well as the whole plant species itself is commonly sold in South Africa amongst its informal medicine markets within KwaZulu-Natal and Gauteng (Sagbo *et al.*, 2019, Maroyi, 2020,). These extracts of *A. ecklonii* are often ingested in an aqueous form such as a tea or liquid concentrate (Hutchings, 1996).

1.1.2 Phytochemical composition

Compounds identified in the Iridaceae family include bioflavonoids, xanthones, anthocyanins, bufadienolides, proanthocyanides, phenolic compounds, sterols, flavanones and isoflavones (Asen *et al.*, 1970, Kumar *et al.*, 1985, Hanawa *et al.*, 1991, Harborne & Williams, 2000). While one cannot say whether these are found in significant proportions in *A. ecklonii* yet, it is likely that these secondary metabolites/compounds will be present in *A. ecklonii* as a member of the Iridaceae family. However, compounds such as α-spinasterol, sitosterol, 3,3'-biplumbagin, 8,8'-biplumbagin, and hydroxynaphthoquinone have been identified in both the root and leaf extracts of *A. ecklonii*, with plumbagin being the major constituent, while separate quinones (specifically hydroxynaphthoquinone) have been found as secondary metabolites with similar medicinal effects to those of plumbagin (Kumar *et al.*, 1985; Harborne & Williams, 2000, Balachandran *et al.*, 2021, Dahlem Junior *et al.*, 2022).

1.1.3 *In vitro* studies undertaken on *A. ecklonii*

In vitro studies showing the potent antimicrobial effects of *A. ecklonii* have changed how researchers view *A. ecklonii* as a phytomedicine and has motivated further research on the medicinal effects *A. ecklonii* is believed to hold. Table 1.1 includes a summary of some of the key *in vitro* studies undertaken to date investigating the antimicrobial properties of the crude *A. ecklonii* extracts. Some of the most influential findings on *A. ecklonii* came following the work done by Pretorius *et al*. (2002) where they were testing the antifungal potential of 39 plant species. The antifungal potential of the plant extracts was determined by measuring their effect on the radial mycelial growth of different pathogenic plant fungi, such as hyphomycetes, agonomycetes, oomycetes and ascomycetes. The radial mycelial growth of fungi is an indirect measure of cellular activity and filamentous spread of fungi perpendicularly (Hendricks *et al*., 2017). Of the plants tested, the most significant mycelial growth inhibition was noted with extracts from *A. ecklonii*. The crude *A. ecklonii* extracts totally inhibited the mycelial growth of all the plant pathogenic test organisms and even outperformed the inhibition produced by a

broad-spectrum synthetic fungicide commonly used in the treatment of plant fungi (difenoconazole and carbendazim) (Pretorius *et al.*, 2002).

With the potent antifungal properties of *A. ecklonii* extracts noted against plant fungi, it is no surprise that there is a desire to investigate its other claimed effects on pathogens causing disease in humans and animals. Mabona *et al.* (2013) conducted antimicrobial studies on South African medicinal plants specifically relating to the treatment of skin infections (Table 1.1). Both organic and aqueous root and leaf *A. ecklonii* extract preparations were tested for their antimicrobial efficacy. Organic extracts were prepared by submerging the dried, crushed plant material in a 1:1 mixture of dichloromethane and methanol (D:M) while the aqueous extracts were prepared by submerging the macerated plant material in sterile distilled water. Thereafter, the liquid extracts were filtered and stored at −80 1C before lyophilisationFrom this study it was found that the organic root and leaf extracts of *A. ecklonii* had notable antimicrobial efficacy against common skin pathogens such as *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Candida albicans, Cutibacterium acnes, Microsporum canis* and *Trichophyton mentagrophytes.* For the aqueous extract preparation, only the root extracts of *A. ecklonii* had noteworthy antimicrobial efficacy against *S. epidermidis*, *P. aeruginosa, T. mentagrophytes* and *M. canis.* In a follow-up study it was also noted that the organic root and leaf extracts of *A. ecklonii* had substantial antimicrobial efficacy against a ATCC 9763 strain of *Saccharomyces cerevisiae (S. cerevisiae)* (Madisha, 2024). Furthermore, with the organic extract preparations, the root displayed the greatest antimicrobial effect, which was comparable to the antifungal abilities of pharmaceutical antifungals such as amphotericin B and nystatin (Mabona *et al.*, 2013). Overall, aqueous extracts of *A. ecklonii* had more moderate antimicrobial effects compared to organic extracts (Madisha, 2024, Mabona *et al.*, 2013). In the study by Mabona *et al.*, 2013, fractionation of *A ecklonii* resulted in the isolation of the known bio-active compound, plumbagin. Plumbagin within *A. ecklonii* was found to exhibit antibacterial activity against *P. aeruginosa, S. aureus,* methicillin resistant *S. aureus,* gentamycin – methicillin-resistant *S. aureus, S. epidermidis* and *C. albicans.* Results observed from other studies on plumbagin demonstrated very low minimum inhibitory concentration (MIC) values (1.56 µg/mL and 0.78 µg/mL against *S. aureus* and *C. albicans*, respectively) (De Paiva *et al*., 2003, Mabona *et al*., 2013). The ability of *A. ecklonii* to inhibit bacterial and fungal growth of pathogens involved in human disease further supports its use beyond a plant fungicide and potentially into a multifunctional antimicrobial.

Table 1.1 Summary of *in vitro* antimicrobial studies undertaken on crude *A. ecklonii* extracts and plumbagin.

Plant material used	Experimental design	Results	Reference
Crude whole plant aqueous extract of both leaf and root combined	• Mycelial radial growth inhibition. • The radial mycelial growth of fungi is an indirect measure of cellular activity and filamentous spread of fungi perpendicularly. • Pathogens tested: *Botrytis cinerea, Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia solani, Verticillium dahlia, Botryosphaeria dothidea* and *Pythium ultimum.*	*A. ecklonii* totally (100%) inhibited mycelial growth of all tested fungal plant pathogens and even outperformed the inhibition by a broad-spectrum synthetic fungicide commonly used in the treatment of plant fungi (difenoconazole and carbendazim).	Pretorius *et al.*, 2002
Plumbagin	• MIC concentration of 50 µg/mL plumbagin dissolved in 30% dimethyl sulfoxide using a microdilution technique. • Pathogens tested: *C. albicans, Escherichia coli, S. aureus* and *Salmonella typhimurium.*	Plumbagin displayed specific antimicrobial activity, completely inhibiting the growth of *C. albicans* and *S. aureus* (MIC of 0.78 µg/mL and 1.56 µg/mL respectively) while being ineffective against Gram-negative bacteria.	Paiva *et al.*, 2003
Whole plant, both leaf and root separately as aqueous and organic extracts Aqueous extracts Organic extracts mixed with dichloromethane and methanol Plumbagin isolated from whole plant by fractionation	• Serial micro-dilution assay was used to quantify the MIC* values for plant extracts. • Dermatologically relevant pathogens such as *S. aureus,* gentamycin-methicillin-resistant *S. aureus, S. epidermis, Brevibacillus agri, C. acnes, P. aeruginosa, T. mentagrophytes, M. canis* and *C. albicans.*	• The organic root extract showed potent antimicrobial effects against all tested pathogens, except *B. agri* and *M. canis*, with MIC values ranging from 0.01 to 0.2 mg/mL. Similarly, the leaf extract exhibited potent antimicrobial effects against all pathogens, except *B. agri* and *M. canis*, with MIC values ranging from 0.05 to 0.75 mg/mL. • Aqueous leaf extracts were the least effective of *A. ecklonii* extracts, only being effective against *B. agri* and *T. mentagrophytes* with MIC values between 0.75 and 1.0 mg/mL. • Plumbagin displayed antimicrobial effects with a low minimum inhibition activity between 2.00 and 16.00 µg/mL comparable to commercial antifungals such as Amphotericin B.	Mabona *et al.*, 2013

Organic and aqueous extracts of leaves and root separately Aqueous extracts	• Serial micro-dilution assay was used to quantify the MIC values for plant extracts. • Pathogens tested: *C. albicans* and *S. cerevisiae.*	• Organic *A. ecklonii* root extracts displayed noteworthy antimicrobial effects for both pathogens with a low minimum inhibition activity between 0.05 and 0.13 mg/mL. • Organic *A. ecklonii* leaf extracts displayed noteworthy antimicrobial effects for both pathogens with a low minimum inhibition activity between 0.13 and 0.25 mg/mL. • Aqueous *A. ecklonii* leaf extracts displayed noteworthy antimicrobial effects for only *S. cerevisiae* with a minimum inhibition activity of 1 mg/mL.	Madisha, 2024

*MIC, minimum inhibitory concentration: The MIC is the lowest concentration of an antimicrobial agents, like plant extracts or bacteriostatic or bactericidal or an antifungal agent, that inhibit the growth of microbial pathogen after overnight incubation.

1.1.4 Toxicology findings

While investigating the antimicrobial potential of *A. ecklonii*, studies have also done a toxicity analysis, as it is not only important to explore the potential efficacy of the plant as a phytomedicine but also its safety. The first report of toxicity data for *A. ecklonii* used the brine shrimp lethality assay and showed that organic preparations of the root and leaf plant extract demonstrated toxicity (percentage mortality) ranging from 96% to 100%, depending on exposure time and plant sample (Dumisa *et al.*, 2020). In a following study also using the brine shrimp lethality assay, a similar percentage of mortality was noted with organic preparations of the extract (Madisha, 2024). In contrast, using the brine shrimp lethality assay, the aqueous extracts of *A. ecklonii* demonstrated a lower percentage of toxicity (percentage mortality) ranging from 10% to 20%, depending on exposure time and plant sample (Madisha, 2024). The high levels of toxicity observed in organic extracts of *A. ecklonii* could be due to the chemical composition of the plant containing compounds such as bufadienolides and proanthocyanides which are known to be toxic when consumed as well as the dichloromethane and methanol used in the preparation of organic plant extracts (Williams *et al.*, 1986). In addition, with the toxicity of organic extracts of *A. ecklonii* noted previously, the toxicity of plumbagin has been tested in human peripheral blood lymphocytes, as well as mouse macrophages (Seshadri *et al*., 2011, Checkers *et al.*, 2014). From the cytotoxicity test of plumbagin in human lymphocytes, it was found that plumbagin was toxic toward the lymphocytes in a time and dose-dependent manner (Seshadri *et al*., 2011). However, despite plumbagins toxicity in human lymphocytes, a lower concertation of plumbagin (1 μM) seemed to have anti-inflammatory effects, as plumbagin reduced lipopolysaccharides (LPS)-induced inflammation in mouse macrophages without inducing any cell death (Checkers *et al.*, 2014). In particular, pre-treating mouse macrophages with plumbagin reduced the LPS-induced increase in tumour necrosis factor alpha (TNF-α), interleukin-1ß (IL-1β), interleukin-6 (IL-6) and prostaglandin E2 (PGE_2) (Checkers *et al.*, 2014). A summary of the toxicity studies for *A. ecklonii* from *in vivo* studies can be found in Table 1.2.

Table 1.2 Summary of *in vitro* toxicity studies undertaken on crude *A. ecklonii* extracts or plumbagin.

Sample studied	Experimental design	Results	Reference
Plumbagin	• Lymphocytes were isolated from peripheral blood obtained from adult healthy volunteers and treated with plumbagin at various concentrations (0-50 μM) for different periods of time. • An MTT* assay assessed cell viability to determine cytotoxicity of plumbagin. • Flow cytometry determined apoptotic cells using annexin V-FITC and propidium iodide staining. • ROS was measured by dihydroethidium fluorescence using a fluorescence detector. • Western blot analysis was used to quantify Bcl-2, Bax and cytochrome-c.	• After 2, 5, and 10 μM treatment with plumbagin the viability values were 69, 53, and 41%, respectively, after 24 h and decreased to 38, 31, and 19% after 72 h. • At higher concentrations of plumbagin (20 and 50 μM), the viability values after treatment for 24 h were less than 40%, which fell to around 10% after 72 h treatment. • Within 6 h of plumbagin administration, 14-30% of cells entered an early apoptotic phase. • On increasing the exposure duration to 12 and 24 h, a decrease in early apoptotic cells and an increase in late apoptotic and necrotic cells were observed. • ROS** were increased by plumbagin as early as 30 min following plumbagin incubation and increased 2.5-fold after 1 h with 2 μM of plumbagin. • Bcl-2*** was reduced by plumbagin whereas Bax increased, increasing the Bax-Bcl-1 ratio following plumbagin incubation. This increase in Bax and plumbagin incubation increased cytochrome-c expression up to 3.9-fold compared to control cells.	Seshadri *et al.*, 2011
Plumbagin	• Mouse macrophage cell line (RAW 264.7 cells) treated with 1 μM plumbagin for 24 h.	• Plumbagin up to 1 μM didn't induce an increase in RAW cells death .	Checkers *et al.*, 2014

Aqueous extracts of leaves and root Organic extracts (dichloromethane and methanol)	• Brine shrimp lethality assay to determine substance toxicity by mortality rate.	• Organic *A. ecklonii* root and leaf extracts were both highly toxic with organic root extracts having a mortality rate of 99% and 100% at 24 h and 46 h respectively, the organic leaf extract had similar findings with a mortality rate of 96% at 24 h and 100% at 48 h. •	Dumisa *et al.*, 2020
Organic and aqueous extracts of leaves and root separately	Brine shrimp lethality assay to determine substance toxicity by mortality rate.	• *A. ecklonii* aqueous extract had low level of toxicity below 50% nearly matching control levels of toxicity within a 1% to 7% range while the organic extract had high toxicity levels of 100%. • Therefore, the aqueous extracts were less toxic with < 50% of brine shrimp dying compared to 100% mortality when organic extracts are used.	Madisha, 2024

*MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide;**ROS, reactive oxygen species ; ***Bcl-2, B-cell lymphoma 2 protein; Bax, Bcl-2 associated X apoptosis regulator

As outlined in Table 1.1, *in vitro* studies undertaken investigating the antimicrobial (specifically antifungal) properties of *A. ecklonii* have shown that the plant has potential as an antifungal and antibacterial with its main component plumbagin likely acting as the chemical compound giving the plant these potent abilities. However, *A. ecklonii* organic extracts seem to be extremely toxic (Table 1.2). The aqueous extract has a more tolerable limit of toxicity and seems to still provide some antimicrobial effects. In terms of plumbagin, *in vitro* studies indicate there seems to be a dose-dependent effect whereby doses above 1 μM appear to be toxic to immune cells (Checker *et al*., 2014). In line with the lower dose of plumbagin (1 μM) being non-toxic, plumbagin also seemed to demonstrate anti-inflammatory potential (Checkers *et al*., 2014).

1.1.5 *In vivo* studies undertaken on *A. ecklonii*

While the antimicrobial and toxicity studies of *A. ecklonii* are known, little/no research has been undertaken from an *in vivo* perspective which further restricts the understanding of the real-world use of this potential phytomedicine. It is not just important but a fundamental part of working with *A. ecklonii* that researchers study the plant further using an *in vivo* model because of its proposed effects regarding fever attenuation and reducing sickness behaviours as believed traditionally. In terms of the *in vivo* research of *A. ecklonii* it is beneficial to start with an animal model as a basis for determining whether treatment using *A. ecklonii* is a probable, safe and clinically appropriate medication. While there are many potential animal models that can be used, rats and mice are often the sample population generally used in most projects due to rats (as well as mice) having a physiological and genetic similarity with humans, especially with regards to the process of developing an immune response and the effects of treatments on the immune system (Wildner, 2019). The physiological similarities of thermoregulation between rats and humans allows us to use the rats' molecular pathways and biomarkers of fever as a comparison to what is expected to be observed in human use of a potential phytomedicine such as *A. ecklonii* extract (Bryda, 2013, Taylor & Gordon, 2019). Rodents are also far more practical and affordable to breed, house and conduct surgeries on than other animals making them appealing laboratory models of human illness. However, when using rodents as a model of human illness it is important to remember that thermoneutrality for rodents occurs at higher ambient temperatures compared to humans (Maloney *et al.*, 2014). Thus, when using rodents as a model for human illness it is important to ensure that their environment falls within their thermoneutral zone as it seems that multiple steps in the immune response pathway are altered when mice are housed below their thermoneutral zone (Maloney *et al*., 2014).

The only *in vivo* research of *A. ecklonii* seems to be from our research group, yet for the purpose of this literature review I will also focus on some *in vivo* analysis of plumbagin, as plumbagin is a known main constituent of *A. ecklonii*. Table 1.3 shows the *in vivo* studies done by our research group investigating the effects of intraperitoneal injection of crude *A. ecklonii* root and leaf aqueous extract preparations on abdominal temperature and body mass of male Sprague Dawley rats. Organic extract preparation could not be used in the *in vivo* studies due to the toxicity findings noted with the brine shrimp toxicity assay (Table 1.2). A pilot study was undertaken to investigate the *in vitro* effects of *A. ecklonii* since no prior research had been done. The leaf extract was administered at a starting dose of 175 mg/kg, following the Organization for Economic Co-operation and Development (OECD) guidelines, while a lower dose of 55 mg/kg was used for the root extract due to its higher toxicity potential identified in brine shrimp assays (OECD, 2008, Madisha, 2024). When the leaf and root aqueous extracts were administered intraperitoneally, rats exhibited signs of illness, including hypothermia followed by fever, and a decrease in body mass. The illness related effects of the leaf and root extracts on abdominal temperature and body mass were dose-dependent. The observed effects of *A. ecklonii* on abdominal temperature were interesting, considering the use of *A. ecklonii* in South Africa as an herbal medicine for fever, and cough-related ailments according to traditional sources (Gerstner, 1939, Zondi, 2013). To investigate the potential of *A. ecklonii* as an herbal medicine to treat fever and sickness behaviour studies using an animal model of fever and sickness behaviour would be required. Considering the observed antifungal properties of *A. ecklonii* and the common presentation of fever in patients with fungal infections, an animal model simulating immune responses to fungal infections may be a good starting point (Guo *et al.*, 2019).

Zymosan, a ligand derived from the cell wall of *S. cerevisiae*, is commonly used to simulate fungal infections and evaluate antipyretic efficacy (Dangarembizi *et al.*, 2019). Thus, using an animal model of zymosan-induced fever, Cottino 2020 investigated the antipyretic properties of *A. ecklonii* leaf and root aqueous extracts. In rats with a zymosan-induced fever, the *A. ecklonii* root extract (55 mg/kg) resulted in a short-lived decrease in abdominal body temperature (~ 2 h) (Figure 1.2 A). With administration of the leaf *A. ecklonii* extract inducing a similar but milder response to that noted with the *A. ecklonii* root extract. The initial decrease in abdominal body temperature induced with administration of the aqueous *A. ecklonii* root

extract was comparable to that noted with intraperitoneal injections of paracetamol in rats with zymosan-induced fevers (Figure 1.2 B).

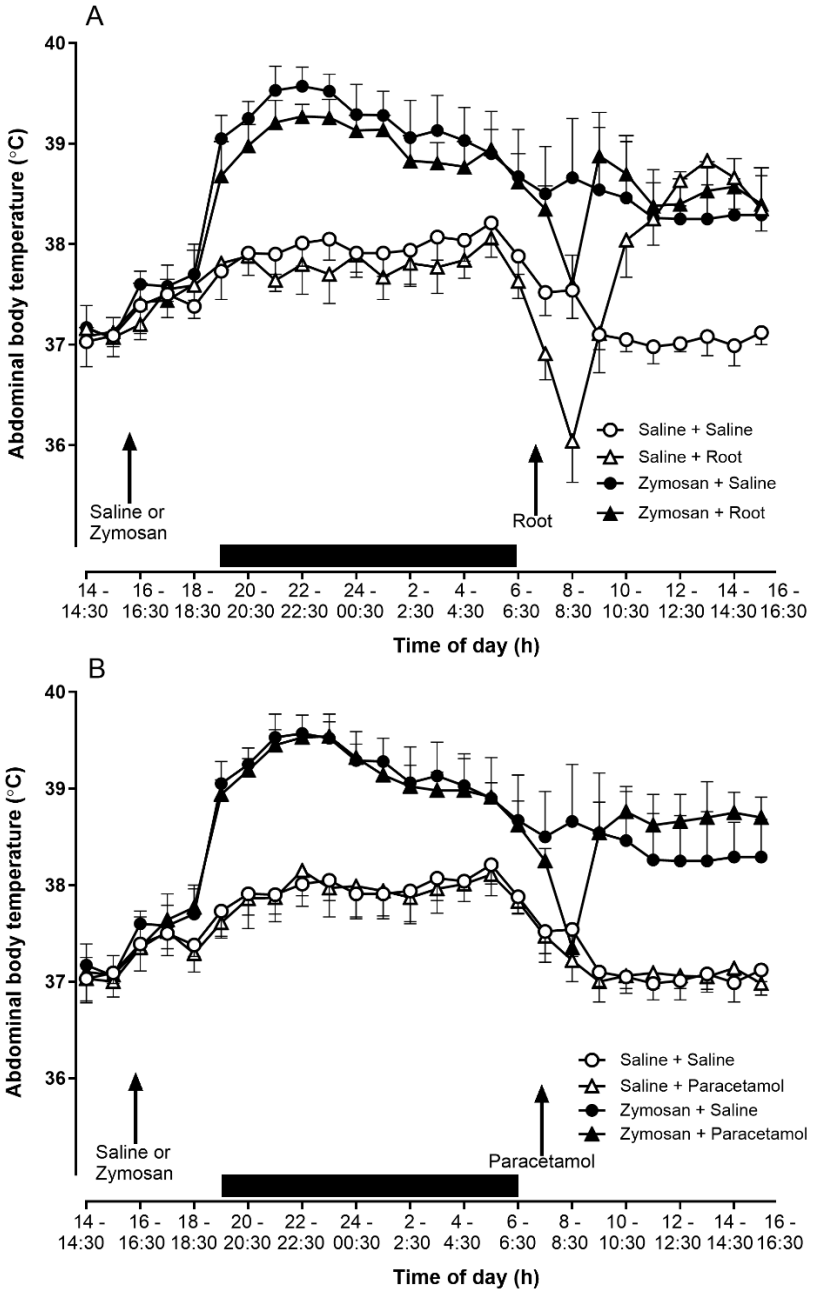


Figure 1.2: Rat's abdominal body temperature (mean and SD) recorded over a 24-hour period.

