



**Evaluation of Selected South African Medicinal Plants
in The Treatment of Pre-eclampsia**

by

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PREFACE

The experimental work described in this thesis was carried out at the Durban University of Technology, the Biomedical Resource Unit, and the Optics and Imaging Centre, Doris Duke Medical Research Institute, University of KwaZulu, Durban, South Africa, between February 2020 and December 2022, under the supervision of Dr N. Govender and Professor T. Naicker.

This study is an original work by the author and has not otherwise been submitted in any form for any degree to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

Rebecca Reddy

8 December 2022

Date

As the candidate's supervisor/co-supervisor, I have approved this thesis for submission.

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8 December 2022

Date

DECLARATION- PLAGIARISM

I, Rebecca Reddy, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.
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Signed:

ABSTRACT

Background: The growing prevalence of pre-eclampsia (PE) has been recognized as a serious global health threat being a key contributor to maternal and foetal morbidity and mortality. Locally, the effects of PE are multiplied due to strained public healthcare resources. The need for novel therapeutic strategies has gained significant importance, as conventional options may be inaccessible and costly to a large percentage of the population and are often associated with side effects. The scientific validation of alternative approaches, such as phytotherapy, has become a major focal point in the treatment and management of PE, as it is perceived to be cheap, accessible, and possess minimal side effects. Medicinal plants are a rich source of phytochemicals that display various biological activities. Hence, these plants have gained interest within the field of novel drug discovery and may offer potential therapeutic benefits in managing PE and its associated complications.

Aim: This study aimed to determine the potential of applications of South African medicinal plants in the management of PE by investigating their anti-oxidative and angiotensin-converting enzyme (ACE) inhibitory activities. The plants used in this study were *Artemisia afra*, *Clausena anisata*, *Dombeya rotundifolia*, *Rhus chirendensis*, *Sclerocarya birrea* and *Warbugia salutaris*, as well as phytochemicals 3 β -taraxerol, β -amyrin, oleanolic acid, hesperidin, nicotinamide, and quercetin for the treatment of PE. We then explored the anti-hypertensive properties of hesperidin in a novel rodent model of PE as a potential treatment option.

Methodology: The methanolic extracts of the selected plants and phytochemicals were initially evaluated *in vitro* for their antioxidant potential and ACE inhibition activities using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, the nitric oxide (NO) scavenging assay and the ACE inhibition assay, respectively. To investigate *in vivo* effect of the hesperidin, Sprague-Dawley rats were surgically implanted with mini-osmotic pumps to deliver arginine vasopressin (200 ng/h) subcutaneously to create a PE phenotype. Animals were treated with hesperidin (200mg/kg.b.w) via oral gavage for 14

days and the physiological effects were characterized by evaluating the clinical, biochemical, hematological, and foetal parameters across all experimental groups.

Key findings: Of the medicinal plants and phytochemicals evaluated, *Dombeya rotundifolia*, hesperidin, and nicotinamide demonstrated significant ACE inhibitory and antioxidant activity, showing the best potential for management of PE related symptoms. Hesperidin administration alleviated the AVP-induced hypertension associated with PE development and improved maternal and foetal outcomes. Placental and individual pup weights were significantly increased in the pregnant hesperidin-treated groups. Urinary protein and urine creatine levels were also significantly improved following treatment with the phytochemical. In addition, hesperidin improved several biochemical and hematological markers including white blood cell counts and lymphocyte levels.

Conclusion: Our findings suggest the potential of medicinal plants to ameliorate oxidative stress-associated disorders. Furthermore, hesperidin improves physiological outcomes in a novel AVP-induced rodent model and support its potential use in the treatment of PE. We provide significant physiological evidence for the use of hesperidin as an alternate anti-hypertensive agent, in resource-limited areas where conventional drugs are often costly and inaccessible. Moreover, we provide a workflow for evaluating other potential phytochemicals that may be valuable in the treatment of hypertensive disorders of pregnancy.

DEDICATION

To My Lord and Saviour **Jesus Christ**

The Author of Life

The Giver of Every Good and Perfect Gift

The Inexhaustible Fountain of Wisdom, Knowledge and Understanding

The Omniscient, The Omnipresent, and above All, The Omnipotent.

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PUBLICATIONS AND PRESENTATIONS

Details of contribution to publications that form part and/or include research presented in this thesis (include Published articles, manuscripts submitted and manuscripts in preparation) as well as other significant research outputs.

PUBLISHED ARTICLES IN THIS THESIS

- **Reddy R.**, Baijnath S., Singh S., Moodley R., Naicker T., Govender N. Hesperidin improves physiological outcomes in an arginine vasopressin rodent model of pre-eclampsia. *Fundamental and Clinical Pharmacology* (Impact factor: 2.9; DOI:10.1111/fcp.12952)
- **Reddy R.**, Baijnath S., Dwarka D., Mellem J., Moodley R., Naicker T., Govender N (2022). Antioxidant and angiotensin-converting enzyme inhibitory potential of South African traditional medicinal plants and plant-derived compounds. *South African Journal of Botany*, (152), p. 85-91 (Impact factor: 3.1; DOI:10.1016/j.sajb.2022.11.042).
- **Reddy, R.**, Pillay, V., Baijnath, S., Singh, S.D., Ramdin, S., Naicker, T. and Govender, N., (2022). Mating success of timed pregnancies in Sprague Dawley rats: Considerations for execution. *Reproductive Biology*, 22(3), p.100682 (Impact factor: 2.2; DOI:10.1016/j.repbio.2022.100682).
- **Reddy, R.**, Baijnath, S., Moodley, R., Moodley, J., Naicker, T. and Govender, N., (2022). South African medicinal plants displaying angiotensin-converting enzyme inhibition: Potential use in the management of pre-eclampsia. *Journal of Ayurveda and Integrative Medicine*, 13(2), p.100562 (Impact factor: 1.28; DOI:10.1016/j.jaim.2022.100562).