The 5-minute interval measurements were averaged into 30-minute intervals. The black bars indicate the night phase (19h00 - 06h00). Black arrows represent the subcutaneous (s.c.) injection being either saline (10 ml/kg) or zymosan (300 mg/kg) given at 16h00 (day 0). The second injection (i.p.) being either root (55 mg/kg), saline (5 ml/kg), or paracetamol (50 mg/kg) was administered (5 ml/kg) at 07h00 (day 1) (Cottino, 2020). Graph A displays the thermal effects of *A. ecklonii* root extract administration in normothermic and febrile rats, while graph B shows paracetamols effects on normothermic and febrile rats.

In addition to the *in vivo* studies undertaken with *A. ecklonii* leaf and root aqueous extracts, Table 1.3 shows findings from *in vivo* studies undertaken with plumbagin where Swiss mice were given either an intraperitoneal injection of plumbagin (2 mg/kg) or vehicle (phosphate buffered saline) 2 h prior to intraperitoneal injection of LPS (15×10^5 endotoxin units/mice) (Checker *et al.*, 2014). Plumbagin administration reduced mortality caused by LPS-induced endotoxic shock in the mice (Checker *et al.*, 2014). In addition, mice treated with LPS and plumbagin also had lower levels of pro-inflammatory cytokines (TNF-α, IL-6, interferon gamma (IFN-γ), and interleukin-12 (IL-12)) compared to mice that received LPS only (Checker *et al.*, 2014). Further evidence of the anti-inflammatory effects of plumbagin were noted in a rat model of inflammation, induced by injecting carrageenan in the paw. Mice that received oral administration of plumbagin (5-20 mg/kg) demonstrated a reduced expression of cyclooxygenase-2 (COX 2), TNFα, IL-1ß, and IL-6 in paw oedema fluid compared to mice that received LPS only (Luo *et al*., 2010).

Based on the information presented in Table 1.1, *A. ecklonii* shows promising potential as an antimicrobial agent that could contribute to the development of new antifungal and antibacterial medications. Results from *in vitro* studies indicate that organic preparations of *A. ecklonii* can be toxic, while aqueous preparations show minimal toxicity (Table 1.2). In terms of *in vivo* studies aqueous preparations of the root and leaf of *A. ecklonii* seem to have the potential to induce a complex presentation of hypothermia, fever and sickness behaviour in rats (Table 1.3). Moreover, when the aqueous preparation of the *A. ecklonii* root extract was administered during zymosan-induced fever in rats, the root extract reduced abdominal temperature to a similar degree to that noted with the use of paracetamol, a known antipyretic (Figure 1.2). To try and unravel these seemingly contradictory properties of *A. ecklonii* such as *A. ecklonii* having both pyrogenic and antipyretic properties, I will systematically cover the current understanding of the fever pathway, fever-hypothermia switch observed during systemic inflammation and potential mechanism of action, of known antipyretics, such as paracetamol.

Table 1.3 Summary of *in vivo* studies undertaken on crude *A. ecklonii* extracts or plumbagin.

Plant material used	Experimental design	Effect	Reference
Plumbagin	• Acute inflammation in the hind paws of male Sprague-Dawley rats was induced by subcutaneous injection carrageenan. • Inflammatory makers were measured in the paw exudates obtained from the rats injected with carrageenan or the vehicle.	• Four hours after injection, carrageenan induced an increase in the expression of TNF-α[1], IL-1ß[2], IL-6[3] and COX-2[4] in paw oedema fluid. • Plumbagin attenuated the increased expression of TNF-α[1], IL-1ß[2], IL-6[3] and COX-2[4]	Luo *et al*., 2010
Plumbagin	• Swiss mice (20 g) received plumbagin (2 mg/kg body weight) or vehicle (PBS[5]) intraperitoneally 2 h prior to i.p. LPS[6] (15×10^5 endotoxin units/mice) administration. • Mice from different treatment groups were sacrificed at 2, 6 and 24 h to examine various parameters associated with LPS-induced endotoxemia.	• Administration of plumbagin effectively prevented mortality caused by endotoxic shock. • Mice from the LPS[6] group showed a significant increase in the levels of TNF-α[1], IL-6[3], IFN-γ[7], and IL-12[8] compared to the control group. • Plumbagin treatment significantly reduced the serum levels of all pro-inflammatory cytokines in mice challenged with LPS[6] compared to the LPS-treated group.	Checker *et al.*, 2014
Aqueous leaf and root	• Intraperitoneal administration of aqueous *A. ecklonii* root or leaf extract in adult male Sprague Dawley rats with abdominal temperature monitoring. • The first dose of leaf extract was 175 mg/kg and then reduced by a factor of 3.2 if the rat showed signs of illness to determine a safe appropriate dosage without endangering rats. • The first dose of root extract was 55 mg/kg and then reduced by a factor of 3.2 if the rat showed signs of illness to determine a safe appropriate dosage without endangering rats.	• Following i.p.[9] administration of the aqueous leaf and root extract the rat's showed signs of ill health which was confirmed by a pronounced short-lived hypothermia (~2 h) followed by fever over a 24 h period. The effects of the leaf and root *A. ecklonii* extracts on abdominal temperature were dose-dependent.	Madisha, 2024
Aqueous leaf and root	• Intraperitoneal administration of aqueous root or leaf extracts of *A. ecklonii* (55 mg/kg) in adult male Sprague Dawley rats with a zymosan-induced fever (300 mg/kg) with abdominal temperature monitoring. • Saline (5 mL/kg) was used as a negative control with paracetamol (50 mg/kg which was administered as a volume of 5mL/kg) as a positive control. • Rats were first given saline or zymosan subcutaneously, then both groups were treated with either saline, paracetamol or *A. ecklonii* extracts intraperitoneally 15 h after their first intervention.	• In rats with a zymosan-induced fever, the *A. ecklonii* root extract (55 mg/kg) resulted in a short-lived decrease in abdominal body temperature (~ 2h). • Administration of the leaf *A. ecklonii* extract induced a similar but milder response to that noted with the *A. ecklonii* root extract. • The initial decrease in abdominal body temperature induced with administration of the aqueous *A. ecklonii* root extract was comparable to that noted with i.p.[9] injection of paracetamol in rats with zymosan-induced fever.	Cottino, 2020

[1]TNF-α, Tumour necrosis factor α; [2]IL-1β, Interleukin-1β; [3] IL-6, Interleukin-6; [4]COX-2, Cyclooxygenase-2; [5]PBS, Phosphate buffered saline; [6]LPS, lipopolysaccharides; [7]IFN-γ, Interferon γ; [8]IL-12, Interleukin-12; [9]i.p., intraperitoneal

1.2 Zymosan-induced fever

1.2.1 Classical mechanisms known to mediate fever

The immune system is the body's natural defence towards pathogens or foreign material entering the body. What is of interest in this study is the aspect of the immune response that induces inflammation and thus a fever, as the febrile response directly relates to the claimed therapeutic effect of *A. ecklonii.* The concept of a fever has been present since sixth century BC due to Sumerian documents describing inflammation and fever (Majno, 1998). Since this first report of fever our understanding has expanded with the advent of microbiology and the growth of medical literature detailing theorems with empirical evidence. The process of fever induction is linked to the mechanisms and process of the innate immune system. The innate immune system, which includes the skin, cellular junctions, mucosa, phagocytes, and phagocytic enzymes, is the frontline barrier against pathogenic infection (Aristizábal & González, 2013). Pattern recognition receptors (PRR) present on phagocytic host cells (such as macrophages, neutrophils, natural killer and mast cells) detect pathogen-associated molecular patterns (PAMPs) found on the surface of exogenous pyrogens (Figure 1.3) (Hoffmann & Akira, 2013, Aristizábal & González, 2013). These PAMPs are polysaccharide and polynucleotide ligands found within or on the surface of pathogens. An example of a PAMP being LPS from Gram-negative bacteria, mannose from yeast and β-glucan from fungal cell walls (Takeuchi & Akira, 2010). When the reaction of PRR and PAMP activation occurs, the host will further activate their macrophages to synthesize cytokines and inflammatory components to enhance the immune system. These secretions include but are not limited to IL-1β, IL-6, IL-10, TNF-α and macrophage inflammatory protein 1 alpha (MIP-1α). The collection of these cytokines, as well as many others, increase the hosts capabilities to kill pathogens, as well as for the onset of inflammation by increasing vascular permeability by nitric oxide and ROS production which complements inflammation by further recruiting lymphocytes at the site of infection (Mosser, 2003). Besides the recognition of PAMPs that initiate the cytokine cascade there are also signals originating from damaged host cells. These damaged cells can augment the immune response towards infection by expressing or releasing damage-associated molecular patterns (DAMPs) which are often intracellular components reacting with the host cells PRRs (Ojcius & Saïd-Sadier, 2012).

Macrophages and monocytes activated from the interaction between DAMPS/PAMPS and PRRs begin synthesizing and secreting cytokines, the main transitional component between the beginning of the immune response and the brains catalyst for fever induction. These cytokines are endogenous pyrogens expressed highly within endothelial cells, liver, spleen and therefore blood (Blomqvist & Engblom, 2018). These endogenous pyrogens or inflammatory mediators such as IL-6, IL-1β and TNF-α promote the synthesis of cyclooxygenase-1 (COX-1), COX-2 and microsomal PGE_2 synthase-1 (mPGES1) within parenchymal cells which themselves are responsible for the production and secretion of PGE_2 (Steiner *et al.*, 2011, Lima *et al.*, 2017, Blomqvist & Engblom, 2018). Once there is circulation of pro-inflammatory cytokines and PGE_2 in the blood of the periphery, both can pass through the blood-brain barrier (BBB) by influx transporters (prostaglandin transporter), saturable transport systems and retrograde axonal transport systems to enter the brain due to the large PGE_2 and cytokine gradient across the blood and BBB, IL-6 also binds to endothelial cells of the BBB stimulating endothelial production of PGE_2 where PGE_2 can be secreted into brain (Banks *et al.*, 1995, Ushikubi *et al.*, 1998, Yarlgadda *et al.*, 2009, Vasilache, 2015, Garami *et al.*, 2018).

In addition to cytokines and PGE_2 directly crossing the BBB, there are separate pathways through which the peripheral immune response can signal the brain and initiate fever (Figure 1.3). Cytokines can activate afferent fibres of the vagus and sensory nerves to generate a fever via the stimulation of cytokine receptors found in vagal sensory neurons (such as tumour necrosis factor receptor and EP_3 receptors) which stimulate immune-to-brain communication inducing fever. This vagal mechanism does not include the BBB whatsoever and is thus placed in the peripheral circulation as a part of immune-to-brain communication (Figure 1.3) (Roth *et al.*, 2001, Chang, 2019). Cytokines can also be transported into the circumventricular organs located near the organum vasculosum of the lamina terminalis, which is neighbouring the hypothalamus providing easy access of PGE_2 and cytokines to the hypothalamus (Roth *et al.*, 2004). The capillaries of the circumventricular organ are fenestrated, allowing cytokines from the blood plasma to cross over into the circumventricular organ easily, providing direct access to the cerebrovascular system, which lacks a BBB between the circumventricular organs and brain (Blatteis *et al.*, 1983, Roth *et al.*, 2001, Roth *et al.*, 2004, Eskilsson *et al.*, 2014). Therefore, cytokines can initiate fever through BBB-independent mechanisms of immune-to-brain communication, promoting central PGE_2 release without necessarily crossing the BBB. These mechanisms of peripheral immune-to-brain communication may not occur independently but rather overlap during an immune response. With TNF-α, IL-1β and IL-6 acting as the main

pro-inflammatory cytokines responsible for facilitating the central inflammatory and febrile response to peripheral immune reactions (D'Mello *et al.*, 2017).

Following cytokine production in the periphery or endothelial stimulation by said cytokines, PGE_2 will be synthesized in the blood, CSF and hypothalamus as the main mediator inducing a febrile response by the hypothalamus (Ivanov *et al.*, 2004). However, PGE_2 can only be synthesized after mPGES1 is expressed along with a heightened COX-2 concentration (Engblom *et al.*, 2003, Li *et al.*, 1999, Ivanov *et al.*, 2004). The synthesis of PGE_2 starts with the release of arachidonic acid from phospholipids from cellular membranes due to phospholipases such as phospholipase A_2 and endocannabinoids catalysed by monoacylglycerol lipase (Sanchez-Alavez *et al.*, 2015). The second step of PGE_2 synthesis is the now released arachidonic acids being converted into prostaglandin G_2 and then prostaglandin H_2 by COX isoenzymes (however during a febrile response the conversion is primarily done by COX-2). COX-1 has been deemed as less noteworthy than COX-2 in fever induction, as COX-1 has been identified as a house-keeping enzyme of the COX isoenzymes, with no upregulation during inflammatory events but rather a decrease of COX-1 to facilitate COX-2 upregulation during inflammatory events (Liu *et al.*, 1996, Smith *et al.*, 2000, Ivanov *et al.*, 2004, Font-Nieves *et al.*, 2012). Finally, PGE_2 is synthesized once prostaglandin H_2 is isomerized by microsomal or cytosolic PGE synthase (mPGES1 or cPGES). For the sake of this study I will look at the rate-limiting agents mPGES1 and COX-2 as precursors of PGE_2 synthesis as mPGES1 has been directly linked as a substrate required for PGE_2 synthesis while COX-2 has been identified as the main COX isoenzyme stimulating PGE_2 activation during febrile responses (Yamagata *et al.*, 2001, Engblom *et al.*, 2003, Font-Nieves *et al.*, 2012, Engström *et al.*, 2012). The firing of warm-sensitive neurons in the hypothalamus is reduced by PGE_2, which leads to an increase in endogenous heat production and heat conservation which will result in the typical rise of core body temperature seen in fever. The major autonomic responses that allow heat to be produced and conserved is the onset of peripheral vasoconstriction, sickness behaviours and non-shivering as well as shivering thermogenesis (Garami *et al.*, 2018). Once fever is maintained the PGE_2 in the brain is synthesized directly at the blood brain barrier by endothelial cells to preserve the fever until the infection is attenuated, thereby reducing COX-2 and mPGES1 expression (Coceani *et al.*, 1986, Engström *et al.*, 2012).

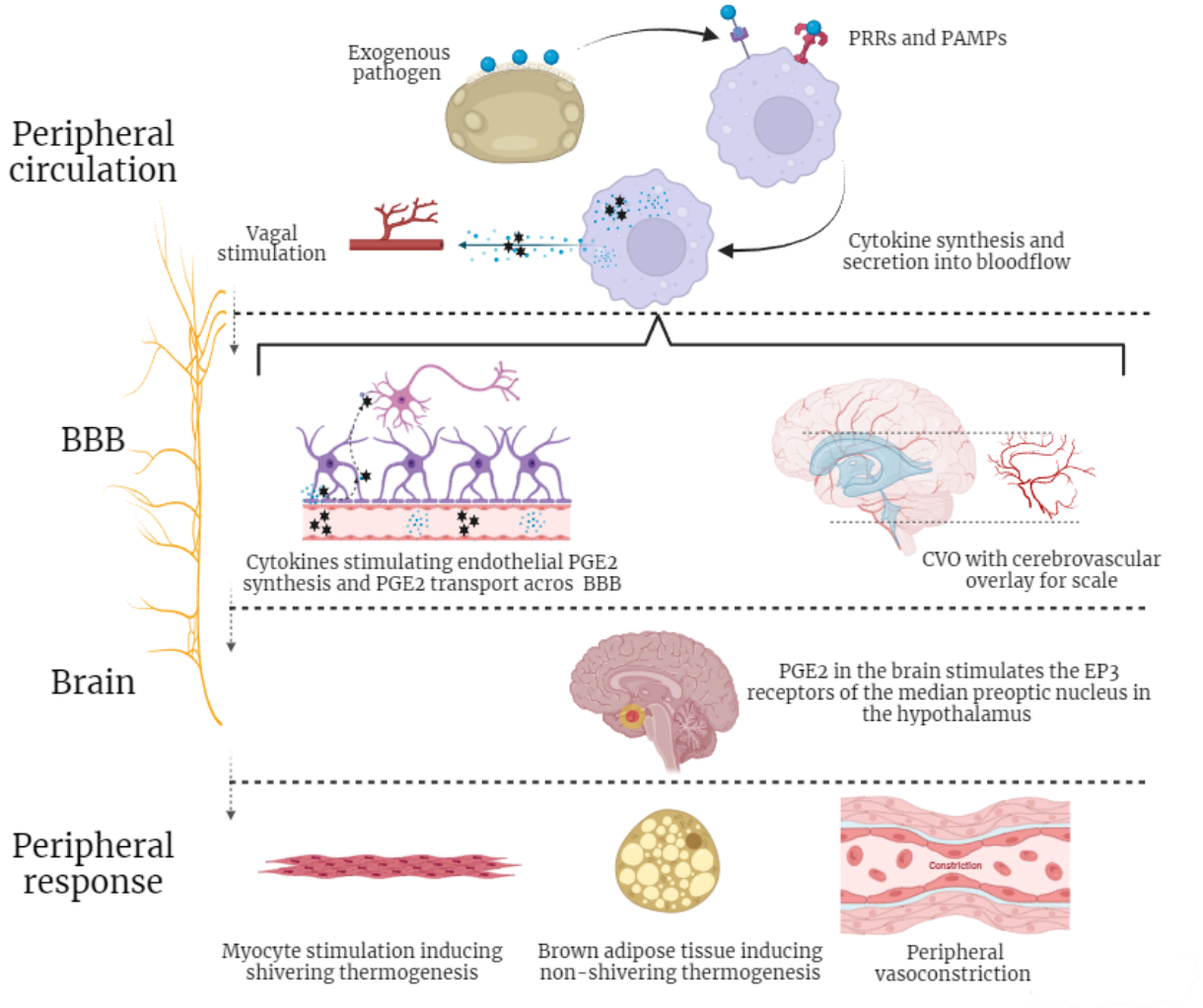


Figure 1.3: Figure of the pathways involved in fever induction, created with BioRender.com.

The PAMPs bind to the PRRs of a macrophage as shown above. The immune cell (macrophage) is activated following PRR stimulation, causing the synthesis and release of pyrogenic cytokines, COX-2 and PGE_2 into surrounding tissue and most importantly, peripheral blood. Pro-inflammatory cytokines IL-6, TNF-α and IL-1β as well as PGE_2 stimulate the vagus nerve which can directly initiate a febrile response in the hypothalamus. Pro-inflammatory cytokines have two other major known BBB pathways to the hypothalamus, by binding to the endothelial cells at the BBB promoting central PGE_2 synthesis as well as peripheral PGE_2 directly crossing over the BBB to bind directly with the EP_3 receptor of the hypothalamus. And lastly, peripheral cytokines as well as PGE_2 move into the CSF via the fenestrated capillaries of the circumventricular organs which lacks a BBB allowing for passive flow of cytokines and PGE_2 into the brain equalizing the PGE_2 gradient between the blood and brain. Once PGE_2 is within the brain, PGE_2 can bind to the EP_3 receptors of the median preoptic nucleus at the hypothalamus decreasing the firing of warm-sensitive neurones. EP_3 stimulation by PGE2 will result in noradrenaline release which promotes vasoconstriction of peripheral blood vessels minimizing heat loss as well as activation of brown and beige adipose tissue resulting in non-shivering thermogenesis (Garami *et al.,* 2018). Lastly, acetylcholine is released to excite myocytes causing shivering to occur promoting heat gain.

1.2.2 Zymosan as a pyrogenic model

Brewers or baker's yeast to the layman otherwise known as *S. cerevisiae* , is a single celled yeast found globally in food products (such as in baking and brewing), probiotics as well as plants and soil (Peter *et al*., 2018). The prevalence of *S. cerevisiae* within human environments and settings makes *S. cerevisiae* a potent opportunistic pathogen in patients who are immunocompromised (Algazaq *et al.*, 2017). While the infections caused by *S. cerevisiae* are often mild and even asymptomatic and thus overlooked by most of the world's healthy population, within those that are immunocompromised *S. cerevisiae* has been noted to induce a wide array of severe symptoms such as urinary tract infections, epidermal infections, peritonitis, esophagitis, fungemia, pneumonia and endocarditis (Algazaq *et al.*, 2017). The main immune response brought on by *S. cerevisiae* is due to the surface ligand (zymosan) of *S. cerevisiae* binding to TLR2/TLR6 and Dectin-1 (Keystone *et al*., 1977, Brown *et al*., 2003). Recently, with the discovery that zymosan induces fever following similar mechanisms to those outlined in Figure 1.3 (Bastos-Pereira *et al*., 2014), it has been suggested that zymosan's use in animal studies may be a good model for simulating fungal infections and testing antipyretic agents (Dangarembizi *et al.*, 2019).

One issue regarding *S. cerevisiae* when used as the pyrogenic pathogen model in a fever study is that it is ethically questionable to infect patients or subjects with said pathogen. The concern is due to the effects of *S. cerevisiae* infection posing a risk towards the host as live yeasts often results in the host experiencing a fever, lethargy, mass stunting via anorexia, abscess development as well as pain (Kanashiro *et al.*, 2009, Bastos-Pereira *et al.*, 2017, Lima *et al.*, 2017, Dangarembizi *et al.*, 2018). Ethically speaking researchers must follow the principle of refining or modifying techniques so as to improve the welfare of research subjects by reducing pain, distress and mortality when studied (Baumans, 2004). By using the ligand (zymosan) from the cell wall of *S. cerevisiae* it is possible to initiate a fever response without the mortal effects of *S. cerevisiae* as the only component of the pathogen is the signalling agent for our immune system to respond towards.

Recent findings have proven that zymosan when administered subcutaneously can be used as an experimental pyrogen for screening the efficacy and mechanism of action of anti-inflammatories and more importantly with reference to our work, antipyretics (Dangarembizi *et al.*, 2019). When administered interperitoneally or as an intra-articular injection, zymosan

has been observed to only result in short-term sickness behaviours and fevers that limit the pyrogenic model of zymosan-induced fevers to acute response studies. However, when given at high enough concentrations (300 mg/kg as a 10 mL/kg volume) as a subcutaneous injection, zymosan can induce a fever as well as associated sickness behaviours such as lethargy and anorexia over a 24 h period (Dangarembizi *et al.*, 2019). The fever associated with zymosan administration was accompanied by an increase in peripheral TNF-α and IL-6 concentrations along with an increased expression of TNF-α, IL-6, IL-1β, COX-2 and mPGES1 within the hypothalamus (Dangarembizi *et al.*, 2019). The expression of these pro-inflammatory substances within the periphery and brain proves zymosan's status as a PAMP. The cytokines TNF-α and IL-6 are the first peripheral cytokines to be expressed following zymosan administration with IL-6 peaking during the plateau phase of fever. Therefore, TNF-α and IL-6 are believed to be responsible for the early development of a zymosan-induced fever while IL-6 has a suspected maintenance role in zymosan-induced fever (Dangarembizi *et al.*, 2019). While not investigated during subcutaneous administration, intraperitoneal injections of zymosan are known to increase PGE_2 concentration within the CSF indicating a central reaction to zymosan administration which induces a fever (Doherty *et al.,* 1985, Kanashiro *et al.*, 2009, Bastos-Pereira *et al*., 2014). The inflammation associated with zymosan- is due to the stimulation of the NF-kB pathway inducing an inflammatory response similarly to LPS administration, making zymosan an ideal phlogistic agent replicating known inflammation and fever pathways (Sahan-Firat *et al.,* 2019). Findings demonstrating zymosans ability to replicate the sickness responses observed with administration of *S. cerevisiae* while reducing mortality risk, suffrage of animals and dose irregularity make zymosan a desirable pyrogenic substance to test fungal-induced fevers against anti-inflammatories and antipyretics (Dangarembizi *et al.*, 2018).

Thanks to research optimizing fungal pyrogen models for fever studies, zymosan can be used (when given subcutaneously) to model a fever over a 24 h period allowing the study of all phases and even treatment of a fever (Dangarembizi *et al.*, 2019). With respect to the treatment of zymosan-induced fevers it has also been found that paracetamol, ibuprofen as well as indomethacin (known antipyretic and anti-inflammatory drugs) all have the potential to act as effective medications in the attenuation of said fever, thus treating and preventing sickness behaviours (Kanashiro *et al.*, 2009, Bastos-Pereira *et al.*, 2017). The combination of safety provision for the host, reliability of zymosan to initiate a fever over 24 h and the known ability

to be treated by antipyretic and anti-inflammatory drugs make zymosan an optimal choice to study fungal-induced fevers.

1.3 Generation of the fever-hypothermia switch during inflammation

Fever is the most common and better researched thermoregulatory response to our body's reaction to infection and inflammation. However, hypothermia has been identified in patients who experience severe systemic inflammation prior to the induction of a fever (Leon, 2004, Romanovsky *et al*., 2005, Steiner *et al.*, 2011, Garami *et al.,* 2018). One hypothesis for the fever-hypothermia switch is that fever represents a disease-fighting strategy to remove pathogens from the body while using host energy reserves, whereas hypothermia is a disease tolerance strategy to reduce the harmful effects of fever in a critically weakened and infected patient who needs to simply survive an infection rather than clear it immediately (Medzhitov *et al.*, 2012). The theory of hypothermia promoting survival has been linked to metabolic suppression, reducing oxygen consumption which prevents hypoxia and cardiac stress experienced during endotoxic shock often inducing a decrease in cardiac output (Corrigan *et al.*, 2014). The exact mechanisms of the fever-hypothermia switch are not known currently, yet we understand systemic inflammation and its biomarkers are key contributors to the hypothermia seen during infection.

The start of hypothermia during systemic inflammation is the initiation of cold-seeking behaviours paired with autonomic effectors inducing peripheral vasodilation and especially inhibition of thermogenesis (Romanovsky, 2004). The vasodilation that occurs during hypothermia because of systemic inflammation is often due to the synthesis of histamine, nitric oxide and prostanoids which are upregulated by the presence of pro-inflammatory cytokines, such as TNF-α and IL-1β (Romanovsky, 2004). With TNF-α being hypothesized to possess cryogenic properties at high concentrations and febrigenic properties at low to moderate concentrations, and IL-1β possessing hypotensive properties (Bibby *et al.*, 1989). It is believed that both TNF-α and IL-1β play a role in the induction and maintenance of hypothermia and hypotension identified in extreme cases of systemic inflammation (Bibby *et al.*, 1989, Kozak *et al.*, 1995). However, once both TNF-α and IL-1β are expressed in the periphery, they promote the synthesis of IL-6 which is the most important endogenous pyrogen in the induction of central PGE_2 and therefore fever, hence it is likely that IL-6 subsides the hypothermia and initiates the switch back to the induction of a fever during infection (van Damme *et al.*, 1986, Eskilsson *et al.*, 2014). In addition to the involvement of cytokines in the fever-hypothermia switch, COX isoforms have also been implicated. During fever induction it is known that COX-

2 is the COX isoform mediating fever, yet during hypothermia COX-1 is the main COX isoenzyme responsible for LPS-induced hypothermia (Steiner *et al.*, 2009). COX-1 is known to be expressed within tissues throughout the body as a housekeeping enzyme with no excess production during inflammation (Garami *et al.*, 2018). Yet, following LPS administration inducing hypothermia, rats given a COX-1 inhibitor (valeryl salicylate or SC-560) had their body temperatures return to normal levels as well as prevented hypothermia while leaving the fever expected from LPS intact (Steiner *et al*., 2009, Garami *et al.*, 2018). It is believed that COX-1 is stimulated due to hydroperoxide and peroxynitrate from systemic inflammation allowing for COX-1-mediated PGE_2 synthesis following LPS-induced hypothermia, which is one of the pathways switching the hypothermia over to a fever (Steiner *et al*., 2009). With COX-1 inducing hypothermia believed to be caused by early production of prostaglandin D_2 and its metabolites (Ueno *et al.*, 1982, Garami *et al.*, 2018).

Therefore, it appears that TNF-α, IL-1β and COX-1 are likely catalysts involved in inducing hypothermia during severe systemic inflammation (Liu *et al.*, 2012, Harden *et al*., 2014, Garami *et al.*, 2018). In terms of how systemic inflammatory mediators, in particular TNF-α, acts within the brain to induce hypothermia, a role for the cannabinoid-1 (CBD-1) receptor has been identified (Steiner *et al.*, 2011). Rats who had their CBD-1 receptor blocked during LPS administration never experienced hypothermia, yet rather had body temperatures akin to that of saline treated rats, showing how CBD-1 is essential for hypothermia to take place during systemic inflammation (Steiner *et al.*, 2011). Peripheral administration of exogenous endocannabinoids, which are known to stimulate CBD-1 receptors, was also found to induce hypothermia through tail-skin vasodilation via transient receptor potential vanilloid 1 (TRPV1) channel stimulation in rats and not CBD-1 receptor stimulation (Steiner *et al.*, 2011). TRPV1 receptors are expressed among sensory neurons throughout the body and are known to be stimulated by heat, noxious stimuli, vanilloids, cannabinoids and inflammatory mediators (Shuba, 2021). While TRPV1 receptors have numerous functions, they can when stimulated cause vasodilation by endothelial relaxation and nitric oxide production (Steiner *et al.*, 2011, Shuba, 2021). Thus, the complex and misunderstood relationship of the fever-hypothermia switch is more of an adaptive response of our immune system to pathogens and the severity of one's reaction to infection. Through the combination and fluctuations of cytokines (such as IL-6, TNF-α and IL-1β), COX 1 and 2 and endocannabinoids during systemic inflammation, hypothermia can be generated through peripheral vasodilation, hypotension, inhibition of thermogenesis and cold seeking behaviours.

1.4 Antipyretic mechanism of action of paracetamol

Paracetamol is one of the main antipyretics used globally for fevers and infections and has been deemed the gold-standard as a primary treatment for not just fever attenuation, but pain and sickness behaviour reduction (Prescott, 2000, Ayoub, 2021). Paracetamol has a known ability to work as a central analgesic by activating descending serotonergic pathways (Anderson, 2008). However, the mechanism of action of paracetamols effect as a central antipyretic is the topic of much debate (Anderson, 2008). Besides central activity, paracetamol has a major peripheral effect of COX isoenzyme inhibition, often within the endothelium and blood (Hinz & Brune, 2012). The leading theory behind the antipyretic effect of paracetamol, is that paracetamol inhibits and reduces PGE_2 synthesis, the terminal mediator of fever in the hypothalamus (Anderson, 2008). However, while the exact pathway is unknown, the leading theory is that paracetamol irreversibly binds to COX preventing PGE_2 synthesis, yet the particular form of COX bound is not fully understood as to whether it's COX-1, COX-2 or COX-3 (Hanson & Maddison, 2008). While the understanding is that paracetamol acts centrally, paracetamol has also been shown to inhibit prostaglandin-synthesising COX enzymes within the periphery of the body, not only by binding to COX but by reducing the already high oxidation state of COX enzymes thus reducing their chemical stability (Hinz & Brune, 2012). Fever research looking into paracetamol's mechanism of action has shown that paracetamol has a greater probability of binding to COX-2 in most clinically relevant situations (Hinz & Brune, 2012). The knowledge of the function of COX-2 has been known for some time in the space of pathophysiology, with COX-2 being a known rate-limiting catalyst for PGE_2 synthesis. Therefore, COX-2 is the primary target of medication attempting to treat inflammation and fever. While COX-3 is a more recently discovered isozyme of COX that's function not fully understood within humans, however, the expression of COX-3 has been shown to be selectively inhibited by analgesic and antipyretic drugs consistently and is therefore a primary target for studies looking into fever and its mechanisms (Chandrasekharan *et al.*, 2002). While antipyretic studies have shown paracetamol to exert its antipyretic action mainly by COX-2 inhibition, the evidence that paracetamol alters peroxidase sites of other COX enzymes means that paracetamol should theoretically affect all COX isoenzymes (Anderson, 2008, Mirrasekhian *et al.*, 2018). Therefore, it can be summarized that the antipyretic properties of paracetamol are reported to be due to inhibition of COX-2 mediated production of PGE_2 within the hypothalamus (Afsar *et al.*, 2015). By decreasing the production of PGE_2 in the hypothalamus during fever, antipyretics decrease core body temperature by increasing heat loss (e.g.,

peripheral vasodilation) and decreasing heat production (e.g., inhibition of shivering and non-shivering thermogenesis).

1.5 Aim and objectives

The aim of the study was to determine the mechanism of action for the suspected antipyretic properties of aqueous extracts of the root of *A. ecklonii* using an animal model of zymosan-induced fever in male Sprague Dawley rats.

The objectives were:

1. To prepare an aqueous extract of the root of *A. ecklonii*.
2. To measure the abdominal temperature of male Sprague-Dawley rats injected with zymosan or saline and aqueous *A. ecklonii* root extract or paracetamol.
3. To measure the concentration of pro-and anti-inflammatory cytokines within the blood plasma of male Sprague-Dawley rats injected with zymosan or saline and aqueous *A. ecklonii* root extract or paracetamol.
4. To measure the mRNA expression of pro-and anti-inflammatory cytokines and enzymes involved in the synthesis of PGE_2, namely COX-1, COX-2 and mPGES1, within the hypothalamus of male Sprague-Dawley rats injected with zymosan or saline and aqueous *A. ecklonii* root extract or paracetamol.
5. To determine the concentration of PGE_2 within the cerebrospinal fluid (CSF) of male Sprague-Dawley rats injected with zymosan or saline and aqueous *A. ecklonii* root extract or paracetamol.

Chapter 2

Methods

2.1 Animals and housing

Male Sprague-Dawley rats (n = 73) were procured from the University of Witwatersrand Research Animal Facility (Johannesburg, South Africa). All animals were housed individually in polypropylene cages lined with sawdust, wood shavings and shredded paper in a temperature-controlled room (25°C ± 1°C and relative humidity of 35-50%). For the enrichment of the rats, toys such as balls and plastic tunnels were placed in each cage. The lighting conditions were set to a 13-hour light/11-hour dark cycle, with lights on from 06:00 to 19:00. Rats were allowed to acclimatise to their environment for 14 days before surgery. The rats had access to rodent pellets (LabChef Rodent Breeder, Nutritionalhub (Pty) Ltd, Stellenbosch, South Africa) and water *ad libitum*. On the day of injection, the body mass of the rats used in this study ranged between 250–300 g. All experiments were performed in accordance with the animal care regulations of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by its Animal Ethics Research Committee (ethics clearance number 2018/11/56/C; Appendix)

2.2 Plant selection, procurement and extraction of plant material

Whole plants of *A. ecklonii* were procured from Random Harvest Nursery (Muldersdrift, Krugersdorp) where harvesting occurred during the warm summer season in February 2019. These plants were housed in the Department of Pharmacy and Pharmacology, University of the Witwatersrand. The root of the plant was separated from the upper plant and carefully rinsed with filtered tap water and left to dry for two weeks at room temperature (ambient temperature of 25°C). The exact duration required for drying varied depending on the thickness of the *A. ecklonii* roots, as well as the humidity and temperature variations of the room. Once the roots had been sufficiently dried it was then pulverized using a high-speed grinder (Pulverissete 14, Fritsch, South Africa). The root extract was then submerged in sterile water (1:1 ratio) and left in a shaker incubator at 37°C for 24 h (FSIM 24, Labcon Ltd, South Africa). Sterile cotton wool was used as a filter to separate the supernatant of the mixture into sterile test tubes. The aqueous extract was then placed into pre-weighed sterile bottles. These bottles were placed in a freezer at -80°C overnight before lyophilisation using a benchtop freeze dryer (Virtis, SP Scientific, BenchTop Pro with Omnitronics). Once lyophilized, the aqueous extracts were exposed to ultra-violet light for 24 h to reduce possible microbial contamination which may have been introduced during the extraction process and thereafter stored at 25°C in a sealed container with

no exposure to sunlight until needed. All aqueous extracts of the root were prepared in a primary laboratory area, under sterile conditions at the University of the Witwatersrand.

2.3 Plant extract preparation for injections

On the day of injection, the root was removed from storage and prepared for injection. Within the sterile environment of a biosafety class II cabinet (BIOBASE, High-tech Zone, Jinan, Shandong, China) the root extract was weighed (AS 82/220.R2 PLUSAnalytical Balance, Poland) out as 0.11 g. Following the weighing of the root, 2 mL of 0.9% sterile non-pyrogenic saline was added to the root extract (Sabbax, Johannesburg, South Africa) to make a concentration of 55 mg/mL. Thereafter the root extract was sonicated using a sterilised hand sonicator (Misonix XL-2000 Series Ultrasonic Liquid Processors, American Laboratory Trading, San Diego) for 10 s. After sonication, the root extract was autoclaved for 15 min at 121°C to sterilize the mixture, bottle and lid thus preventing any cross-contamination or risk of pyrogen addition to the plant extract (Hashemi, 2008). Autoclaving was chosen over filtration to sterilize the plant, as autoclaving is less damaging to the antibacterial activity of aqueous plant extracts and thus retains the plants bioactive compounds to the peak probable concentration (Hashemi *et al.*, 2008). After the autoclave procedure the bottle containing the extract was removed and allowed to cool at room temperature. The rats received an intraperitoneal (i.p.) injection of the aqueous root extract of *A. ecklonii* at a dose of 55 mg/kg. A previous study undertaken within our research group has shown this dose to briefly attenuated zymosan-induced fever in rats (Cottino, 2020). Concerns regarding the precipitation of solid plant matter in the root extract solution arose from the limited homogenization or dissolution capability of the saline suspension. To enhance uniformity, a method of gentle shaking was employed on the storage bottle prior to drawing up the root extract solution, aiming to achieve ideal mixing of the solution and solutes.

To test the root extract for contamination, it was plated on to agar (Tryptone Soya agar CM0131, ThermoFisher Scientific). Agar plates were sealed in a sterile manner and checked daily for a week to identify possible contamination. In addition ~50 μL of root extract was aseptically pipetted into a broth (Tryptone Soya broth CM0129, ThermoFisher Scientific) and the broth tube was sealed and incubated to be checked in the same manner as the agar plate.

2.4 Preparation of zymosan

Zymosan A from *S. cerevisiae* (Z4250, lot number BCBP2641V, Sigma Aldrich, St. Louis, Missouri, USA) was reconstituted in 0.9% sterile, non-pyrogenic saline (Sabax, Johannesburg, South Africa). After reconstituting the zymosan in saline, 30 mg/mL solution was vortexed, placed in a water bath at 37 °C for 10 min, and then sonicated (Fisher Scientific Model 50 Sonic Dismembrator, Waltham, Massachusetts, U.S.) in 30 s intervals, with 10 s pauses in between for a total of 2 min. The rats used in this study received a single subcutaneous (s.c.) injection, in the dorsum of their neck, of 300 mg/kg of zymosan A; the volume injected was 10 mL/kg of a 30 mg/mL solution. The dose of zymosan used, was based on a previous study undertaken in our research group which showed that injecting rats subcutaneously with 300 mg/kg zymosan, induced fever and sickness behaviour for 24 h (Dangarembizi *et al.*, 2019).

2.5 Body mass

The body mass of the rats was measured daily using an electronic scale (Diamond, USA) accurate to 5 g. Measurements were taken daily at 16:00. Body mass was also measured immediately before interventions, such as injections and surgeries. The electronic scale was calibrated before use.