CONFERENCE PRESENTATIONS

Reddy R, Baijnath S, Singh SD, Moodley R, Naicker T, Govender N (2022). The *in vivo* anti-hypertensive effects of plant-derived compounds and plant extracts on blood pressure in an AVP-induced rat model of pre-eclampsia. Presented at the *29th Scientific Meeting of the International Society of Hypertension*, Kyoto, Japan, 12-16 October 2022.

Awarded the International Society of Hypertension Travel Grant to present this work in the above-mentioned congress.

OTHER SIGNIFICANT RESEARCH OUTPUT

- **Reddy, R.**, Baijnath, S., Singh, Sanil., Naicker, T. and Govender, N., 2023. The effects of plant-derived compounds and plant extracts on blood pressure in an AVP induced rat model of pre-eclampsia. *Journal of Hypertension*, 41(suppl 1), p.e362 (Impact factor: 4.77; DOI:10.1097/01.hjh.0000916472.93957.46).
- Govender, N., Ramdin, S., **Reddy, R.** and Naicker, T., 2021. Transforming growth factor-beta and liver injury in an arginine vasopressin-induced pregnant rat model. *Clinical and Experimental Reproductive Medicine*, 48(2), p.124 (Impact factor: 2.27; DOI:10.5653/cerm.2020.04035).
- Erukainure, O.L., **Reddy, R.** and Islam, M.S., 2019. Raffia palm (*Raphia hookeri*) wine extenuates redox imbalance and modulates activities of glycolytic and cholinergic enzymes in hyperglycemia-induced testicular injury in type 2 diabetic rats. *Journal of food biochemistry*, 43(3), p.e12764 (Impact factor: 2.72; DOI:10.1111/jfbc.12764).

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACEI	Angiotensin-converting enzyme inhibitors
Ang-I	Angiotensin 1
Ang-II	Angiotensin 2
ARBs	Angiotensin 2 receptor blockers
AT-1	Angiotensin 2 type 1 receptor
AVP	Arginine vasopressin
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
HDP	Hypertensive disorders of pregnancy
LB	Lee-Boot
LMICs	Low- and middle income countries
LPS	Lipopolysaccharide
PE	Pre-eclampsia
PIGF	Placental growth factor
ROS	Reactive oxygen species
RUPP	Reduced uterine perfusion pressure
SD	Sprague-Dawley
sENG	Soluble endoglin
sFIT-1	Soluble Fms-like Tyrosine Kinase-1
SOD	Superoxide dismutase
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

THESIS OVERVIEW

This thesis is organized into 6 chapters. The general structure of the thesis and the focal point of each chapter is described below: The principal findings of this PhD research study have been compiled and presented by a manuscript format.

CHAPTER 1: Introduction

This chapter provides a general background of the study, explaining the problem statement, the rationale, and the aims and objectives of the study.

CHAPTER 2: Literature review (Section A and B)

Section A provides a detailed literature review pertaining to the pathogenesis of pre-eclampsia development, its association with the renin-angiotensin-aldosterone system, treatment/management of pre-eclampsia, the use of medicinal plants and phytochemicals as alternatives to synthetic drugs as well as the use of animal models for the study of pre-eclampsia. Section B (published manuscript in the Journal of Ayurveda and Integrative Medicine) highlights the use of indigenous medicinal plants found in South Africa displaying angiotensin-converting enzyme inhibitory activity whilst also evaluating their potential use in the treatment of pre-eclampsia.

CHAPTER 3: Published in the *South African Journal of Botany*

This chapter is associated with objective 1 and 2. It investigated the *in-vitro* antioxidant and anti-hypertensive activity of South African medicinal plant extracts and bioactive compounds and the potential use in the management of pregnancy-related hypertension.

CHAPTER 4: Published in the *Journal of Reproductive Biology*

This chapter is associated with objective 3 and involves the laboratory methods undertaken to achieve successful mating. It discusses the importance of timed pregnancies, and provides recommendations for timed-pregnancy experiments to achieve an appropriate sample size for the study of human pregnancy disorders such as pre-eclampsia.

CHAPTER 5: Manuscript accepted in the *Journal of Fundamental and Clinical Pharmacology*

This chapter provides a complete physiological report of hesperidin for the treatment of pre-eclampsia.

CHAPTER 6: Synthesis (conclusion) and recommendations

This chapter provides an overview of the study based on the results of the previous chapters. It also provides recommendations for future research.

CHAPTER 1

INTRODUCTION

1.1. Background

Hypertensive disorders of pregnancy (HDP) are a significant cause of maternal and perinatal morbidity and mortality (1) and affect approximately 10% of all pregnant women (2). Pre-eclampsia (PE), the predominant form of HDP, complicates almost 2–4 % of all pregnancies worldwide (3). The vast majority (>95%) of an estimated 76,000 maternal and 500,000 foetal and neonatal fatalities occur in low- and middle-income countries (LMICs) (4-6). Due to limited resources and a lack of access to adequate obstetric care and family planning services, LMICs bear the greatest burden of key complications compared to high-income countries (7, 8).