2.6 Abdominal temperature

Abdominal temperature was measured using sterile wax-coated temperature-sensitive radiotransmitters (TA10TA-F40 Implant, Data Science, St. Paul, MN USA). The radiotransmitters were implanted intra-abdominally into the rats. All the radiotransmitters were calibrated by the manufacturer (Data Science, St. Paul, MN USA) to an accuracy of 0.1°C in a 35°C and 39°C water bath. The radiotransmitters were placed in F10 sterilant (Health and Hygiene (Pty) Ltd, VetEx, South Africa) prior to surgical insertion to prevent risk of infection. The rats were placed under general anaesthesia induced by an intramuscular injection of ketamine hydrochloride (80 mg/kg) (Anaket-V, Bayer, South Africa) and xylazine (20 mg/kg) (Chanazine, Bayer, South Africa). Once the rats were unconscious, the sterile wax coated temperature sensitive radiotransmitters were implanted intra-abdominally. Directly before and 4 h after the surgery the rats were given an analgesic in the form of an intramuscular injection of 0.05 mg/kg Buprenorphine (Temgesic®: Schreign-Plough, Johannesburg, South Africa).

Once the surgery was completed the rats were placed in a warm room to recover for approximately 4 h, before being transferred back into their original polypropylene cages. The cage bedding was replaced with a paper towel to prevent inhalation of dust or wood shavings by the recovering rats. The bedding was replaced with wood shavings the following morning after surgery, once the rats had started moving around the cage. The rats were all given a minimum of a week post-surgery to recover before any interventions could continue. During their recovery, the rats were monitored for any signs of infection or discomfort. To detect the output frequency (Hz) of each radiotransmitter, a receiver plate (RPC-1, Data Sciences, St Paul, MN, USA) was placed beneath each cage. The output frequency from each plate was transmitted to a peripheral processor (DP-24 DataPort, VitalView, Minimitter, Sunriver, OR, USA) and connected to an external computer where the signal was converted to ℃ and recorded every 5 min.

2.7 Experimental procedures

Rats were randomly assigned to receive one of the following combinations of injections:

- Saline (s.c.) + Saline (i.p.) (n=10)
- Saline (s.c.) + Paracetamol 50 mg/kg (i.p.) (n=10)
- Saline (s.c.) + Aqueous *A. ecklonii* root extract 55 mg/kg (i.p.) (n=12)
- Zymosan 300 mg/kg (s.c.) + Saline (i.p.) (n=9)
- Zymosan 300 mg/kg (s.c.) + Paracetamol 50 mg/kg (i.p.) (n=10)
- Zymosan 300 mg/kg (s.c.) + Aqueous *A. ecklonii* root extract 55 mg/kg (i.p.) (n=11)

The initial s.c. injection of either zymosan or saline was given at timelines between 15:45 and 16:45. The second i.p. injection was administered 15 h later. The rationale for using this timepoint was based on a previous study undertaken in our research group which showed that fever induced by s.c. administration of zymosan (300 mg/kg) was stable during 15 - 18 h after administration (Dangarembizi *et al*., 2019). Furthermore, this time point also corresponds to a period where afebrile rats have stable abdominal temperature, and there are minimal fluctuations caused by changes in circadian rhythm. As the current study is investigating the effects of *A. ecklonii* root extract on abdominal temperature, it was important to select a timepoint where abdominal temperature in both febrile and afebrile rats was stable and not influenced by changes in circadian rhythm.

All s.c. injections were administered at a volume of 10 mL/kg body mass. Paracetamol (Lot #P181012C, Cipla, Activis Pharma (Pty) Ltd, Bryanston, South Africa) was used as a positive control (Ayoub, 2021) 15 h after the initial s.c. injection. Rats received a single i.p. injection of 50 mg/kg paracetamol. The volume injected was 5 mL/kg of a 10 mg/mL solution. The dose was based on previous studies that displayed how paracetamol reduces zymosan-induced fever at this dose (Cottino, 2020.). Saline was injected i.p. at a volume of 5 mL/kg body mass.

The first surgical intervention involved radiotransmitter insertion, followed by the first injection 14 days later (Figure 2.1). This two-week period allowed for full surgical recovery and recording of abdominal temperature before any interventions began. For the second injection, rats received an i.p. injection of saline, paracetamol (50 mg/kg) or *A. ecklonii* root extract (55 mg/kg). Saline and paracetamol were administered at a volume of 5 mL/kg body mass. Blood, cerebrospinal fluid (CSF) and hypothalamic tissue was collected under terminal anaesthesia induced with an i.p. injection of 1 mL sodium pentobarbital (Euthanase, 200 mg/mL; Kyron Laboratories (Pty) Ltd., South Africa) 90 min after rats received their second injection. Previous studies within our research group have shown that administering *A. ecklonii* root extract (55 mg/kg) can attenuate the fever response ~ 90 min after injection, thus it was decided it would be best to collect samples at this time point (Cottino, 2020).

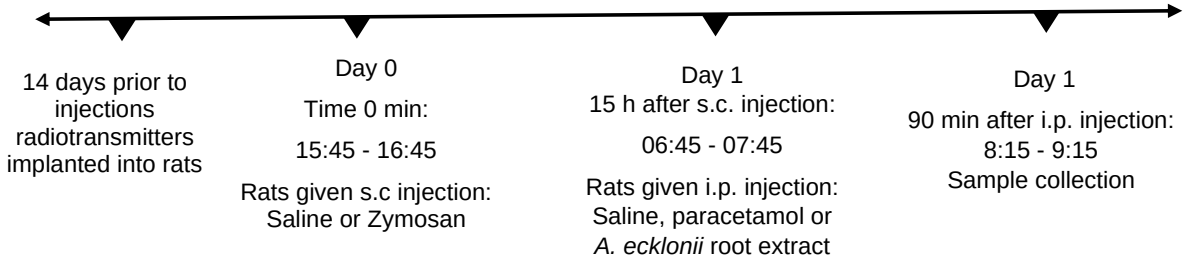


Figure 2.1: The experimental timeline for the different interventions used in the study.

Once the rat was unresponsive, CSF was collected according to the method described by Consiglio and Lucion, 2000. This method involves holding the rat upright and rotating their head forward so that a sterile 25-gauge needle may be inserted into the spinal cord 2 mm below the occipital bun. Approximately 20-60 µL of CSF was pipetted from the basin of the needle head . The CSF was stored in Eppendorf tubes containing 2 µL indomethacin (2.5 µg/µL) to stop prostaglandin production. The tube containing CSF was kept on ice until centrifuged at

4775 x g for 10 min at 4°C. After centrifugation, the supernatant was removed and frozen at -80°C. Blood samples were collected by means of cardiac puncture into sterile ethylene diamine tetra acetic acid. The blood was kept on ice until centrifuged at 4766 x g for 10 min at 4°C. The blood plasma was stored at -80°C in a sterile Eppendorf tube until cytokine measurements were completed for that sample. After blood collection, rats were transcardially perfused with ice-cold 0.9% saline for 2 min. The hypothalamus was carefully dissected from the brain of each rat and all collected tissues were snap-frozen in liquid nitrogen and stored at -80°C until real-time polymerase chain reaction (RT-PCR) was performed.

2.8 Cytokine and prostaglandin E2 measurement

The concentrations of IL-1β, IL-6, IL-10 and TNF-α within the blood plasma was measured in duplicate using a commercially available R & D systems (MN, USA) rat magnetic Luminex performance assay according to protocols provide by the manufacturer. Plasma samples were diluted 1:1 and assayed in duplicate. The assay was performed using a 96-well plate. The standard or sample (50 µL) was added to each well, followed by 50 µL diluted microparticle cocktail. Thereafter, the plate was incubated for 2 h at room temperature on a shaker at 800 rpm. After the incubation the plate was washed with wash buffer three times. Diluted biotin-antibody cocktail (50 µL) was then added to each well and the plate was incubated for 1 h at room temperature on a shaker at 800 rpm. After the incubation the plate was washed with wash buffer three times. Diluted streptavidin-PE (50 µL) was then added to each well and the plate was incubated for 30 min at room temperature on a shaker at 800 rpm. After the incubation the plate was washed with wash buffer three times. On completion of the final wash, 100 µL wash buffer was added to each well and the plate was incubated for 2 min at room temperature on a shaker at 800 rpm. After the incubation the plate was read using a Luminex® analyser. The Luminex® 200™ System (Luminexcorp, Austin, Texas, United States) was validated monthly using a Bio-Plex® validation kit (Bio-rad, California, United States, 04.0 #171203001) and calibrated on the day of the assay using a Bio-Plex calibration kit. A five-parameter logistic (5-PL) standard curve for the TNF-α, IL-6, IL-10 and IL-1β data was created. The results were expressed as picograms per millilitre (pg/mL). The lowest detectable concentration, termed the limit of detection (LOD), for each cytokine was (all units in pg/mL): TNF -α = 26.34, IL-6 = 20.84, IL-10 = 8.78, and IL-1β = 12.64. Several quality parameters were examined, including fit probability of the standard curve and coefficient of variation. All fit probabilities and coefficient of variation met the quality criteria. Where the values obtained were below the

detection limit, the detection limit of the assay divided by the square root of two was assigned (Corghan and Egeghy, 2003).

The PGE_2 was measured in the CSF using a PGE_2 ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). The PGE_2 assay was performed using a 96 well plate. Samples were diluted 1:2 and assayed in duplicate. The ELISA buffer (100 µL) was added to the non-specific binding wells and 50 µL to the maximum binding wells. Thereafter 50 µL of the standard or sample was added to the appropriate well followed by 50 µL PGE_2 tracer to each well except the total activity and blank wells. The PGE_2 monoclonal antibody (50 µL) was then added to each well except the total activity well, non-specific binding well and the blank wells. The plate was then incubated for 18 h at 4°C. After the incubation the plate was washed five times with wash buffer and then Ellman's reagent (200 µL) was added to each well as well as 5 µL PGE_2 tracer to the total activity wells. The plate was incubated on an orbital shaker in the dark for 60 min. After the final incubation the sample optical density was determined using a microplate reader set at 414 nm. The PGE_2 concertation was determined using a four-parameter logistic fit standard curve.

2.9 Real-time polymerase chain reaction (RT-PCR)

Approximately 35 mg of hypothalamic brain tissue was sonicated in 350 µL lysis buffer and 3.5 µL beta-mercaptoethanol for 10 s using a hand sonicator (Misonix XL-2000 Series Ultrasonic Liquid Processors, American Laboratory Trading, San Diego). Total RNA was then extracted using an Illustra minispin RNA extraction kit (GE Healthcare, 25050070, USA), as per the manufacturer's instructions. Following the sonification, the lysate was filtered through a RNAspin filter. The filtrate was then centrifugation for 1 min at 11 000 x g. Ethanol (350 µL) was then added followed by vortexing twice for 5 s each. The lysate was then loaded onto a RNAspin column and centrifuged for 30 s at 8000 × g. A desalting buffer (350 µL) was added to the column and then it was centrifuged at 11 000 × g for 1 min to dry the membrane. DNase I reaction mixture (95 µL) was added directly onto the centre of the silica membrane of the column and allowed to incubate for 15 min. After the incubation, the column was washed three times starting with 200 µL wash buffer 1 (GE Healthcare, 25050070, USA, 70% ethanol and 30% NucliSENS lysis buffer) which was added and centrifuged for 1 min at 11 000 x g , 600

µL wash buffer 2 (GE Healthcare, 25050070, USA, 70% ethanol and 30% RNase-free water) was then added and centrifuged for 1 min at 11 000 x g with the last wash step being 250 µL of wash buffer 2 which was centrifuged for 2 min at 11 000 x g. The RNA was then eluted in 50 µL RNase-free H_2O and centrifuged for 1 min at 11 000 x g. A sample (1 µL) was analysed so that RNA could be quantified (as ng/µL) using a NanoDrop spectrophotometer (NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer, Thermo Scientific, Waltham, Massachusetts, U.S.) This measures the sample absorbance at different ranges of ultraviolet-visible light. This quantification was done as a quality assessment measure prior to RT-PCR to ensure enough RNA was extracted and that there was an acceptably low range of contaminants such as proteins, salts, phenol and guanidine from each sample (measured as the A260/A280 and A260/A230 ratios being as low as possible) before reverse transcription (NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer, Thermo Scientific, Waltham, Massachusetts, U.S.). Once each sample had a high enough RNA yield as well as minimal contaminants using NanoDrop spectrophotometry (NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer, Thermo Scientific, Waltham, Massachusetts, U.S.), total RNA was reverse-transcribed into cDNA by incubation of 1 µL total RNA with 4 µL SuperScript™ IV VILO™ MasterMix and nuclease-free water (Applied Biosystems, Foster City, USA), in a total reaction volume of 20 µL, at 25°C for 10 min and then 50°C for 10 min, followed by inactivation at 85°C for 5 min in a thermocycler (T100 Thermal Cycler 1861096, Bio-Rad, Johannesburg, South Africa). The resultant cDNA (1 µL) was quantified using a NanoDrop spectrophotometer (NanoDrop OneC, Thermo Scientific, South Africa) in the same manner as RNA quantification with the only difference being the spectrophotometry settings being calibrated to cDNA analysis. After reverse-transcription, quantitative real-time PCR was performed in duplicate with a pre-optimised primer/probe mixture (Taqman Gene Expression Assay; Applied Biosystems, Foster City, USA) and Taqman universal PCR Master Mix (Applied Biosystems, Foster City, USA) using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA.). The PCR cycle condition used included an initial denaturation at 95 °C for 10 min, 40 cycles of annealing at 95 °C for 15 s and elongation at 60 °C for 1 min. The values for measuring the number of PCR cycles known as cycle threshold (Ct) were obtained using software called StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA). The reference gene used to normalize the cDNA quantities between different reactions was β-actin (labelled as Actb by Applied Biosystems as Rn00667869_m1). The reason for using β-actin as the reference gene was due to its prominence in eukaryotic cells giving a high expression in most tissue samples (Bustin, 2000). The β-actin values between the saline and zymosan groups in our experimental set-up remained relatively constant. By

calculating the difference between the mean Ct value of the reference gene and the mean Ct value of the target gene, the average ΔCt value was determined for each sample. Subsequently, the ΔΔCt value was obtained by subtracting the average ΔCt value for each sample from the average ΔCt values for all rats injected with saline. Finally, the relative quantification (RQ) was calculated as 2 to the power of negative ΔΔCt. This was done for each sample individually (Livak & Schmittgen, 2001). The assay IDs for the genes that were measured are IL-6 (Rn01410330_M1), IL-10 (Rn01644839_M1), IL-1β (Rn00580432_M1), TNF-α (Rn99999017_M1, COX-1 (Rn00566881_M1), COX-2 (Rn00568225_M1) and mPGES1 (Rn00572047_M).

2.10 Data analysis

All statistical analyses were conducted using GraphPad Prism 10 (GraphPad Software Inc, USA). The sample size used in the study was based on previous work undertaken in our research group where we were able to detect significant differences in abdominal temperature using 9 – 12 rats per intervention (Cottino, 2020). For statistical purposes, we averaged the original five-minute abdominal temperature recordings over successive 30-min periods. Changes in abdominal temperature were analysed by a two-way repeated measures analysis of variance (ANOVA) with treatment and time as the main effects. The ANOVA was followed by either a Šídák's or Tukey's multiple comparisons test for *post hoc* analysis, in order to identify differences between the groups. Since there were no significant differences between the saline + saline, or saline + paracetamol treatment groups, data from both groups were combined and used as one control group. Previous studies undertaken in our research group found that rats injected i.p. with aqueous *A. ecklonii* root extract (55 mg/kg) showed either no change in abdominal temperature or a decrease in abdominal temperature (~1°C) ~ 90 min after injection (Madisha, 2024). Thus, as the aim of the study was to investigate the potential antipyretic properties of *A. ecklonii* root extract or if the plant induces hypothermia, we examined the changes in abdominal temperature of the rats that received injections of saline + aqueous *A. ecklonii* root to identify rats which did and did not show a decrease in abdominal temperature. Rats were classified as not having a decrease in abdominal temperature if the abdominal temperature remained within ± 0.3 °C of the pre-injection values after the stress-induced hyperthermia. When animals are confronted with a stressor, like an injection, they initiate a stress response which includes an increase in body temperature, known as stress-induced hyperthermia (Olivier *et al*., 2005). The hyperthermia induced by injection is a rapid response

reaching a maximum within 10 – 15 min after the start of the stress-inducing stimulus (Olivier *et al*., 2005). A value of 0.3 °C was selected based on studies showing that the mean daily body temperature of rats is 37.1 ± 0.2°C at all ages (Refinetti, 1990). Data were expressed as means and standard deviations (SD).

The blood plasma cytokine concentrations and PGE2 concentrations in the CSF, as well as the hypothalamic mRNA expression of TNF-α, IL-1β, IL-10, IL-6, COX-1, COX-2 and mPGES1 was analysed using non-parametric tests (Mann-Whitney U test or a Kruskall-Wallis test), as the data followed a non-normal distribution. Data were expressed as median and interquartile range. A P value of < 0.05 was considered statistically significant.

Chapter 3

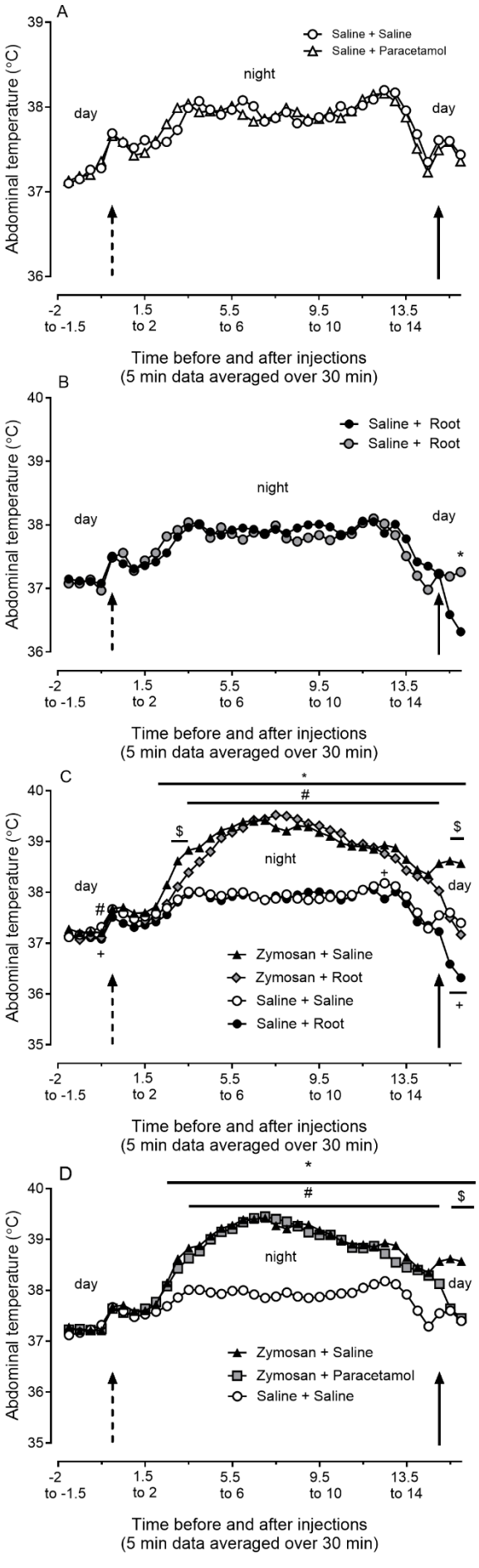
Results

Abdominal temperature was measured in rats treated according to a double injection protocol (s.c. injection between 16:00–17:00 followed 15 h later with an i.p. injection). The different experimental conditions will be identified in the results section below as follows:

- Saline (10 ml/kg) s.c. + Saline (5 ml/kg) i.p. = saline + saline
- Saline (10 ml/kg) s.c. + Paracetamol (50 mg/kg) i.p. = saline + paracetamol
- Saline (10 ml/kg) s.c. + Root (55 mg/kg) i.p. = saline + root
- Zymosan (300 mg/kg) s.c. + Saline (5 ml/kg) i.p. = zymosan + saline
- Zymosan (300 mg/kg) s.c. + Paracetamol (50 mg/kg) i.p. = zymosan + paracetamol
- Zymosan (300 mg/kg) s.c. + Root (55 mg/kg) i.p. = zymosan + root

3.1 Abdominal temperature

Figure 3.1A shows there were no significant differences in the abdominal temperature of rats that received saline + saline, or saline + paracetamol and thus the data from both groups was combined as one control group for figures 3.1B – D. The main effects of treatment were ($F(1, 18) = 0.041$, $P = 0.84$), time ($F(6, 110) = 33.78$, $P < 0.0001$) and interaction ($F(36, 648) = 1.05$, $P = 0.38$). Figure 3.1B shows that of the 12 rats that received an i.p. injection of *A. ecklonii* aqueous root extract, five rats maintained their abdominal temperature around 37 °C for the 90 min period after the injection, while 7 rats had a ~ 1°C decrease in abdominal temperature (main effects of treatment ($F(1, 10) = 6.77$, $P = 0.026$), time ($F(1.9, 19.0) = 16.45$, $P < 0.0001$) and interaction ($F(2, 20) = 17.62$, $P < 0.0001$). Figure 3.1C shows that in comparison to rats injected with saline + saline, rats injected with zymosan + saline developed a fever, which started after a latent period of 2 h, peaked at 39.42 (0.14)°C and continued for 16.5 h after injection (main effects of treatment ($F(4, 52) = 119.9$, $P < 0.0001$), time ($F(8.5, 445.6) = 305.7$, $P < 0.0001$) and interaction ($F(144, 1872) = 22.26$, $P < 00001$). Rats injected with zymosan + *A. ecklonii* root extract showed a similar fever profile to rats injected with zymosan + saline, with the exception of a ~ 1°C decrease in abdominal temperature after injection of the *A. ecklonii* root extract (Tukey *post hoc* $P < 0.001$). A similar ~ 1 °C decrease in abdominal temperature after injection of the *A. ecklonii* root extract was also noted for rats that received saline + *A. ecklonii* root extract, compared to rats that received saline + saline (Tukey *post hoc* $P < 0.01$). Figure 3.1D shows that rats injected with zymosan + paracetamol showed a similar fever profile to rats injected with zymosan + saline, with the exception of a ~ °C decrease in abdominal temperature after injection of paracetamol (Tukey post hoc $P < 0.01$).