Current management options for PE involve the premature delivery of the foetus and placenta, and the use of synthetic antihypertensive agents such as diuretics, beta (β)-blockers, calcium-channel blockers, as well as blockers of the renin-angiotensin-aldosterone system (i.e., angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers) (9, 10). However, the majority of these drugs are accompanied by various side effects viz., renal failure, heart failure, and pregnancy angioneurotic edema (11). As a result, the treatment focus has shifted to naturally-derived compounds rather than synthetic drugs since they potentially have fewer toxic side effects and more potent positive therapeutic effects (12).

Approximately 80% of the population in developing countries, utilises traditional medicine as the first-line of treatment to address their primary healthcare needs (13). In South Africa, an estimated 200,000 native traditional healers report the use of 3000 plant species as traditional herbal remedies (14). Plant-based medicines and plant-derived compounds have also been used for many decades to alleviate the complications associated with pregnancy and childbirth (15). Traditional medicinal plants are reported to contain several naturally occurring phytochemicals that have the potential to address a large number of healthcare concerns, which have not yet entered the drug discovery pipeline (16). In South Africa, we have access to one of the largest varieties of indigenous plants that are possible unexploited reservoirs of phytochemicals that may confer antihypertensive potential in the management of PE (17).

1.2. Motivation and rationale of the study

Natural product research is an exciting platform for novel drug discovery initiatives in the pharmaceutical industry. Since PE is a growing public health threat, it may negatively impact the economy and increase the disease burden of South Africa. Medicinal plants are easily

accessible, demonstrate minimal side effects, and may confer novel medicinal options as a treatment and preventive preference. Despite the increasing evidence of approximately 30 000 plant species found in South Africa, only less than 10% have been scientifically evaluated (18). Nevertheless, the current use of herbal preparations by the public for basic health care management may impose various health risks, as they may contain harmful properties. Since the local population in South Africa actively utilises several of these plants, improving the current understanding of their physiological effects in the management of PE is essential. Extensive investigations on the biological properties are of utmost importance to validate the use of phytomedicine and its compounds. It is within this framework that this study was performed since there is little or no scientific validation of the selected medicinal plants for the treatment of hypertensive disorders of pregnancy.

The results of this study will unmask the mechanism of action and assist in determining the anti-hypertensive effects of indigenous medicinal plants and phytochemicals, which may be further investigated as potential therapeutic agents for the management of hypertensive disorders of pregnancy such as PE. We provide an *in vitro* and *in vivo* approach that can be applied to the future evaluation of other potential PE/hypertension treatments. Our study proposes new possibilities in the search for improved therapeutic advances for the treatment of PE. Hence, the outcomes from this study are novel and contribute to the global understanding of the pathophysiology and treatment of PE and its associated complications. The use of natural products may serve as a precursor for the possible development of drugs within the world health organization (WHO) approved recommendations and guidelines. Furthermore, this information provided could be helpful for patients who are continuously looking for cost-effective alternatives to synthetic drugs, especially in Africa, where the emergence of PE is reaching high rates. This study therefore aimed to explore the anti-oxidative and anti-hypertensive properties of selected indigenous medicinal plants and phytochemicals in a novel rodent model of PE as a potential treatment option.

1.3. Aims and objectives

This study aimed to conduct a detailed investigation on the anti-oxidative, and anti-hypertensive potentials of medicinal plants found in South Africa, namely, *Artemisia afra*, *Clausena anisata*, *Dombeya rotundifolia*, *Rhus chirendensis*, *Sclerocarya birrea*, and *Warbugia salutaris*, and phytochemicals; 3 β -taraxerol, β -amyrin, oleanolic acid, hesperidin, nicotinamide, and quercetin for the treatment of PE.

The specific objectives of this research are:

1. To assess the *in vitro* anti-oxidative activities of the crude extracts and phytochemicals using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and ferric reducing antioxidant power (FRAP) assay.
2. To determine the *in vitro* angiotensin-converting enzyme (ACE) inhibitory potential of the selected plant extracts and phytochemicals using the ACE inhibitory enzyme assay.
3. To develop a suitable mating protocol to achieve an appropriate sample size of pregnant rats with an accurate gestational day 0 for the study of PE.
4. To investigate the *in vivo* anti-hypertensive effects of the phytochemical (Hesperidin) that demonstrated potent antioxidant and ACE inhibitory activity, using a novel arginine vasopressin (AVP)-induced rodent model of PE.
5. To determine the maternal and foetal outcomes in Sprague-Dawley (SD) rats with and without treatment of the phytochemical using the AVP-induced rodent model of PE.

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CHAPTER 2

LITERATURE REVIEW

This chapter is divided into sections A and B. Section A provides a detailed literature review pertaining to the pathogenesis of PE development, its association with the renin-angiotensin-aldosterone system, treatment/management of PE, the use of medicinal plants and phytochemicals as alternatives to synthetic drugs as well as the use of animal models for the study of PE. Section B (published manuscript in the *Journal of Ayurveda and Integrative Medicine*) highlights the use of indigenous medicinal plants found in South Africa displaying angiotensin-converting enzyme inhibitory activity whilst also evaluating their potential use in the treatment of PE.

SECTION A

2.1. Clinical definition and prevalence of pre-eclampsia

PE is defined as new-onset hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg), with/without proteinuria (urinary protein ≥ 300 mg per 24 h), maternal organ dysfunction or foetal growth restriction; such clinical characteristics are usually detected at or after 20 weeks of gestation (1). Organs affected by PE include the brain, resulting in severe headaches, visual disturbances, or eclamptic seizures; the liver, causing epigastric pain or abnormal liver function tests; the kidneys, causing abnormal renal function tests or proteinuria; the hematological system, causing hemolysis, thrombocytopenia, or coagulopathy; the lungs, producing low oxygen saturation or pulmonary oedema; and the placenta, causing foetal growth restriction (1-3).