A
Saline + Saline
Saline + Paracetamol
night
day
day
Abdominal temperature (°C)
39
38
37
36
-2 to -1.5
1.5 to 2
5.5 to 6
9.5 to 10
13.5 to 14
Time before and after injections
(5 min data averaged over 30 min)
B
Saline + Root
Saline + Root
night
day
day
*
Abdominal temperature (°C)
40
39
38
37
36
-2 to -1.5
1.5 to 2
5.5 to 6
9.5 to 10
13.5 to 14
Time before and after injections
(5 min data averaged over 30 min)
C
*
#
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night
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Zymosan + Saline
Zymosan + Root
Saline + Saline
Saline + Root
Abdominal temperature (°C)
40
39
38
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13.5 to 14
Time before and after injections
(5 min data averaged over 30 min)
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Zymosan + Saline
Zymosan + Paracetamol
Saline + Saline
Abdominal temperature (°C)
40
39
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36
35
-2 to -1.5
1.5 to 2
5.5 to 6
9.5 to 10
13.5 to 14
Time before and after injections
(5 min data averaged over 30 min)

Figure 3.1: Abdominal temperature (mean) measurements before and after injection of saline or zymosan and saline, *A. ecklonii* root extract or paracetamol. The 5-min abdominal temperature recordings were averaged over 30 min and plotted over 18.5 h. The 1st black arrow (dashed line) on the left indicates the point at which the s.c. injection of saline (10 mL/kg) or zymosan (300 mg/kg) was administered (16:00 – 17:00), with the 2nd black arrow (solid line) indicating the i.p. injection of saline (5 mL/kg), *A. ecklonii* root extract (55 mg/kg) or paracetamol (50 mg/kg) (07:00 – 08:00). Day and night cycles are shown by text within each figure with the label "day" and "night"; lights on at 06:00 and off at 19:00. Figure 3.1 A, since there were no significant differences between the saline + saline or saline + paracetamol treatment groups, both interventions have been combined and referred to as saline + saline. For Figure 3.1B * differences in abdominal temperature between rats given *A. ecklonii* root extract (n = 7 and n = 5; P = 0.003). Figure 3.1 B shows responders (black) and non-responders (grey) to *A. ecklonii* root extract administration 15 h after receiving saline. For Figure 3.1C * zymosan + saline (n = 9) significantly different from saline + saline (n = 20); # zymosan + root (n = 11) significantly different from saline + saline (n = 20); $ zymosan + *A. ecklonii* root extract (n = 11) significantly different from zymosan + saline (n = 9); + saline + *A. ecklonii* root extract (n = 7) significantly different from saline + saline (n = 20). For Figure 3.1D * zymosan + saline (n = 9) significantly different from saline + saline (n = 20); # zymosan + paracetamol (n = 10) significantly different from saline + saline (On = 20); $ zymosan + paracetamol (n = 10) significantly different from zymosan + saline (n = 9).

3.2 Plasma cytokine concentrations

Overall, there were no significant differences in the plasma cytokine concentrations of rats 90 min after they received an i.p. injection of saline or paracetamol:

- plasma IL-1ß concentration - saline (12.64 (12.64 – 24.59)) or paracetamol (15.35 (12.64 - 23.90)) (Mann-Whitney U = 47; P = 0.84).
- plasma IL-6 concentration - saline (988.3 (239.0 - 1881.0)) or paracetamol (57.29 (34.48 - 99.27)) (Mann-Whitney U = 42.5; P = 0.49).
- plasma TNF-α concentration - saline (26.34 (26.34 – 41.58)) or paracetamol (41.58 (26.34 - 66.91) (Mann-Whitney U = 24; P = 0.05).
- plasma IL-10 concentration - saline (8.78 (8.78 – 16.45) or paracetamol (15.63 (8.78 - 24.66) (Mann-Whitney U = 37; P = 0.30).

Thus, the data from both groups was combined as one control group for figures 3.2 – 3.4.

Figure 3.2 shows that the seven rats that had a decrease in abdominal temperature after the i.p. injection of *A. ecklonii* root extract, had a significantly greater plasma IL-1ß, IL-6, TNFα and IL-10 concentration compared to the five rats that did not show a decrease in abdominal temperature (IL-1β Mann-Whitney U = 4.5; P= 0.032; IL-6 Mann-Whitney U = 0; P = 0.002; TNFα Mann-Whitney U = 0; P = 0.002; IL-10 Mann-Whitney U = 0; P = 0.002).

Figure 3.3A and B shows that in comparison to rats injected with saline + saline, rats injected with zymosan + saline or zymosan + *A. ecklonii* root extract had significantly greater plasma IL-1ß and IL-6 concentrations (IL-1β Kruskal-Wallis statistic = 32, $P < 0.0001$; IL-6 Kruskal-Wallis statistic = 45.13, $P < 0.0001$). Figure 3.3C shows that there were no significant differences in the plasma TNF-α concentration between rats injected with saline + saline and rats injected with zymosan + saline or zymosan + *A. ecklonii* root extract (Kruskal-Wallis statistic = 29.13, $P < 0.0001$, Dunn's *post hoc* test $P > 0.05$). Rats injected with zymosan + *A. ecklonii* root extract did, however, have a greater plasma concentration of TNF-α compared to rats injected with zymosan + saline (Dunn's *post hoc* test $P = 0.008$). Figure 3.3D shows that in comparison to rats injected with saline + saline, rats injected with zymosan + *A. ecklonii* root extract had significantly greater plasma IL-10 concentrations (Kruskal-Wallis statistic = 45.13, $P < 0.0001$). There was no significant difference in the plasma IL-10 concentration between rats injected with saline + saline and zymosan + saline (Dunn's *post hoc* test, $P = 0.32$). Furthermore, a significant increase in plasma cytokine concentrations were also noted after injection of the *A. ecklonii* root extract as the seven rats that received saline + *A. ecklonii* root extract and had a decrease in abdominal temperature had greater plasma IL-1ß, IL-6, TNF-α and IL-10 concentrations compared to rats that received saline + saline (IL-1β Dunn's *post hoc* test, $P = 0.0025$; IL-6 Dunn's *post hoc* test, $P < 0.0001$; TNF-α Dunn's *post hoc* test, $P = 0.001$;IL-10 Dunn's *post hoc* test, $P < 0.0001$).

Figure 3.4A and B shows that rats injected with zymosan + paracetamol showed an increase in plasma IL-1β, and IL-6 concentrations compared to rats injected with saline + saline (IL-1β Dunn's *post hoc* test, $P = 0.012$; IL-6 Dunn's *post hoc* test, $P = 0.006$). There were no significant differences in plasma IL-1ß and IL-6 concentrations between rats injected with zymosan + paracetamol and rats injected with zymosan + saline (IL-1β Dunn's *post hoc* test, $P > 0.99$; IL-6 Dunn's *post hoc* test, $P > 0.99$). Figure 3.4C and D shows that there were no significant differences in the plasma TNF-α and IL-10 concentration for rats injected with zymosan + paracetamol and rats injected with zymosan + saline or saline + saline (TNF-α Dunn's *post hoc* test, $P > 0.99$; IL-10 Dunn's *post hoc* test, $P > 0.99$).

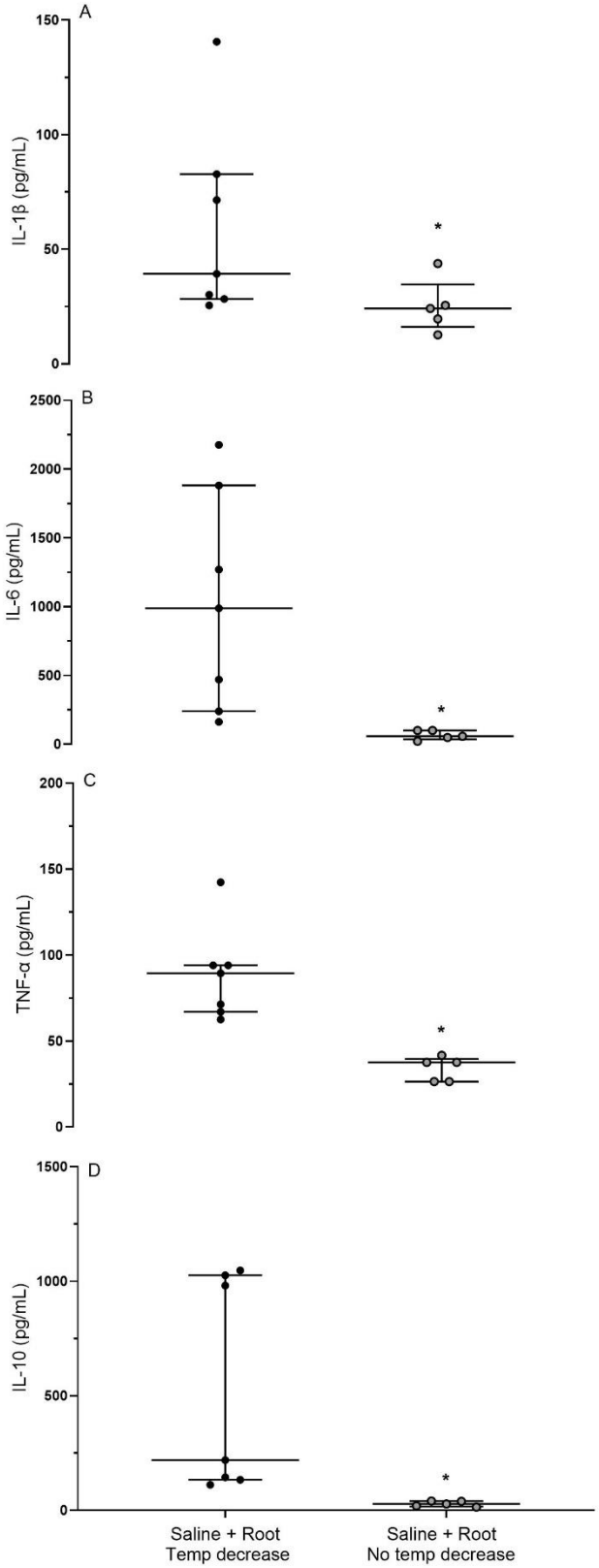

Figure 3.2: Scatterplot of the IL-1β, IL-6, TNFα and IL-10 plasma concentrations (median and IQR) 90 min after rats received an i.p. injection of *A. ecklonii* root extract (55 mg/kg). The i.p. injection was preceded by a s.c. injection of saline (10 mL/kg) 15 h earlier. *P < 0.05 between rats given *A. ecklonii* root extract with a decrease in abdominal temperature (n = 7) and rats without a decrease in abdominal temperature (n = 5).

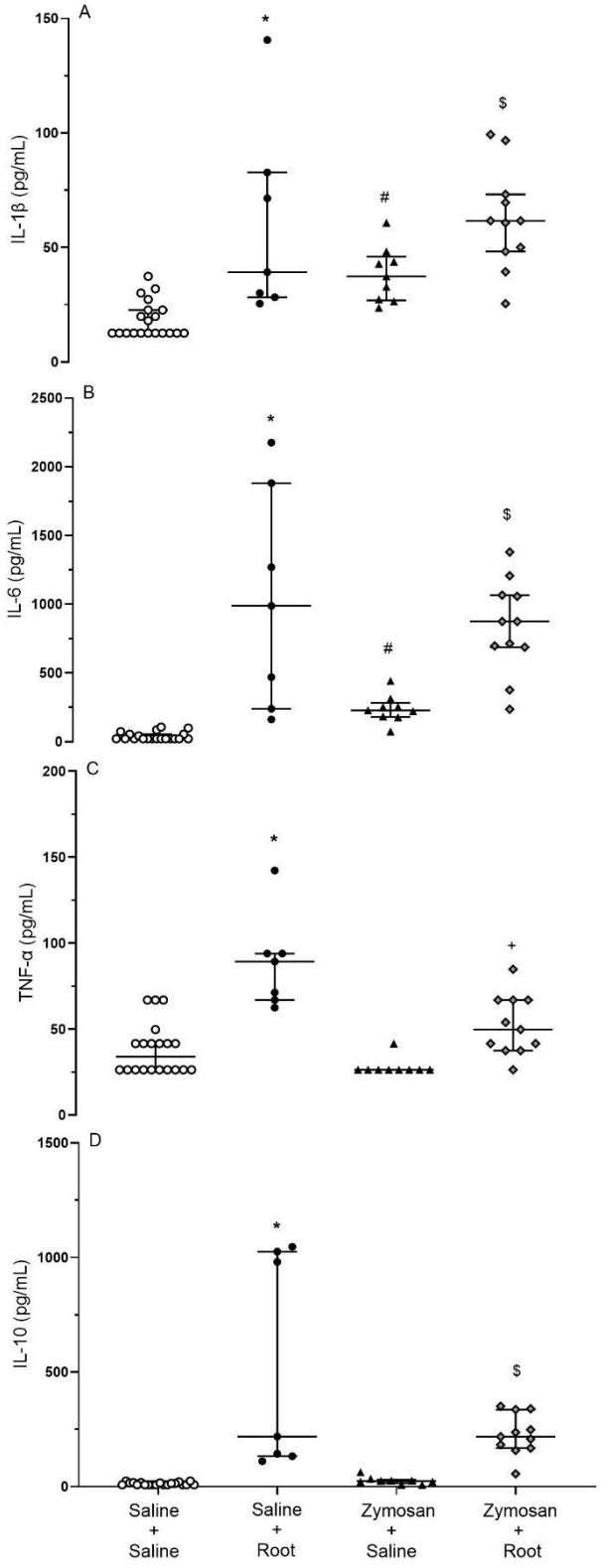

Figure 3.3: Scatterplot of the IL-1β, IL-6, TNF-α and IL-10 plasma concentrations (median and IQR) 90 min after rats received an i.p. injection of *A. ecklonii* root extract (55 mg/kg) or saline (5 mL/kg). The i.p. injection was preceded by a s.c. injection of zymosan (300 mg/kg) or saline (10 mL/kg) 15 h earlier.* $P < 0.05$ between rats given saline + saline (n=20) and saline + *A. ecklonii* root extract with a decrease in abdominal temperature (n = 7); # $P < 0.05$ between rats given saline + saline (n=20) and zymosan + saline (n = 9); $ $P < 0.05$ between rats given saline+ saline (n=20) and zymosan + *A. ecklonii* root extract (n = 11); + $P < 0.05$ between rats given zymosan + saline (n = 9) and zymosan + *A. ecklonii* root extract (n = 11).

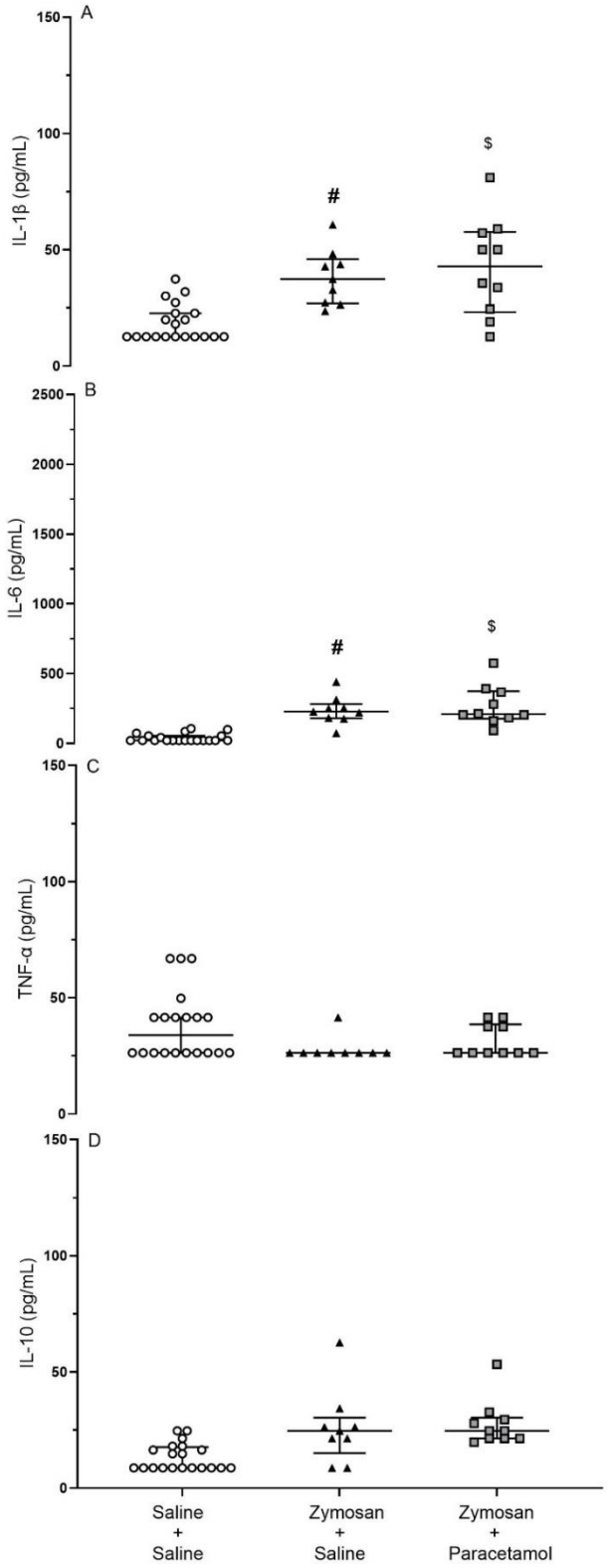

Figure 3.4: Scatterplot of the IL-1β, IL-6, TNF-α and IL-10 plasma concentrations (median and IQR) 90 min after rats received an i.p. injection of paracetamol (50 mg/kg) or saline (5 mL/kg). The i.p. injection was preceded by a s.c. injection of zymosan (300 mg/kg) or saline (10 mL/kg) 15 h earlier. # $P < 0.05$ between rats given saline + saline (n=20) and zymosan + saline (n = 9); $ $P < 0.05$ differences between rats given saline + saline (n = 20) and zymosan + paracetamol (n = 10). Since there were no significant differences between the saline + saline or saline + paracetamol treatment groups, both interventions have been combined and referred to as saline + saline.

3.3 PGE_2 concentrations in cerebrospinal fluid (CSF)

The concentration of PGE_2 in the CSF of rats 90 min after they received an i.p. injection of saline (32.30 (11.25 - 49.05)) or paracetamol (36.50 (21.13 - 49.43)) was not significantly different (Mann-Whitney U = 13; P = 0.79); thus, the data from both groups was combined as one control group for figures 3.5A and B. We were not able to measure the PGE_2 concentration in the CSF of all the rats due to technical difficulties with the CSF collection; CSF was collected from 5 of the 7 rats which showed a decrease in abdominal temperature and 2 of the 5 rats which did not show a decrease in abdominal temperature. Figure 3.6A shows that there were no differences in the CSF PGE_2 concentration between rats that had a decrease in abdominal temperature (n = 5) after the i.p. injection of *A. ecklonii* root extract, compared to rats that did not show a decrease in abdominal temperature (n = 2) (Mann-Whitney U = 0; P = 0.09). Figure 3.6B shows that in comparison to rats injected with saline + saline, rats injected with zymosan + saline or zymosan + *A. ecklonii* root extract had significantly greater CSF PGE_2 concentrations (Kruskal-Wallis statistic = 28.15, P < 0.0001). There was no significant difference in the CSF PGE_2 concentrations for rats that received saline + saline or saline + *A. ecklonii* root extract (Dunn's *post hoc* test, P = 0.12). Figure 3.6C shows that there were no significant differences in CSF PGE_2 concentrations between rats injected with zymosan + paracetamol and rats injected with saline + saline (Dunn's *post hoc* test, P = 0.19).