PE is responsible for about 2-4 % of all pregnancy complications worldwide and is estimated to cause at least 76 000 maternal deaths (4). LMICs bear the greatest burden of major complications owing to inadequate resources and poor access to suitable obstetric care and family planning services than high-income countries (5). In Africa, PE occurs in 10 % of pregnancies, which is considerably greater than the global average (6). Hence the development of preventive options using natural therapies in low-income countries is of utmost importance.

2.2. Risk factors

The risk factors associated with PE development are illustrated in Fig. 1, with chronic hypertension and a history of PE posing the greatest risks (7). The risk of developing PE is highest in those with a history of preterm pre-eclampsia and about 25–30% of women who have recurrent illnesses (8). Obstetric complications from a previous pregnancy, such as foetal growth restriction, stillbirth, and abruption, also carry an elevated risk of PE (7). Some pre-pregnancy risk factors for PE such as weight loss and avoiding multiple pregnancies may be more amenable to change than others (7). Hence, raising public awareness of the negative pregnancy outcomes linked to maternal age, as well as managing chronic medical conditions may reduce the risks associated with PE development (7).

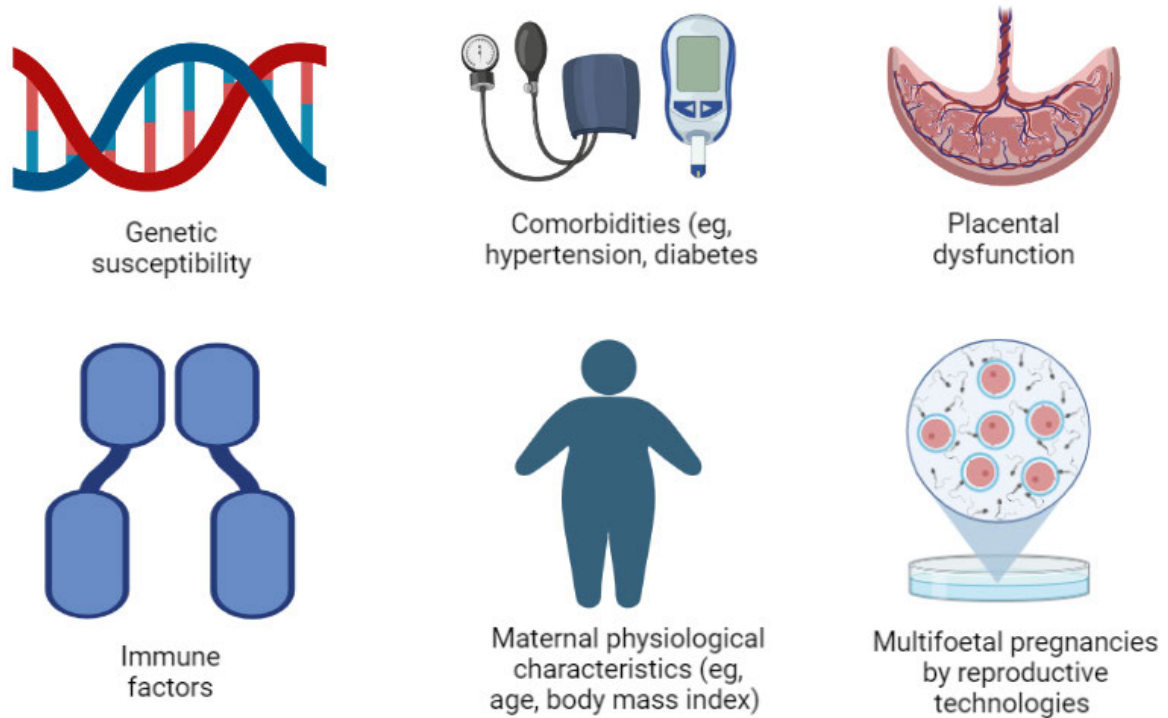


Fig. 1: Risk factors associated with pre-eclampsia. (Image created with BioRender; adapted from (7)).

2.3. Pathogenesis of pre-eclampsia

2.3.1. The classical “2-stage” model

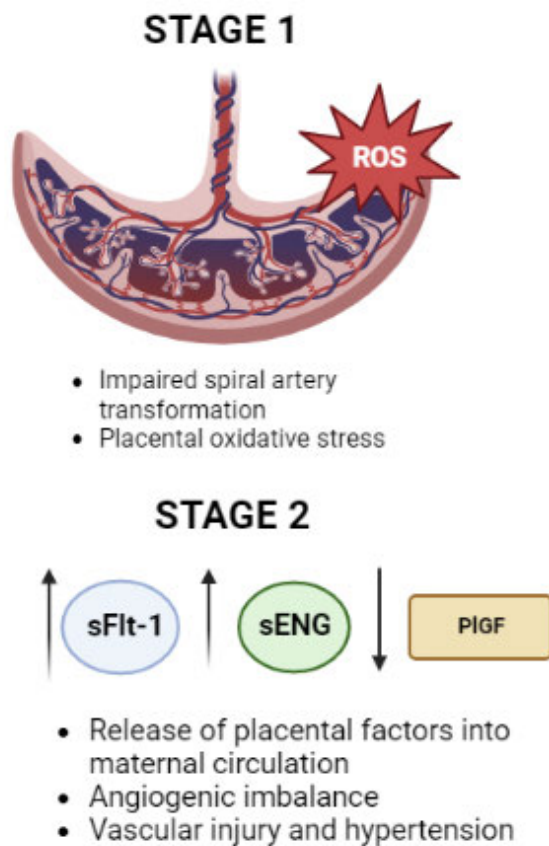
During the early stages of a normal pregnancy, spiral artery remodeling enhances nutrient and oxygen exchange in pregnancy (7). The smooth muscle cells of the media of the spiral artery are replaced by a fibroid-type material mediated in part by extravillous trophoblast cells’ migration into the inner third of the uterine myometrium (9). The terminal portion of the spiral artery is consequently flaccid creating a system with high capacitance and low resistance at the maternal-foetal interface (7). This supports nutrient exchange between the mother and foetus as well as a rich vascular supply to the implantation site (7). In contrast, women with PE experience impaired spiral artery remodeling (stage 1: Fig. 2) (10), and resultant hypoperfusion that is unable to meet the oxygen and nutrient demands of the foetus (7). This promotes a turbulent lower blood flow and subsequent placental ischemia (11). Moreover, the hypoxic microenvironment promotes the release of various factors that cause oxidative stress (12, 13), placental injury, and an angiogenic imbalance (14). As the second trimester of pregnancy progresses, the disproportionate angiogenic levels result in endothelial dysfunction, vascular inflammation, and maternal vascular damage (stage 2: Fig. 2) (12).

Endothelial dysfunction is associated with a dysregulation in placental-derived proinflammatory cytokines (7), exosomes (15), extracellular vesicles (16), anti-angiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sENG) (17, 18). sFlt-1 subsequently binds to the proangiogenic vascular endothelial growth factor (VEGF) functional receptor-1 binding domain and blocks VEGF's ability to signal the endothelial cells to maintain vasorelaxation (7). Furthermore, the elevations in sFlt-1 are accompanied by a suppression of pro-angiogenic placental growth factor (PlGF) (7). Reports confirm elevations in sFlt-1 concentrations several weeks prior to and during the clinical onset of PE (18, 19). This angiogenic imbalance is accompanied by the onset of clinical manifestations of hypertension and maternal organ injury (Fig. 2) (7).

2.3.2. An update of the classic “2-stage” model

For several years PE was considered a 2-stage disease that involves poor placentation (stage 1) followed by the clinical manifestation of the disorder namely hypertension and proteinuria (stage 2) (20), which has subsequently become insufficient and now revised to a 5-stage model (Fig. 2). Robillard and co-workers suggested that “poor tolerization” arising from a short interval between first coitus and conception increases the likelihood of poor placentation indicating that primipaternity is a risk factor for PE development (21). Previous pregnancy with the same conceiving father creates memory regulatory T cells that presumably decline over time demonstrating the loss of protective benefits after a long inter-pregnancy interval (22). The importance of decidual regulatory T cells for maternal immune tolerance to ensure a healthy placenta is emphasized (23). Furthermore, the possible impact of pre-existing maternal chronic vascular inflammation caused by diseases (e.g., obesity, diabetes, hypertension, etc) and pregnancy risk factors (e.g., multiple pregnancies) could increase the risk of poor placentation which constitutes the 2nd stage (24). The resultant compromised immune tolerance associated with the placental oxidative, endoplasmic reticulum, and inflammatory stress leads to placental development dysfunction and promotes impaired uteroplacental perfusion (stage 3), and is associated with excessive or deficient placental derived factors in the maternal circulation (Fig. 2) (20). This is followed by stage 4, which is characterized by the clinical signs of PE (hypertension and proteinuria) (24). The 5th stage is associated with acute atherosclerosis, similar to atherosclerosis, suffered by middle and old-aged, non-pregnant adults (24). This is significant as it can intensify dysfunctional placental perfusion and influences spiral artery thrombosis, thereby worsening the clinical disorder (20, 24) (Fig. 2).

ORIGINAL 2-STAGE MODEL



UPDATED 5-STAGE MODEL

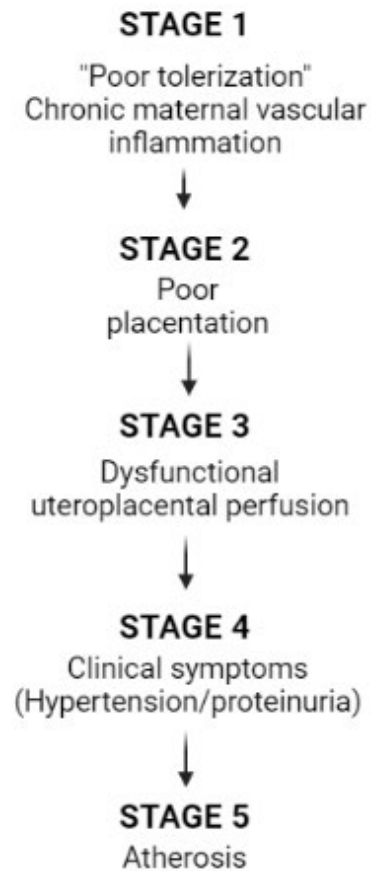


Fig. 2: Pathogenesis of pre-eclampsia depicting the original “2-stage model and the updated 5-stage model. sFlt-1: soluble fms-like tyrosine kinase-1; sENG: soluble endoglin; PlGF: placental growth factor. (Image created with BioRender; adapted from (7, 24)).

2.4. Other contributing factors to the pathogenesis of pre-eclampsia

2.4.1. The role of oxidative stress in the pathogenesis of PE

Pregnancy is known to cause increased oxidative stress induced by a normal systemic inflammatory response, resulting in high levels of circulating reactive oxygen species (ROS) (25). Accordingly, the elevated oxidative stress may result in potential tissue damage (26, 27), however, this is counter-balanced by an upsurge in the synthesis of antioxidants such as superoxide dismutase, carotenoids, tocopherols, and ascorbic acid, hence maintaining a normal state (28). The apparent antioxidant reduction in response to this oxygenation stimulus may contribute to oxidative stress and subsequent trophoblast degeneration and impaired spiral artery remodeling (29).