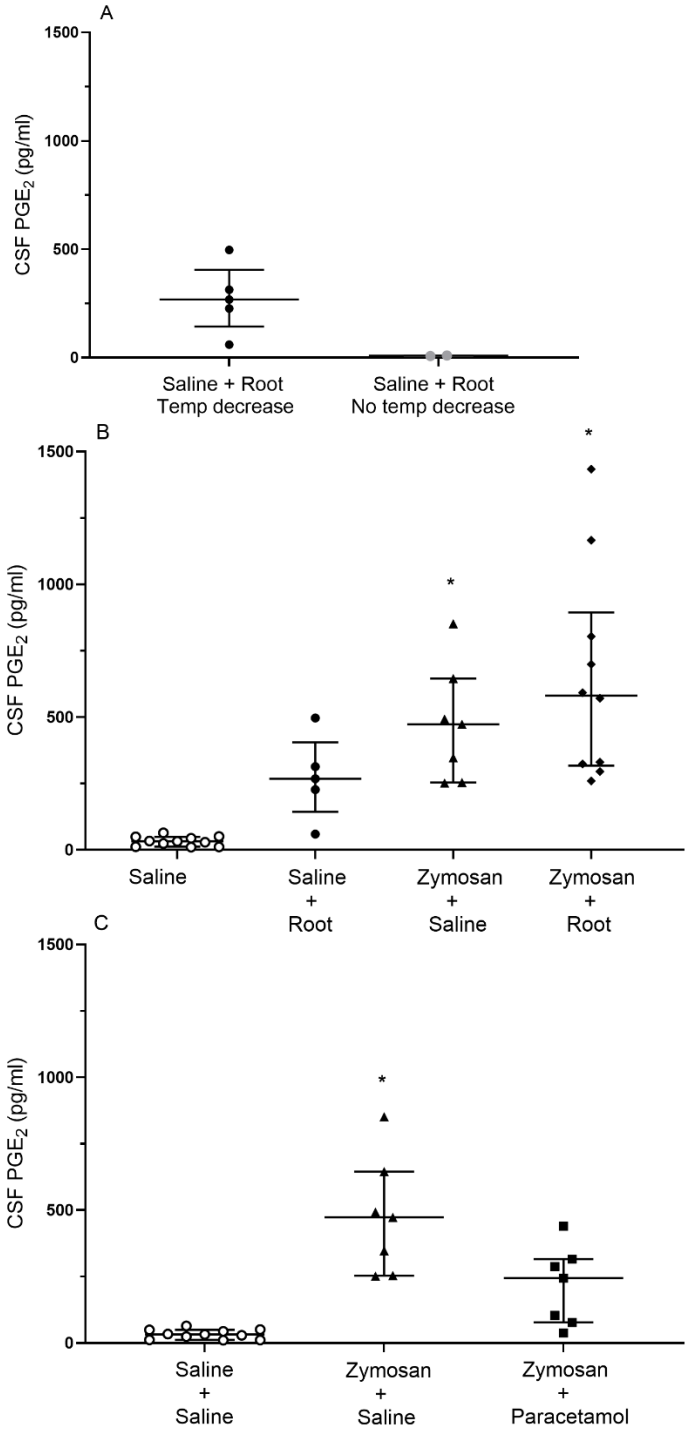

Figure 3.5: Scatterplot of CSF PGE_2 concentrations (median and IQR) 90 min after rats received an i.p. injection of *A. ecklonii* root extract (55 mg/kg) or paracetamol (50 mg/kg). The i.p. injection was preceded by a s.c. injection of zymosan (300 mg/kg) or saline (10 mL/kg) 15 h earlier. # differences between rats given saline + saline (n = 10) and zymosan + saline (n = 7; P = 0.0006); $ differences between rats given saline + saline (n = 10) and zymosan + *A. ecklonii* root extract (n = 10; P < 0.0001). Since there were no significant differences between the saline + saline or saline + paracetamol treatment groups, both interventions have been combined and referred to as saline + saline. CSF was not obtained from all rats, and thus the sample size per group is reduced compared to the numbers outlined in the methods section.

3.4 Relative mRNA expression of cytokines and enzymes involved in PGE_2 synthesis in the hypothalamus.

Overall, there were no significant differences in the relative hypothalamic mRNA expression of cytokines and enzymes involved in PGE2 rats 90 min after they received an i.p. injection of saline or paracetamol:

- hypothalamic IL-1 mRNA expression - saline (1.04 (0.80 – 1.19)) or paracetamol ((1.08 - 0.91 – 1.30)) (Mann-Whitney U = 44; P = 0.68).
- hypothalamic IL-6 mRNA expression - saline (1.03 (0.91 – 1.15)) or paracetamol (0.95 (0.86 - 1.14)) (Mann-Whitney U = 39; P = 0.43).
- hypothalamic TNFα mRNA expression - saline (1.07 (0.90 – 1.27)) or paracetamol (1.08 (0.89 – 1.15)) (Mann-Whitney U = 50; P > 0.99).
- hypothalamic IL-10 mRNA expression - saline (1.40 (0.59 – 1.69)) or paracetamol (2.00 (1.07 – 6.79) (Mann-Whitney U = 29; P = 0.12).
- hypothalamic COX-1 mRNA expression - saline (1.00 (0.88 – 1.18)) or paracetamol (0.92 (0.90 - 1.00) (Mann-Whitney U = 40; P = 0.48).
- hypothalamic COX-2 mRNA expression - saline (0.95 (0.90 – 1.12)) or paracetamol (1.07 (0.96 - 1.12) (Mann-Whitney U = 39; P = 0.43).
- hypothalamic mPGES1 mRNA expression - saline (1.00 (0.88 – 1.18)) or paracetamol (1.04 (0.96 - 1.25) (Mann-Whitney U = 43; P = 0.63).

Thus, the data from both groups was combined as one control group for figures 3.7 – 3.11.

Figure 3.6A, C and D shows that there were no significant differences in the hypothalamic mRNA expression of IL-1ß, TNF-α and IL-10 for the seven rats with a decrease in abdominal temperature after the i.p. injection of *A. ecklonii* root extract, compared to rats that did not show a decrease in abdominal temperature (IL-1β Mann-Whitney U = 9; P = 0.20; TNF-α Mann-Whitney U = 15; P = 0.75; IL-10 Mann-Whitney U = 7; P = 0.23). Figure 3.6B, shows that the seven rats with a decrease in abdominal temperature after the i.p. injection of *A. ecklonii* root extract, had a significantly greater hypothalamic mRNA expression of IL-6 compared to rats that did not show a decrease in abdominal temperature (Mann-Whitney U = 4; P = 0.03).

Figure 3.7A and D shows that there were no significant differences in the mRNA expression of IL-1ß and IL-10 in the hypothalamus for rats injected with saline + saline and rats injected with zymosan + saline or zymosan + *A. ecklonii* root extract (IL-1β Kruskal-Wallis statistic = 13.44,

P = 0.009; Dunn's *post hoc* test, P > 0.05; IL-10 Kruskal-Wallis statistic = 3.4, P = 0.49). Rats injected with zymosan + *A. ecklonii* root extract had significantly lower mRNA expression of IL-1ß in the hypothalamus compared to rats injected with zymosan + saline (Dunn's *post hoc* test, P = 0.007). Figure 3.7B shows that rats injected with zymosan + *A. ecklonii* root extract had significantly greater hypothalamic mRNA expression of IL-6 compared to rats injected with zymosan + saline or saline + saline (Kruskal-Wallis statistic = 34.66, P < 0.0001). There were no significant differences in the hypothalamic mRNA expression of IL-6 between rats injected with saline + saline and zymosan + saline (Dunn's *post hoc* test, P > 0.99). Figure 3.7C shows that rats injected with zymosan + *A. ecklonii* root extract had significantly lower hypothalamic mRNA expression of TNF-α compared to rats injected with zymosan + saline or saline + saline (Kruskal-Wallis statistic = 32.48, P < 0.0001; Dunn's *post hoc* P = 0.001 and P = 0.012). There were no significant differences in the hypothalamic mRNA expression of TNF-α between rats injected with saline + saline and zymosan + saline (Dunn's *post hoc* test, P > 0.99). Furthermore, a significant increase in hypothalamic mRNA expression of IL-6 was noted after injection of the *A. ecklonii* root extract as the seven rats that received saline + *A. ecklonii* root extract and had a decrease in abdominal temperature had greater hypothalamic mRNA expression of IL-6 compared to rats that received saline + saline (Dunn's *post hoc* test, P = 0.0003). A significant decrease in hypothalamic mRNA expression of TNF-α was noted after injection of the *A. ecklonii* root extract as the seven rats that received saline + *A. ecklonii* root extract and had a decrease in abdominal temperature had lower hypothalamic mRNA expression of TNF-α compared to rats that received saline + saline (Dunn's *post hoc* test, P = 0.02).

Figure 3.8 shows that there were no significant differences in the mRNA expression of IL-1ß, IL-6, TNF-α and IL-10 in the hypothalamus between rats injected with zymosan + paracetamol and rats injected with zymosan + saline (IL-1β Dunn's *post hoc* test, P > 0.99; IL-6 Dunn's post hoc test, P > 0.99; TNF-α Dunn's post hoc test, P > 0.99; IL-10 Kruskal-Wallis statistic = 3.4, P = 0.49).

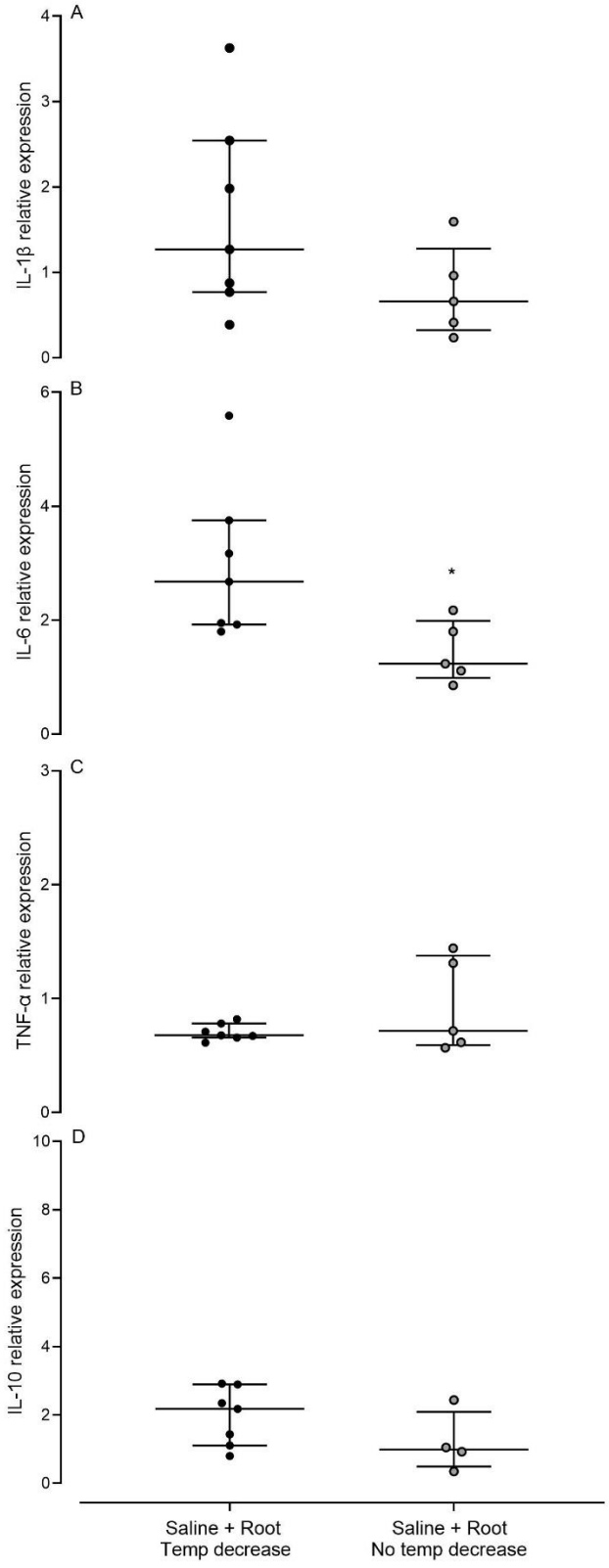

Figure 3.6: Scatterplot of the mRNA expression of IL-1ß, IL-6, TNF-α and IL-10 in the hypothalamus (median and IQR) 90 min after rats received an i.p. injection of *A. ecklonii* root extract (55 mg/kg) or saline (5 mL/kg). The i.p. injection was preceded by a s.c. injection of saline (10 mL/kg) 15 h earlier. *P = 0.03 between rats given *A. ecklonii* root extract (n = 7) with a decrease in abdominal temperature and (n = 5) rats without a decrease in abdominal temperature.

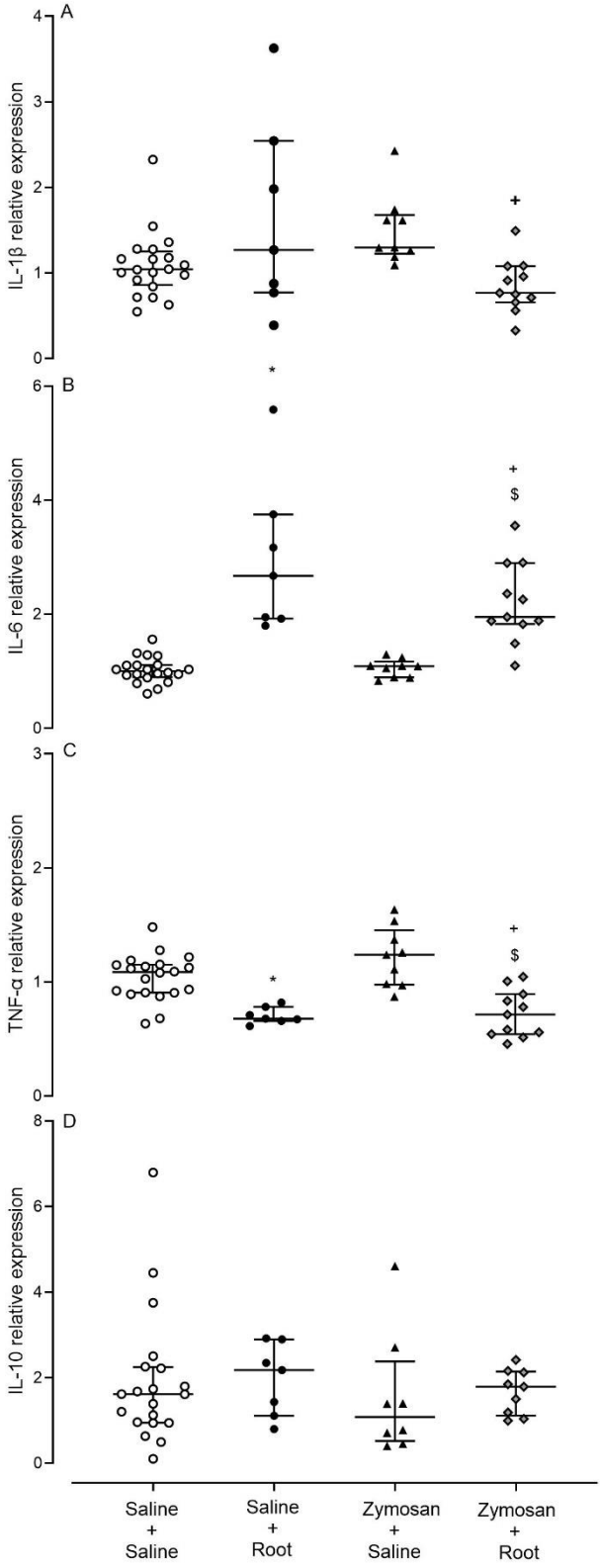

Figure 3.7: Scatterplot of the hypothalamic mRNA expression of IL-1β, IL-6, TNF-α and IL-10 (median and IQR) 90 min after rats received an i.p. injection of *A. ecklonii* root extract (55 mg/kg) or saline (5 mL/kg). The i.p. injection was preceded by a s.c. injection of zymosan (300 mg/kg) or saline (10 mL/kg) 15 h earlier.* P <0.05 between rats given saline + saline (n=20) and saline + *A. ecklonii* root extract with a decrease in abdominal temperature (n = 7); $ P < 0.05 between rats given saline+ saline (n=20) and zymosan + *A. ecklonii* root extract (n = 11). + P < 0.05 between rats given zymosan + saline (n = 9) and zymosan + *A. ecklonii* root extract (n = 11).

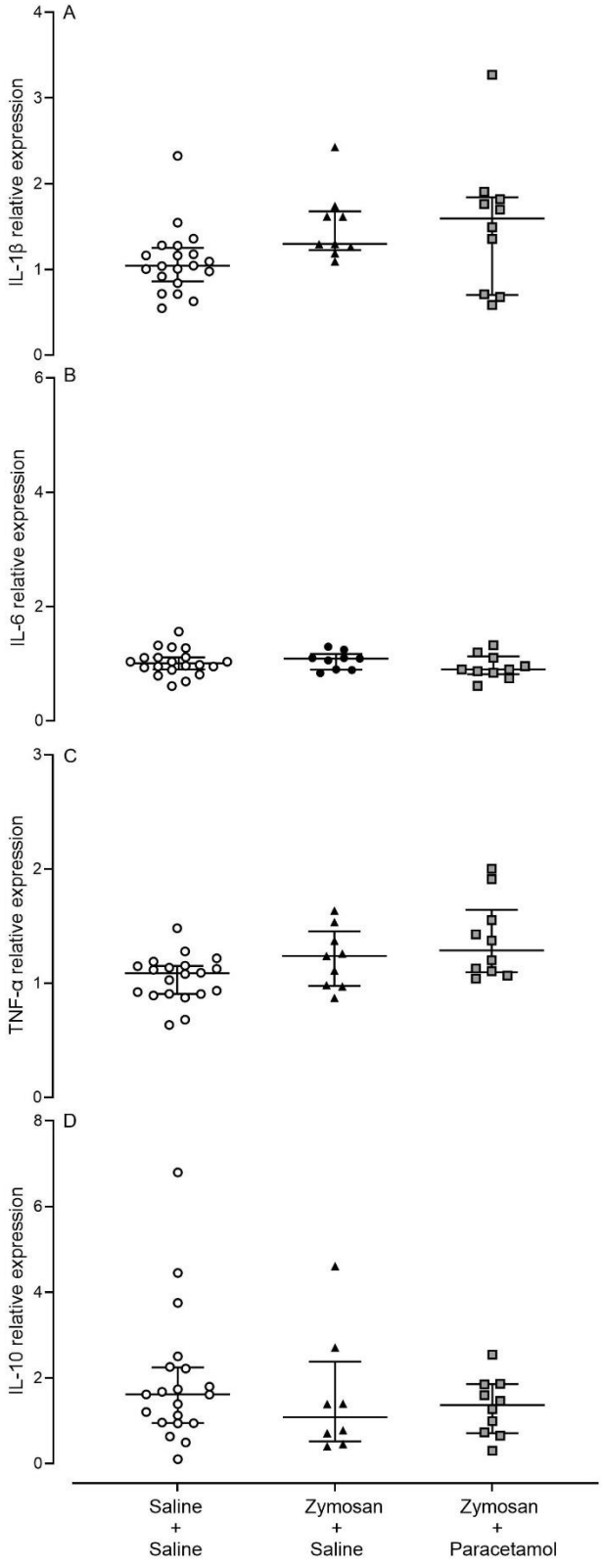

Figure 3.8: Scatterplot of the hypothalamic mRNA expression of IL-1β, IL-6, TNF-α and IL-10 (median and IQR) 90 min after rats received an i.p. injection of paracetamol (50 mg/kg) or saline (5 mL/kg). The i.p. injection was preceded by a s.c. injection of zymosan (300 mg/kg) or saline (10 mL/kg) 15 h earlier.

Figure 3.9 shows that there were no significant differences in the mRNA expression of COX-1, COX-2 and mPGES1 in the hypothalamus for the seven rats with a decrease in abdominal temperature after the i.p. injection of *A. ecklonii* root extract, compared to rats that did not show a decrease in abdominal temperature (COX-1 Mann-Whitney U = 14; P = 0.63; COX-2 Mann-Whitney U = 7; P = 0.11; mPGES1 Mann-Whitney U = 5; P = 0.05).