As discussed above, placental insufficiency induced by defective trophoblast cell migration and the resultant absence of physiological conversion of spiral arteries predisposes a hypoxic/ischaemic microenvironment that promotes the release of cytotoxic factors into the maternal circulation (7). Hypoxia and/or reoxygenation in the placenta is associated with an imbalance in angiogenesis, vascular endothelial damage, cardiovascular complications, an elevated inflammatory response (30-32), and consequent oxidative stress resulting in adverse maternal and foetal consequences (25).

2.4.2. Markers of oxidative stress in PE

Circulating lipid peroxidation products in normal healthy pregnancies (33) are usually counter-balanced by an increase in the activity of antioxidant systems (34, 35). The activity of glutathione peroxidase in maternal erythrocytes and platelets, as well as extracellular superoxide dismutase activity, gradually rises during pregnancy until the third trimester (36, 37). However, this antioxidant ability is deficient in pre-eclamptic women, resulting in an imbalance between the existing pro-oxidant and antioxidant systems and consequent oxidative stress (38-40). This oxidative imbalance is demonstrated by enhanced placental production of ROS, reactive nitrogen species, and reduced antioxidant levels, which act as free radical scavengers and inhibitors of ROS (28, 41). Earlier studies highlight decreased plasma levels of catalase, superoxide dismutase, and increased lipid peroxidation by-products as well as reduced placental activity of glutathione peroxidase, and glutathione S-transferase from women with PE (42, 43). Reduction in non-enzymatic antioxidants such as total thiols in plasma, α -tocopherol, and carotenoids (vitamin A, β -carotene, and lycopene) have also been reported (44-49). Additionally, lower plasma vitamin C concentrations have been reported in PE compared to normotensive pregnancies (44, 46, 49, 50).

In PE, activated neutrophils increase the production of superoxide compared with normal-tension neutrophils in normotensive pregnancies (51-53). Increased placental superoxide levels are also observed in pre-eclamptic women (54-56). Furthermore, a higher expression of F2-isoprostanes, a placental marker for lipid peroxidation occurs in PE compared to normal pregnancies (57). Off note, serum paraoxonase levels, an enzyme found in high-density lipoprotein which protects against oxidative stress, is decreased in PE (58, 59). Furthermore, the cellular concentration of 8-hydroxy-20-deoxyguanosine, a marker of oxidative DNA damage, is higher in placental DNA from pre-eclamptic versus normal pregnancies (60, 61),

suggesting that oxidative stress stimulates DNA and RNA damage. These reports collectively confirm that pregnancies complicated with PE predict lower antioxidant defenses than normal pregnancies (62).

2.4.3. Antioxidant therapy for the treatment of PE

The combination of higher lipid peroxidation markers and decreased antioxidant capacity is a strong indicator of oxidative stress, as evidenced by studies investigating the potential value of antioxidant supplementation in PE (50, 63, 64). Antioxidants protect proteins and enzymes from oxidation and destruction by free radicals, as well as maintain cell membrane integrity (65). They are categorized as either free radical scavengers or cellular and extracellular enzymes (viz., glutathione peroxidase, superoxide dismutase, and catalase), that inhibit peroxidase reactions involved in free radical production (65). An earlier report demonstrated that the intake of vitamin C below the recommended dietary allowance increases the risk of PE development (46). However, the administration of a combination of antioxidants (vitamin E: 800 IU/day; vitamin C:1000 mg/day; allopurinol: 200 mg/day) to women with established PE showed no improvement in symptoms (66). In contrast, Chappell and co-workers showed a reduction in the incidence of PE by 50% in a high-risk population, when a combination of 1000 mg of vitamin C and 400 I of vitamin E was administered daily from 16 to 22 weeks until delivery (63). These investigators later demonstrated a decrease in placental and endothelial dysfunction biomarkers, viz., 8-epi-prostaglandin F_{2α}, leptin, and plasminogen activator inhibitor-1/-2 indicating that vitamin supplementation prevented lipid peroxidation (50).

Furthermore, the antioxidant lycopene when used in the treatment of PE increased mean birth weight and reduced the rate of intrauterine growth restriction (67). Likewise, L-arginine supplementation resulted in higher birth weight and a reduced number of preterm births in high-risk pregnancies, highlighting the effectiveness of L-arginine in reducing the adverse neonatal outcomes associated with PE (68). Additionally, supplementation with 200 mg of co-enzyme Q10 daily from 20 weeks of pregnancy until delivery reduced the risk of developing PE in high-risk women (69). Recent data demonstrate that melatonin, a direct free-radical scavenger, and indirect antioxidant, induces antioxidant pathways in the placenta and endothelial cells as well as reduces trophoblastic sFlt-1 secretion (70). Moreover, resveratrol reduced the secretion of anti-angiogenic sFlt-1 and sENG from primary trophoblasts (64) supporting its clinical use in the prevention and treatment of PE. The data confirms that antioxidant therapy improves

maternal and neonatal outcomes in PE and highlights their potential utility in the prevention and delay of its development (71). Oxidative stress can thus be overcome or prevented by exogenous antioxidant supplementations or by enhancing endogenous antioxidant defense systems of the body (71).