Figure 3.10A shows that rats injected with zymosan + saline or zymosan + *A. ecklonii* root extract had significantly lower hypothalamic mRNA expression of COX-1 compared to rats injected with saline + saline (Kruskal-Wallis statistic = 32.76, P < 0.0001). Figure 3.10B shows that rats injected with zymosan + saline or zymosan + *A. ecklonii* root extract had significantly greater hypothalamic mRNA expression of COX-2 and mPGES1compared to rats injected with saline + saline (COX-2 Kruskal-Wallis statistic = 38.26, P < 0.0001; mPGES1Kruskal-Wallis statistic = 43.75, P <0.0001). Furthermore, there were no significant differences in the hypothalamic mRNA expression of COX-1 and mPGES1 between the seven rats injected with saline + *A. ecklonii* root extract and had a decrease in abdominal temperature and rats injected with saline + saline (COX-1 Dunn's *post hoc* test, P > 0.99; mPGES1 Dunn's *post hoc* test, P > 0.72). A significant increase in hypothalamic mRNA expression of COX-2 was noted after injection of the *A. ecklonii* root extract as the seven rats that received saline + *A. ecklonii* root extract and had a decrease in abdominal temperature had greater hypothalamic mRNA expression of COX-2 compared to rats that received saline + saline (Dunn's *post hoc* test, P = 0.03).

Figure 3.11A shows that rats injected with zymosan + paracetamol had significantly lower hypothalamic mRNA expression of COX-1 compared to rats injected with saline + saline (Dunn's *post hoc* test, P = 0.003). Figure 3.11B and C shows that rats injected with zymosan + paracetamol had significantly greater hypothalamic mRNA expression of COX-2 and mPGES1 compared to rats injected with saline + saline (COX-2 Dunn's *post hoc* test, P = 0.0001; mPGES1 Dunn's post hoc test, P = 0.0006).

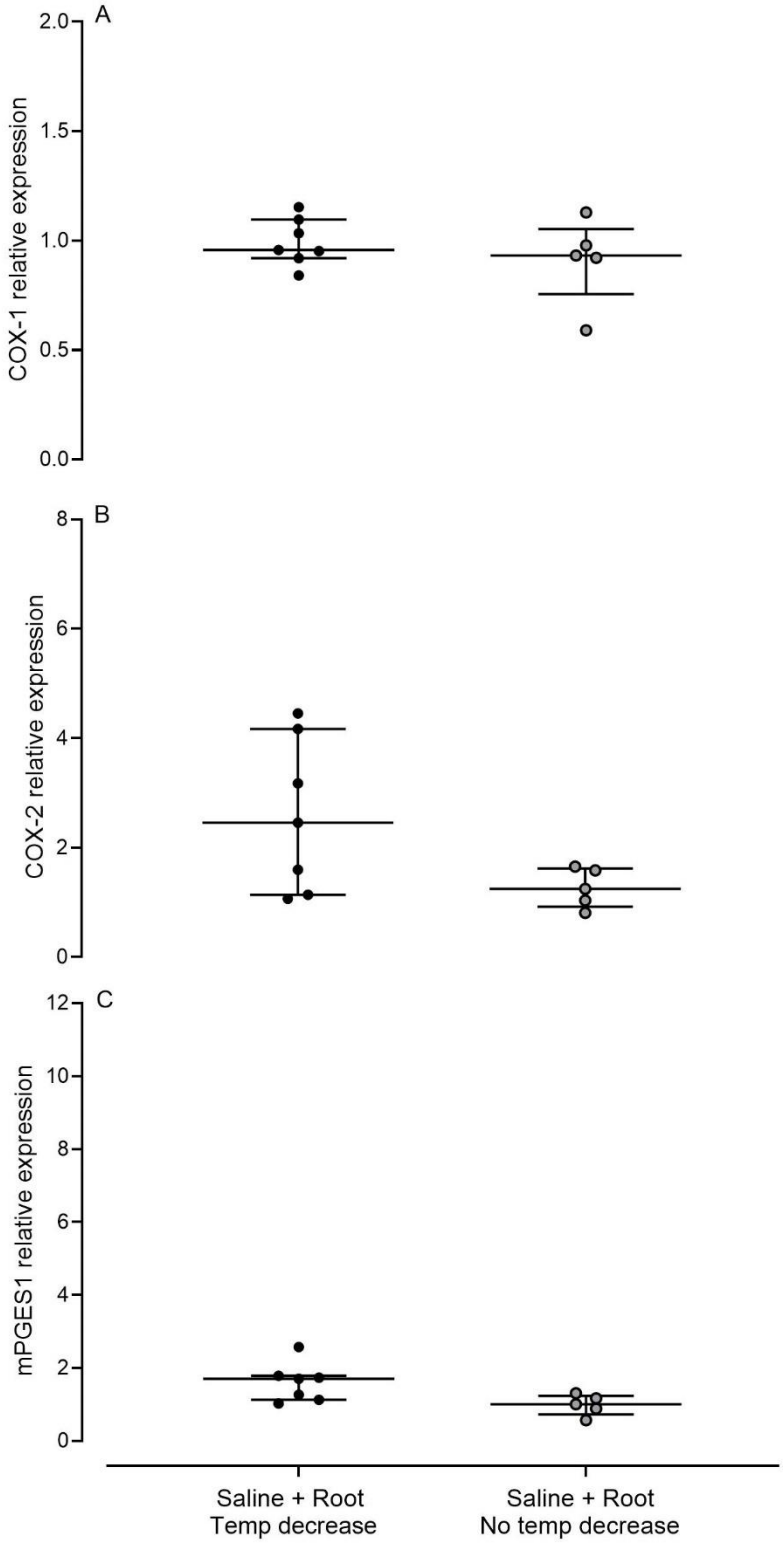

Figure 3.9: Scatterplot of the mRNA expression of COX-1, COX-2 and mPGES1 in the hypothalamus (median and IQR) 90 min after rats received an i.p. injection of *A. ecklonii* root extract (55 mg/kg) or saline (5 mL/kg). The i.p. injection was preceded by a s.c. injection of saline (10 mL/kg) 15 h earlier. Rats given *A. ecklonii* root extract with a decrease in abdominal temperature (n = 7) and rats without a decrease in abdominal temperature (n=5).

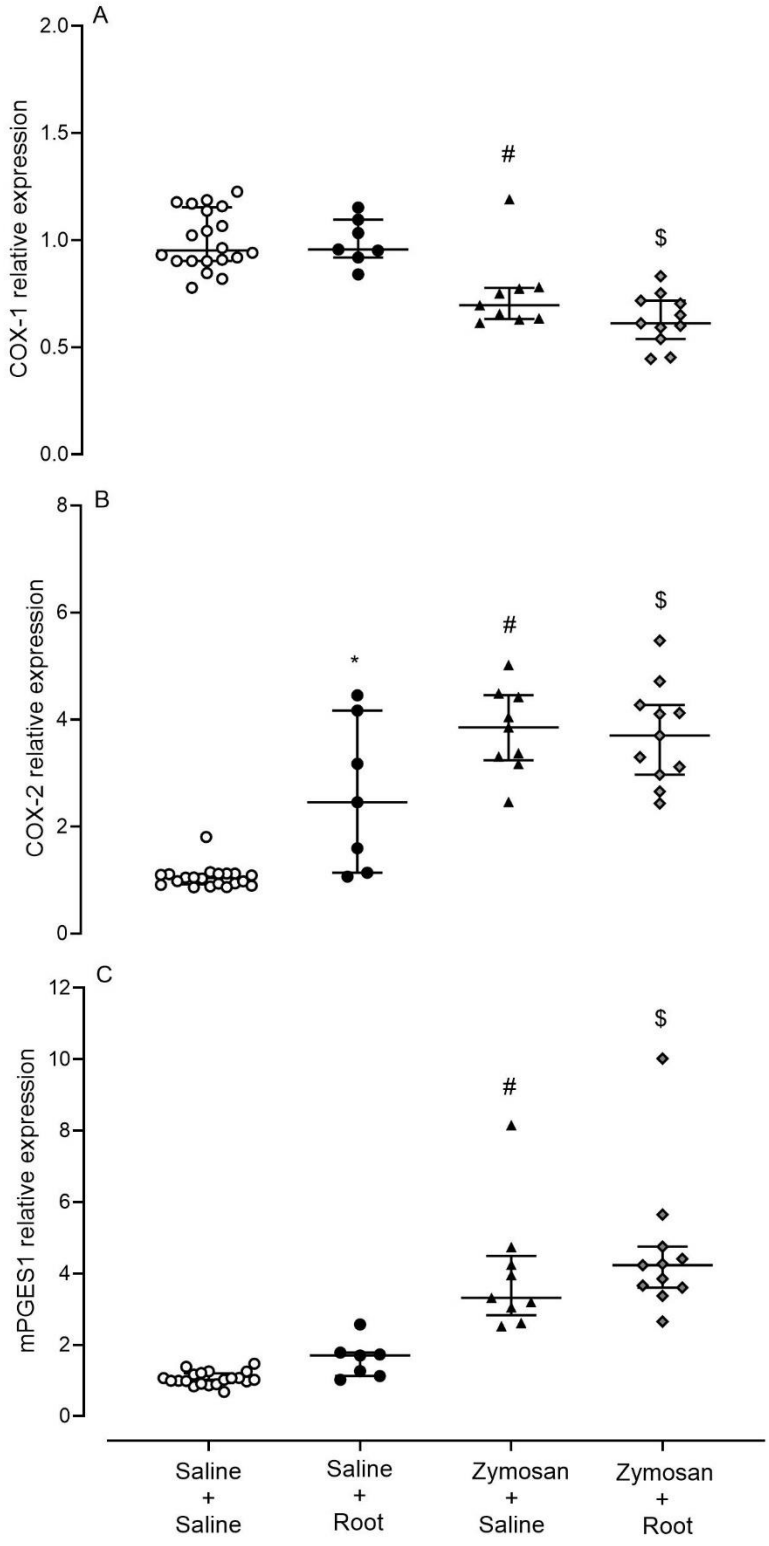

Figure 3.10: Scatterplot of the hypothalamic mRNA expression of COX-1, COX-2 and mPGES1 (median and IQR) 90 min after rats received an i.p. injection of *A. ecklonii* root extract (55 mg/kg) or saline (5 mL/kg). The i.p. injection was preceded by a s.c. injection of zymosan (300 mg/kg) or saline (10 mL/kg) 15 h earlier.* $P < 0.05$ between rats given saline + saline (n=20) and saline + *A. ecklonii* root extract with a decrease in abdominal temperature (n = 7); # $P < 0.05$ between rats given saline+ saline (n=20) and zymosan + saline (n = 9); $ $P < 0.05$ between rats given saline+ saline (n=20) and zymosan + *A. ecklonii* root extract (n = 11).

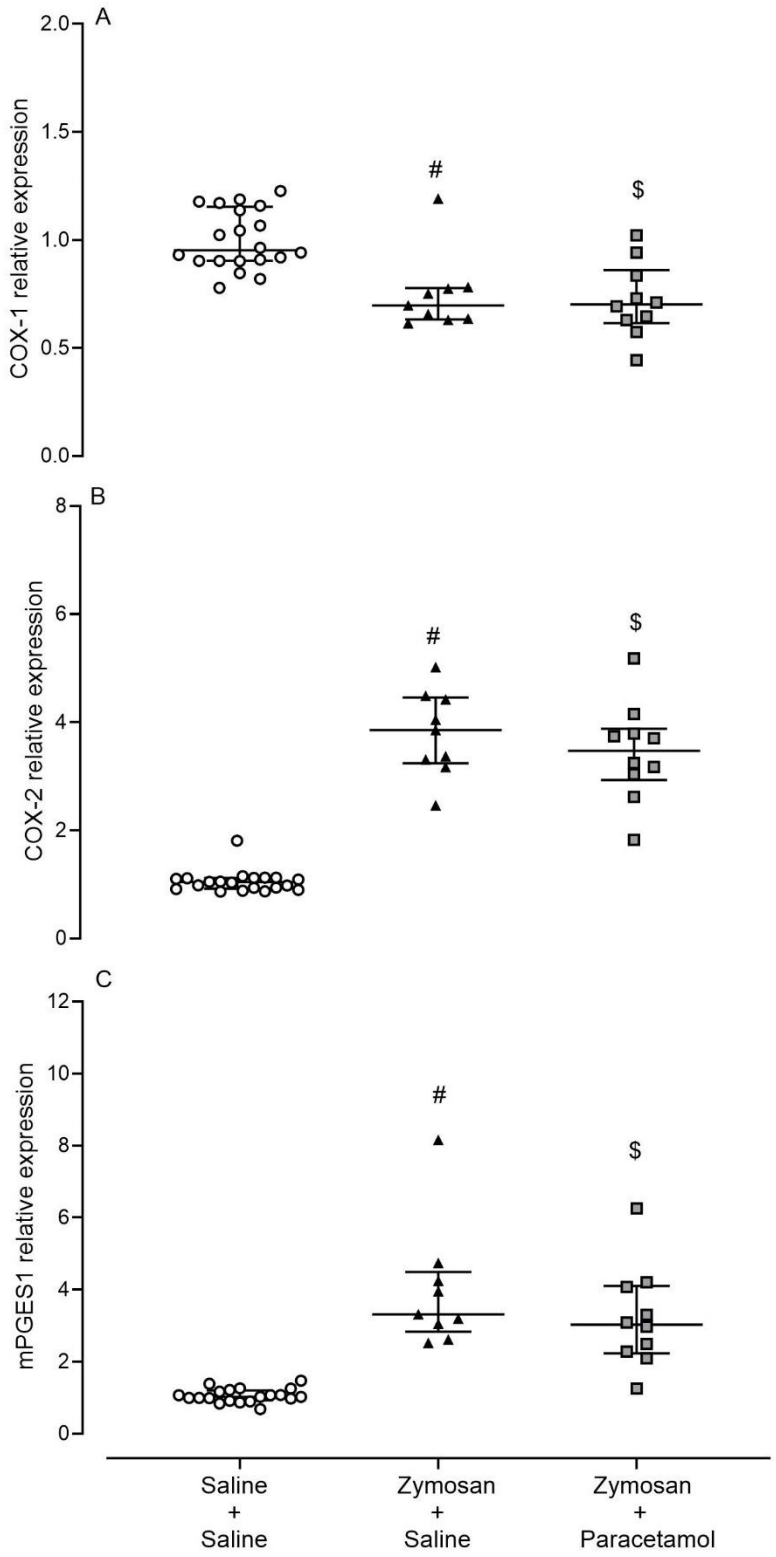

Figure 3.11: Scatterplot of the hypothalamic mRNA expression of COX-1, COX-2 and mPGES1 (median and IQR) 90 min after rats received an i.p. injection of paracetamol (50 mg/kg) or saline (5 mL/kg). The i.p. injection was preceded by a s.c. injection of zymosan (300 mg/kg) or saline (10 mL/kg) 15 h earlier. # $P < 0.05$ between rats given saline+ saline (n=20) and zymosan + saline (n = 9); $ $P < 0.05$ between rats given saline+ saline (n=20) and zymosan + paracetamol (n = 10).

3.5 Summary of results

Table 3.1 indicates the differences in the molecular responses between rats which showed a decrease in abdominal temperature compared to rats which received saline + saline. Overall, rats which had a decrease in abdominal temperature following i.p. administration of the *A. ecklonii* root extract displayed increased pro-and anti-inflammatory cytokines in the periphery. Moreover, they also showed an increase in the hypothalamic expression of IL-6 and COX-2 while TNF-α was decreased.

Table 3.1 Effect of *A. ecklonii* root extract on the inflammatory and febrile biomarkers in rats given saline.

	Saline + Root (Temp decrease)		**Saline + Root (No temp decrease)**	
Cytokines & enzymes	Plasma protein concentration	Hypothalamic mRNA expression	Plasma protein concentration	Hypothalamic mRNA expression
Pro-inflammatory				
IL-1β	↑	-	-	-
IL-6	↑	↑	-	-
TNF-α	↑	↓	-	-
Anti-inflammatory				
IL-10	↑	-	-	-
Fever-associated biomarkers				
COX-1	not measured	-	not measured	-
COX-2	not measured	↑	not measured	-
mPGES1	not measured	-	not measured	-
CSF PGE_2 concentration				
PGE_2	-		-	

↓ indicates that the concentration or expression of the biomarker was lower than that measured within saline + saline rats, ↑ indicates that the concentration or expression of the biomarker was greater than that measured within saline + saline rats, - indicates that there was no difference in the concentration or expression of biomarkers, "not measured" means that the biomarker was not measured in the plasma as a concentration and was only targeted in the hypothalamus as an expressional level.

Table 3.2 shows the molecular comparisons between rats which received zymosan + *A. ecklonii* root extract or paracetamol and rats which received zymosan + saline. While both interventions reduced the zymosan-induced fever by ~ 1°C, they were not associated with the same molecular changes in the periphery and hypothalamus. Paracetamol administration reduced the concentration of PGE_2 in the CSF, while *A. ecklonii* root extract administration increased the peripheral concentration of TNF-α and IL-10.

Table 3.2 Effect of paracetamol and *A. ecklonii* root extract on the inflammatory and febrile biomarkers rats given zymosan.

	***A. ecklonii* root extract**		**Paracetamol**	
Cytokines & enzymes	Plasma protein concentration	Hypothalamic mRNA expression	Plasma protein concentration	Hypothalamic mRNA expression
Pro-inflammatory				
IL-1β	-	↓	-	-
IL-6	-	↑	-	-
TNF-α	↑	↓	-	-
Anti-inflammatory				
IL-10	↑	-	-	-
Fever-associated biomarkers				
COX-1	not measured	-	not measured	-
COX-2	not measured	-	not measured	-
mPGES1	not measured	-	not measured	-
CSF PGE_2 concentration				
PGE_2	-		↓	

↓ indicates that the concentration or expression of the biomarker was lower than that measured within zymosan + saline rats, ↑ indicates that the concentration or expression of the biomarker was greater than that measured within zymosan + saline rats, - indicates that there was no difference in the concentration or expression of biomarkers, "not measured" means that the biomarker was not measured in the plasma as a concentration and was only targeted in the hypothalamus as an expressional level.

Chapter 4

Discussion

Despite that both paracetamol and *A. ecklonii* root extract reduced the abdominal temperature once administered of febrile rats who had been given zymosan previously, *A. ecklonii* root extract resulted in an increase in plasma TNF-α and IL-10 concentrations whereas paracetamol did not. Paracetamol also prevented the increase of PGE_2 CSF concentrations in zymosan treated rats, unlike *A. ecklonii* root extract which had the highest mean PGE_2 CSF concentration among our interventions despite reducing the abdominal temperature of zymosan treated rats. Thus, the ability of *A. ecklonii r*oot extract to lower abdominal temperature seems to be related to its inflammatory potential. The difference in thermal response to *A. ecklonii* root extract administration in rats previously given saline was expected, as the differential temperature response was observed in *A. ecklonii* extract studies done within our research group previously (Cottino, 2020). The rats that received saline + *A. ecklonii* root extract and showed a decrease in abdominal temperature, had an increase in all measured blood plasma cytokine concentrations (IL-1β, IL-6, TNF-α and IL-10) and hypothalamic mRNA expression of IL-6 in comparison to rats that received saline + saline, thus confirming the inflammatory potential of *A. ecklonii*.

4.1 Zymosan-induced fever

Previous studies examining the efficacy of zymosan as a pyrogenic substance for fever studies found that s.c. zymosan (300 mg/kg) increased the concentration of pro-inflammatory cytokines TNF-α and IL-6 in the periphery. Simultaneously, the administration of zymosan increased the hypothalamic expression of TNF-α, IL-6, IL-1β, COX-2 and mPGEs-1 (Bastos-Pereira *et al.*, 2017, Dangarembizi *et al.*, 2019). From these previous findings, s.c. zymosan (300 mg/kg) administration induced a fever over 24 h with plasma TNF-α being believed to be responsible for early fever development and plasma IL-6 for the maintenance of fever during the plateau phase (Dangarembizi *et al.*, 2019). Separate studies examining i.p. zymosan administration found that zymosan caused an increase in PGE_2 concentration within the CSF, indicative of a peripheral to central immune reaction to zymosan (Doherty *et al.,* 1985, Kanashiro *et al.*, 2009). With zymosan stimulating the increase of peripheral cytokine and PGE_2 plasma concentrations after 30 min which possibly stimulate PGE_2 synthesis in the periphery and central systems resulting in a fever (Doherty *et al.,* 1985), while others found an increase in PGE_2 concentrations within the CSF with no peripheral PGE_2 increase 3.5 h after zymosan administration (Kanashiro *et al.*, 2009). The reduction of COX-1 expression in the hypothalamus was expected as COX-1 is known to decrease during inflammatory events to facilitate COX-2 synthesis (Liu *et al.*,

1996, Smith *et al.*, 2000, Ivanov *et al.*, 2004, Font-Nieves *et al.*, 2012). The pro-inflammatory cytokines within the periphery reaches the BBB passing through due to the cytokine gradient (saturable transport system) as well as binding to the BBB, stimulating the endothelial cells within the BBB to synthesise COX-2, mPGES1 and PGE_2 within the central systems reaching the hypothalamus and CSF (Steiner *et al.*, 2011, Lima *et al.*, 2017, Blomqvist & Engblom, 2018).

4.2 Antipyretic properties of *A. ecklonii*

The difference in abdominal temperature responses to the *A. ecklonii* root extract in saline-treated rats could have been related to variations in solute concentration administered to the rats, as the aqueous root extract contained insoluble components which could have resulted in inconsistent amounts of the extract being administered (Schikarski *et al*., 2023). Interestingly, unlike the responses noted for rats that received saline and the aqueous *A. ecklonii* root extract, all the rats that received zymosan and the aqueous *A. ecklonii* root extract showed a decrease in abdominal temperature. The difference in responses between febrile and non-febrile rats to the aqueous *A. ecklonii* root extract may be related to differences in the inflammatory state of the rats at the time of injection. It is possible that the hypothermic phase brought on by potent systemic vasodilation was more consistent in febrile rats who already experienced inflammation at the time of the aqueous *A. ecklonii* root extract injection. The zymosan rats were more prone to experiencing systemic vasodilation compared to saline rats when given aqueous *A. ecklonii* root extract, as febrile rats had less of a required inflammatory response to meet the threshold to induce systemic vasodilation. Rats who were given saline and then *A. ecklonii* root extract and showed a decrease in abdominal temperature experienced a significant increase in their peripheral concentration of both pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10). The presence of the aforementioned pro-inflammatory cytokines and IL-10 which are produced by immune cells such as macrophages is indicative of a notable peripheral inflammatory response (Lyer *et al.*, 2012, Gibson *et al.*,2013). The increased concentration of inflammatory markers suggests the hypothesis that there is PAMP and PRR activation following *A. ecklonii* root extract administration, leading to the inflammatory reaction observed in the study (Takeuchi & Akira, 2010, Blomqvist & Engblom, 2018). Only hypothalamic IL-6 and COX-2 expression was increased with TNF-α expression decreasing following *A. ecklonii* root extract administration in rats. The increased COX-2 expression in the hypothalamus following *A. ecklonii* root extract administration in saline + *A. ecklonii* root extract rats shows that the inflammation is likely about to induce a fever following

the hypothermia. Given time, the fever-hypothermia switch would likely occur following *A. ecklonii* root extract administration. In fact, this was indeed observed in previous studies undertaken in our research group, where abdominal temperature was monitored over 24 h (Cottino, 2020).

The zymosan + *A. ecklonii* root extract group had a potent inflammatory response with a significant increase in the peripheral concentration of both pro-inflammatory cytokines (TNF-α) and anti-inflammatory cytokines (IL-10) when compared to rats which received zymosan + saline. When compared to the control zymosan + saline rats, the zymosan + *A. ecklonii* root extract group had an increased hypothalamic expression of IL-1β, IL-6 and TNF-α. Like in the saline + *A. ecklonii* root extract group, the zymosan + *A. ecklonii* root extract rats experienced a severe peripheral inflammatory response as shown by the heightened cytokine load in the plasma. The reduction of hypothalamic TNF-α expression in both rats that received saline and rats which received zymosan following *A. ecklonii* root extract administration was most likely due to the significant concentration of the anti-inflammatory cytokine IL-10 in the periphery. Endogenous IL-10 has been shown to inhibit fever and reduce the concentration of inflammatory cytokines, such as IL-6 and IL-1 receptor antagonist, in rats injected with LPS (Cartmell *et al.*, 2003). The neutralisation of IL-10 in febrile rats has been proven to increase the severity and duration of fever as well as increasing IL-6 and IL-1β synthesis (Cartmell *et al.*, 2003). Therefore, IL-10 is stimulated because of the inflammation taking place following *A. ecklonii* root administration which ultimately leads to IL-10 upregulation and synthesis (Iyer *et al.*, 2012). Thus, the root appears to act as an antipyretic within zymosan administered rats by lowering their abdominal temperature to that of normothermic rats while also inducing hypothermia in healthy rats who had only received saline. The root extract clearly has some mechanism in which it reduces the core temperature of rats regardless of their health status while also inducing a peripheral inflammatory response and therefore is not acting as an ideal antipyretic.

One noticeable observation was the administration of the *A. ecklonii* root extract increased blood plasma TNFα concentration and decreased abdominal temperature. The mechanism underlying the occurrence of acute hypothermia induced by systemic inflammation is not fully understood, but is believed to involve high levels of cytokines in the plasma inducing a local response, particularly TNF-α which is thought to act as an endogenous cryogen, IL-1β with hypotensive properties, and COX-1 as a cryogenic agent in the fever-hypothermia switch (Bibby *et al.*, 1989, Kozak *et al.*, 1995, Leon, 2004, Steiner *et al.*, 2009, Steiner *et al.*, 2011,

Garami *et al.*, 2018). The high concentrations of TNF-α and IL-1β in the periphery following potent inflammation stimulates the synthesis of histamine, nitric oxide and prostanoids in the peripheral blood plasma leading to systemic vasodilation which causes and is believed to maintain the hypothermia reaction to inflammation (Bibby *et al.*, 1989, Kozak *et al.*, 1995, Romanovsky, 2004). Besides the cytokines TNF-α and IL-1β, COX-1 has been shown to be an agent responsible for LPS-induced hypothermia. No mechanism for hypothermia due to upregulation of COX-1 is known, yet rats given COX-1 inhibitor did not experience hypothermia following LPS administration and could even have their hypothermia attenuated when COX-1 was inhibited (Steiner *et al.*, 2009, Garami *et al.*, 2018). COX-1 is thought to be upregulated and synthesized prior to COX-2 synthesis due to inflammation-induced synthesis of hydroperoxide, peroxynitrate and prostaglandin D_2 stimulating COX-1 synthesis early in the development of hypothermia (Ueno *et al.*, 1982, Steiner *et al.*, 2009, Garami *et al.*, 2018). However, when TNF-α and IL-1β reach sufficiently high concentrations, the hypothermic phase is often attenuated due TNF-α and IL-1β stimulating IL-6 synthesis. A fever ensues, as both IL-6 and IL-1β act as endogenous pyrogens promoting COX-2 and thereafter PGE_2 synthesis switching the hypothermia (induced due to cryogens in the periphery) into a fever once PGE_2 is present in the central system (Leon, 2004; Conti *et al.*, 2004). COX-1 may also be a part of the fever-hypothermia switch due to COX-1-mediated PGE_2 synthesis following hypothermia. Yet within this study, *A. ecklonii* root extract treated rats had a reduced hypothalamic expression of COX-1. However, in this study, hypothalamic expression measurements only represent the central response to the inflammation, and was done at the peak of the hypothermic response likely when the fever-hypothermia switch would take place. Hypothermia due to inflammation is believed to be a peripheral response, so COX-1 would need to be ideally measured in the plasma to make inferences of COX-1's influence in the hypothermia displayed in rats given saline + *A. ecklonii* root extract. The last mechanisms suspected of being responsible for *A. ecklonii*-induced hypothermia is transient receptor potential ankyrin 1 (TRPA1) and TRPV1 stimulation. Stimulation of TRPV1 has been shown to induce hypothermia through tail-skin vasodilation and nitric oxide synthesis due to the presence of exogenous endocannabinoids, noxious stimuli and inflammatory mediators such as cytokines (Steiner *et al.*, 2011). However, while responding to similar stimuli to TRPV1 the TRPA1 pathway is known to be the dependant mechanism that paracetamol uses for its analgesic properties (Mirrasekhian *et al.*, 2018). However, the TRPA1 pathway is also responsible for paracetamol-induced hypothermia when paracetamol is used in high concentrations resulting in excessive metabolites such as N-acetyl-p-benzoquinoneimine (NAPQI) (Bar-or *et al.*, 2015, Gentry *et al.*, 2015). Stimulation of TRPA1 is often due to a reduction in glutathione usually due to an increase in ROS often

experienced during inflammation, as with *A. ecklonii* administration (Aubdool *et al.*, 2011, Bar-or *et al.*, 2015). The inflammation experienced following *A. ecklonii* administration likely increase ROS concentrations in the plasma reducing glutathione concentration making TRPA1 vulnerable to stimulation by *A. ecklonii* metabolites. It was possibly the plumbagin content of *A. ecklonii* root extract that induced inflammation and therefore abdominal temperature reduction as plumbagin has been shown to be toxic towards peripheral blood lymphocytes and macrophages in a dose-dependent manner (Seshadri *et al*., 2011, Checkers *et al.*, 2014). Besides plumbagin, bufadienolides and proanthocyanides could be present in the root extract which are known to be toxic and therefore could induce inflammation when injected (Williams *et al.*, 1986).

The peripheral inflammation experienced following *A. ecklonii* root extract administration explains the reduction of abdominal temperature in both saline and zymosan administered rats due to peripheral vasodilation that occurs in severe inflammatory responses. The inflammatory signals resulting from *A. ecklonii* root extract-induced inflammation trigger a cytokine cascade, leading to the synthesis of COX-2 in the peripheral and central systems and eventually PGE_2 in the central system. The synthesis of PGE_2 would eventually induce a fever once bound to EP_3 receptors in the preoptic hypothalamus. However, before PGE_2 can be synthesized or reach the brain, the large concentration of cytokines in the periphery induce hypothermia due to peripheral vasodilation caused by TRPA1 and TRPV1 stimulation leading to the increased expression of vasodilators such as nitric oxide. Therefore, the systemic inflammation caused due to *A. ecklonii* root extract administration induces hypothermia (similarly to LPS administration) due to peripheral vasodilation leading to the observed reduction in abdominal temperature in zymosan administered rats, as well as the occurrence of hypothermia in rats given saline and then treated with *A. ecklonii* root extract (Steiner *et al*., 2009, Cottino, 2020). The reduction of abdominal temperature because of *A. ecklonii* root extract administration is later followed by the onset of a fever in normothermic rats previously given saline. With the fever aspect of *A. ecklonii* root extract administration likely being due to COX-2, mPGES1 and PGE_2 being synthesized with PGE_2 being present in the brain, matching the thermal findings done previously involving *A. ecklonii* root extract by our lab (Cottino, 2020.).

While the findings from the current study show that the ability of *A. ecklonii* root extract to lower abdominal temperature seems to be related to its inflammatory potential, these findings are based on the use of only one dose of an aqueous *A. ecklonii* root extract preparation, as well

as only one time point of tissue harvesting. Previous studies undertaken within our research group have shown that the effect of an *A. ecklonii* aqueous root extract preparation is dose-dependent, whereby greater doses (175 - 55 mg/kg) induce hypothermia followed by fever, while smaller doses (< 55 mg/kg) induce only fever (Madisha, 2024). Moreover, organic extracts tend to possess more of the active compounds found in plant material in comparison to aqueous plant extracts, this difference in bioactive compounds often render organic extracts as more potent and possibly toxic due to the heightened pharmacological impact the extract would have on the patient (Do *et al.*, 2014). Thus, a different pharmacological profile to that noted in the current study could be observed with different doses and preparations of the *A. ecklonii* root extract. Time-dependent changes in inflammatory mediators in the periphery and the hypothalamus have also been noted following s.c. administration of zymosan (Dangaermbizi *et al.*, 2019). For example, following zymosan administration, TNF-α peaks at 2h in blood plasma and 18h in the hypothalamus. Thus, the mechanism of action of *A. ecklonii* root extract may also differ depending on when it is administered.

4.3 Antipyretic properties of paracetamol

Within this study, the zymosan-induced fever was attenuated by paracetamol as expected and had no effect in altering the abdominal temperature of rats given saline + paracetamol. While paracetamol attenuated the zymosan-induced fever, the drug had no effect on the expression of COX-2 and mPGES1 within the hypothalamus. The lack of COX-2 and mPGES1 expression reduction is unexpected as paracetamol is known to reduce COX-2 synthesis or bio-availability which would in theory reduce mPGES1 expression and therefore PGE_2 concentration within the peripheral and central systems (Anderson, 2008, Hanson & Maddison, 2008, Hinz & Brune, 2012). Paracetamol has also been believed to reduce the already high oxidative stress level of COX-2 enzymes once synthesized, thus lowering COX-2 stability and thereby mPGES1 stimulatory signalling (Hinz & Brune, 2012). If the mechanism by which paracetamol works is oxidative stress and not COX-2 inhibition from binding, a reduction in mPGES1 should still be observed, which is not seen. Yet, paracetamol clearly inhibited PGE_2 within the CSF thereby attenuating the zymosan-induced fever in the current study. Therefore, paracetamol reduced PGE_2 in the CSF but not the hypothalamic expression of COX-2 or mPGES1. Paracetamol likely does not affect the expression or synthesis of COX-2 or mPGES1 in the hypothalamus but rather inhibits PGE_2 synthesis by reducing the active forms of COX-2 enzymes in the plasma either by binding to the enzymes or increasing their oxidative state. By reducing the bio-availability of active COX-2 in the periphery, there is a reduced stimulatory effect at the

BBB endothelial cells to synthesize PGE_2 centrally which would attenuate a fever. The maintained hypothalamic expression levels of COX-2 and mPGES1 are likely due to the high concentrations of IL-1β and IL-6 in the plasma in comparison to the saline + saline intervention. With the maintained hypothalamic COX-2 levels being due to zymosan administration stimulating endothelial cells at the BBB to synthesize COX-2 centrally which in turn would raise mPGES1 expression levels. Yet the active form of COX-2 is likely too low due to paracetamol for PGE_2 to synthesize. Therefore, paracetamol acted as expected of an antipyretic in attenuating a fever by reducing PGE_2 concentrations centrally likely by inhibiting active COX-2.

4.4 Future studies

The use of Sprague Dawley rats within the present study was fundamentally essential for the project, providing invaluable data on inflammatory biomarkers and the efficacy of the proposed effects for medicinal plants such as *A. ecklonii.* Such reliable *in vivo* results could not have been found without the use of animals in this research project. Animal studies remain indispensable for advancing phytomedicine based studies and conducting research and development on safe pharmaceuticals and their prescription strategies. Animal models serve as an affordable, safe, and accessible model for studying various aspects of medical research, contributing significantly to scientific progress even in contemporary times. For future antipyretic studies, it is recommended to analyse the expression and concentration of additional markers such as COX-2, TRPA1 and PGE_2 in both central and peripheral tissues and to include additional time points for sample collection and multiple doses of the medicinal plant. This approach will facilitate a more thorough investigation of the underlying mechanisms of potential treatments or drugs. In addition, conducting primary analyses that records the abdominal temperature during treatments over longer periods, such as 24 hours, can help eliminate false positives and negatives in the results of antipyretic research by preventing impressions that treatments may appear to be antipyretic. Future studies involving *A. ecklonii* root extract could examine the viability of extracting plumbagin from the root as well as the anti-microbial and possible anticarcinogenic effects of the root which seem to have the most support for the plants use as a phytomedicine in the clinical setting. Although the use of *A. ecklonii* as an antifungal, antibacterial and anticarcinogen has been supported previously (Pretorius *et al.,* 2002, Mabona *et al.,* 2013, Taneja *et al*., 2021), the use of *A. ecklonii* should be followed by close monitoring for inflammation of patients in whom the plant is administered. Despite the benefits *A. ecklonii* possesses as a potential medication, if left unchecked patients could experience severe

inflammatory reactions while treating other infections or ailments, worsening their condition and putting patients at risk of cytokine-associated toxicity due to a cytokine storm (Karki *et al.*, 2021). Future *A. ecklonii* research should prioritise identifying the risk of inflammation and a cytokine storm when using *A. ecklonii* for other purposes other than as an antipyretic. Hypothermia is believed to improve survival during infection as hypothermia acts as a disease-tolerance strategy to infection, especially in weakened or critically ill patients (Medzhitov *et al.*, 2012, Corrigan *et al.*, 2014). Extracts of *A. ecklonii* could be used not only as an antimicrobial if used carefully with its inflammatory properties in mind, but could also be used to induce hypothermia while killing off pathogens to reduce the risk of critically ill patients' mortality. Possibly with the ability to inhibit systemic inflammation with additional anti-inflammatories being prescribed, *A. ecklonii* could be used optimally in infected patients whom one might want to induce hypothermia. To maintain the autonomy of those seeking the plant for its supposed medicinal properties, providing this information to South African traditional healers and their customer base is essential for promoting public safety through education.

Chapter 5

Conclusion

Given the reliance of a significant portion of the global population on phytomedicine and traditional practices for healthcare, there is a growing interest in exploring plant-based remedies as alternatives to pharmaceuticals (Sultana *et al*., 2015, Khan & Ahmad, 2019). Phytomedicine has the potential benefits of increased accessibility, affordability, and supposedly fewer side effects compared to conventional medicine for many around the world and especially in rural settings. It is the responsibility of health care researchers and specialists to ensure the safety of medication taken by a population and to develop new treatment options from plant-based sources for the increasing demand for phytomedicine. *A. ecklonii* is used traditionally in South Africa as a phytomedicine to treat numerous diseases and symptoms. The plant is primarily ingested to treat fever and coughing-related illnesses. Yet, the plant has not been tested for its efficacy as an antipyretic or as to how it works when ingested. Therefore, this study aimed to determine the mechanism of action for the suspected antipyretic effect of *A. ecklonii* in a zymosan-induced fever.

The comparison of the known antipyretic paracetamol with *A. ecklonii* displayed the differences in efficacy between the two treatment options. Paracetamol had no effect on the cytokine, COX or mPGES1 levels in normothermic or febrile rats and attenuated the zymosan-induced fever by lowering PGE_2 levels in the CSF. Yet, *A. ecklonii* acted in a very different manner of a supposed antipyretic. When administered, *A. ecklonii* did manage to reduce the abdominal temperature of febrile rats similarly to paracetamol, yet also induced hypothermia followed by a fever in normothermic rats. The root extract of *A. ecklonii* was found to increase the peripheral concentration of pro-inflammatory cytokines and COX-2 expression. Clearly both paracetamol and *A. ecklonii* reduced the fever of rats given zymosan but through different mechanisms. Paracetamol worked as expected by reducing the driver of fever being PGE_2, whereas *A. ecklonii* induced systemic vasodilation due to the increased presence of cytokine in the periphery from inflammation causing the abdominal temperature drop in both normothermic and febrile rats. Findings from this study have shown that *A. ecklonii* root extract could have an inflammatory effect on patients whom the root is administered to, which have been misunderstood as a potential antipyretic effect due to peripheral vasodilation reducing febrile abdominal temperature.

In conclusion, this study has affirmed the antipyretic effects of paracetamol as well as the inflammatory capabilities of zymosan administration. Yet, the most significant finding was that the temperature lowering capabilities of *A. ecklonii* was related to its inflammatory potential. People who use the plant as a form of alternative medicine must practice caution, as the plant is not likely to improve the sickness behaviours of those using it. This study has shown that *A.*

ecklonii does not possess antipyretic properties and is a dangerous form of phytomedicine when used to treat a fever, as the plant itself may make patients using it as an antipyretic more ill. However, future research into the supported claims that *A. ecklonii* can be used as an antimicrobial and anticarcinogenic novel medication hold promise and should be looked into with consideration for the plant's inflammatory potential and the potential impact that the plant could have in critically ill patients (Pretorius *et al*., 2002, Mabona *et al*., 2013, Taneja *et al*., 2021). These studies could use an animal model of *S. cerevisiae* induced infection to investigate *A. ecklonii's* antifungal properties. Lastly, the utilization of animals as a screening tool for phytomedical studies has proven to be highly effective. These animals not only revolutionize medical research but also play a crucial role in ensuring the development and implementation of safe and effective pharmaceutical interventions, thereby saving lives and improving global healthcare.

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APPENDIX

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2018/11/56/C

APPLICANT: Mr P Madisha

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Investigating the antipyretic and anti-inflammatory properties of Aristea ecklonii in a rat model of Saccharomyces cerevisiae-induced fever

Number and Species

256 200-250g male Sprague-Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2018/11/27. This approval remains valid until 2021/02/13.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: _____ (Chairperson, AESC) Date: 18/2/2019

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 14/02/2018

cc: Supervisor: Prof L Harden and Prof S Van Vuuren
Director: CAS

Works 2000/lain0015/AESCCert.wps