2.5. The role of the renin-angiotensin-aldosterone system (RAAS) in pre-eclampsia

2.5.1. The Classical RAAS pathway

The RAAS is a crucial modulator in the regulation of blood pressure and electrolyte balance (72). Renin is synthesized and released by juxtaglomerular cells of the kidney in response to low blood pressure and low circulating sodium chloride (72). Angiotensinogen, produced in the liver, is enzymatically cleaved by renin to form angiotensin-I (Ang-I) (72), which is cleaved by ACE to form angiotensin II (Ang-II), a potent vasoconstrictor (Fig. 3). Ang II subsequently activates the G-protein coupled angiotensin II receptor type 1 (AT1) and increases the peripheral vascular resistance, leading to vasoconstriction and a subsequent rise in blood pressure (73). The effects of Ang-II are also mediated by the AT2 receptor, which is abundantly expressed in foetal tissues, and adult cardiac and renal tissues (74). Furthermore, Ang-II stimulates the adrenal secretion of aldosterone and the subsequent increase in re-absorption of sodium and water, and blood volume (75).

2.5.2. The RAAS in pre-eclampsia

Various characteristics of the RAAS in PE vary from normal pregnancy (72). In the normal pregnancy state, RAAS components (renin, angiotensinogen, Ang-I, Ang-II, and aldosterone) except ACE in the circulation are increased. (72). In contrast, in pre-eclamptic women, the circulating levels of renin, Ang-I, Ang-II, and aldosterone are lower than their normotensive counterparts (72). The maternal symptoms, viz., hypertension and renal damage in PE, may be associated with increased Ang-II or AT1 receptor activation (72). In an earlier study, women with PE were reported to have an autoantibody that stimulates the AT1 receptor (76). This AT1 receptor agonistic autoantibody signifies a key interference with the normal functioning of the RAAS (72).

The clinical significance of sFlt-1 in placental development and PE is widely reported (18, 76) (77, 78). As previously discussed, sFlt-1 is an antagonist of free VEGF and PlGF (79-81) and is subsequently elevated in pre-eclamptic placentas compared with normotensive pregnancies (82, 83). During normal pregnancy, the placenta produces sFlt-1 through Ang-II stimulation of trophoblast cells via the calcineurin-nuclear factor of activated T-cells pathway (84). The overstimulation of the AT1 receptor by the AT1-autoantibody results in excessive sFlt-1 production (72). Hence, AT1-autoantibody purified from pre-eclamptic patient serum is reported to induce sFlt-1 secretion in a pregnant mouse model, human placental villous explants, and human trophoblast cells (84) indicative that AT1-autoantibody may be responsible for the increase in sFlt-1 secretion observed in PE.

2.6. Treating and managing hypertension in pre-eclampsia

Currently, several pharmacologically active compounds/drugs viz., adrenergic inhibitors, diuretics, and direct vasodilators are employed in the treatment and management of hypertensive disorders of pregnancy.

2.6.1. Adrenergic inhibitors

This class of antihypertensive medications inhibits the sympathetic nervous system and subsequently reduces the force and rate of cardiac contraction (85), as well as peripheral vascular resistance, thereby lowering blood pressure (86). Off note, beta-adrenergic blockers affect the renin RAAS pathway by reducing renin production in the kidney's juxtaglomerular cells (87). However, adrenergic inhibitors frequently cause side effects such as orthostatic hypotension and severe hepatotoxicity that can be fatal (88).

2.6.2. Diuretics

Diuretics are effective in the treatment of hypertensive disorders as short-term use results in natriuresis and a concomitant decrease in blood volume, subsequently lowering cardiac output (89). Long-term use of diuretics may also result in a decrease in peripheral vascular resistance, thereby lowering blood pressure (90). Albeit, diuretics frequently cause side effects such as hyperlipidemia, hyperglycemia, glycosuria, and hyperuricemia (91).

2.6.3. Direct Vasodilators

2.6.3.1. Calcium channel blockers

Calcium channel blockers lower blood pressure by attaching to L-type high-voltage calcium channels, preventing calcium ions from entering blood vessels, and resulting in subsequent vasodilation (92). An earlier report suggests that calcium channel blockers may boost nitric oxide bioavailability, thereby enhancing endothelial function (93). Nevertheless, concerns remain regarding the potential negative effects of blocking these channels because of the significant role that calcium plays in a number of cellular processes (94). As such, side effects emanating from its use include excessive hypotension, headaches, tachycardia, and oedema (95).

2.6.3.2. Angiotensin receptor blockers (ARBs)

ARBs are reported to displace Ang II from its target receptor (AT1 receptor) in blood vessels, thereby antagonizing its effect (96). Furthermore, ARBs reduce aldosterone production and lead to an increase in sodium excretion (97). However, long-term use is associated with angioedema and ageusia (98).

2.6.3.3. Angiotensin-converting enzyme inhibitors

To-date, ACEIs have proven to be valuable in the management of hypertensive disorders (99). The mechanism by which ACEIs exert their effects is by inhibiting Ang-II production (Fig.3), which subsequently increases sodium and urine excretion, reduces vascular resistance, increases venous capacity, stimulates vasodilation, and decreases blood pressure (99). Additionally, ACEIs may cause a reduction in arterial wave reflection and an increase in aortic distensibility (100). These hemodynamic changes in blood flow associated with the use of ACEIs help to reverse heart hypertrophy (100). Currently, ACEIs are the drug of choice for preventing end-organ damage caused by hypertension and are recommended in patients at increased risk of developing cardiac and renal abnormalities linked to hypertension (100). In addition to their hemodynamic effects, ACEIs are reported to minimize/stop the build-up of interstitial collagen (101).

Off note, treatment with a low-dose, short-acting ACE inhibitor (captopril, 12.5 to 25 mg/day) in pregnancies complicated by severe hypertension is associated with an increase in cardiac output, reduced total peripheral resistance, and minimal/no foetal complications (102). The clinical value of RAAS inhibitors, as well as ACEIs and ARBs, confirms a significant reduction

in all-cause mortality (103, 104). Albeit, conventional ACEIs such as Captopril, Ramipril, Lisinopril, and Enapril also demonstrate several side effects including increased blood-potassium levels (hyperkalemia), fatigue, dizziness, persistent headaches, and a loss of taste (104). Furthermore, synthetic ACEIs remain contra-indicated during the 2nd and 3rd trimesters of pregnancy despite having an excellent safety profile and being well tolerated (1, 105, 106). Several other limitations are associated with the use of anti-hypertensive pharmaceutical products (107). Hence, the urgency for an alternative treatment that is effective in slowing the development and progression of hypertension and its symptoms in pregnancy with fewer or no side effects is warranted.

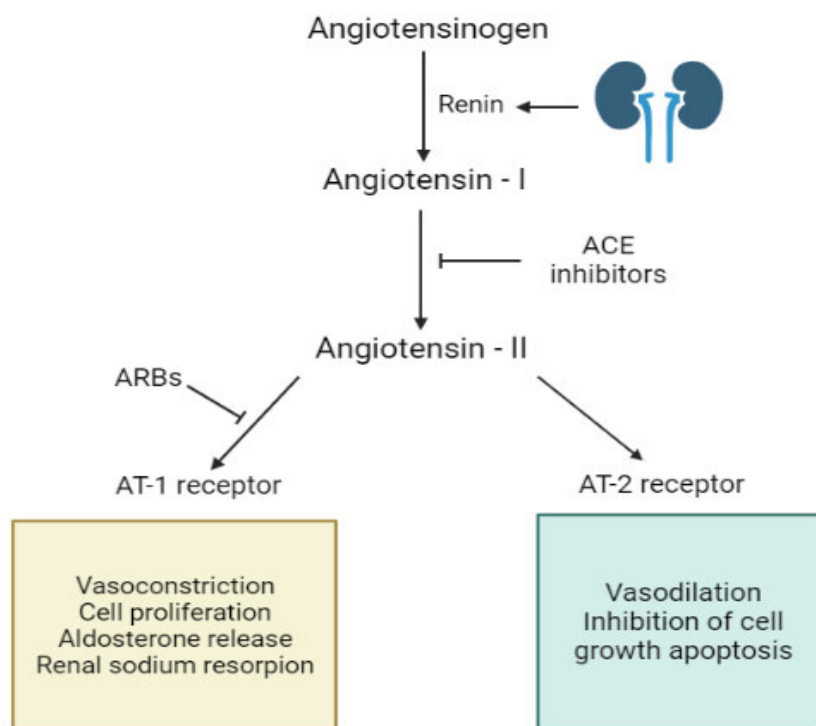


Fig. 3: The renin-angiotensin-system pathway. ACE: Angiotensin-converting enzyme; ARBs: Angiotensin-1 receptor blockers. (Created with BioRender; Adapted from (108)).

2.7. Medicinal plants and phytochemicals: an alternative to synthetic drugs

2.7.1. Medicinal Plants

The use of plants to treat various diseases dates back to when Indian, Chinese, and African civilizations left written records of their use (109-111). In general, medicinal plants refer to the whole or parts of plants that comprise one or more active compounds, that could serve as essential ingredients in drug development (112). They contain various phytochemicals and

have unique nutritional value, that may show potential biological and therapeutic effects. One of the earliest discoveries was the presence of quinine in Cinchona bark which sparked interest in plants and the development of new medicines (113). Several natural substances extracted from higher plants viz., quinine, morphine, codeine, digoxin, atropine, and hyoscine have been explored for their therapeutic utility and continues to be in use today (114). Medicinal plants are currently more widely recognized and in higher demand than ever before, with more than 50 000 plant species being used in pharmaceutical and cosmetic products (115).

In Africa, current research into the treatment of chronic diseases, including PE, is aimed largely at determining the effectiveness of medicinal plants, as a result of the high costs and unaffordability of conventional pharmaceutical drugs. Additionally, several side effects and setbacks are associated with conventional pharmaceutical drugs thus making them more difficult to adhere to (116). Conventional drugs may thus contain a single active compound that works to produce a positive pharmacological effect, however, this is accompanied by several side effects and contraindications with adverse reactions (117). In contrast, medicinal plants contain several bioactive compounds that work synergistically to create a net therapeutic effect (118). Hence these bioactive compounds have the potential to serve as precursors in novel drug development, as 40% of conventional drugs are obtained from plants (117).

Due to the low socioeconomic state of Africa, traditional medicine remains the primary method of healthcare for 80% of the African population (119, 120). The identification and evaluation of medicinal plants in Africa, especially South Africa, will improve the availability, effectiveness, safety, and affordability to improve public health (121). Even in scientifically and economically advanced countries, about 40% of the population reports the use of alternative remedies, including herbal medicines, for disease prevention and treatment (71).

2.7.2. Phytocompounds in the treatment of pre-eclampsia

In the 19th century, morphine was isolated from opium and recognised as the first plant secondary metabolite to be used in medicine (122). Biological studies of secondary metabolites widely demonstrate both physiological and pharmacological properties (123-125). Off note, phenolic compounds such as tannins, flavonoids, and phenolic acids, are found in fruits, vegetables, tea, coffee, fruit juices, wines, and other plant-based foods and have a significant impact on human health (126). Furthermore, they are implicated in physiological repair and

adaptive systems as well as demonstrate antioxidant, anti-inflammatory, and antibacterial activities (127).

Phytomedicine is reported to provide diuretic, sympathetic, and vasodilative effects that benefit the management of hypertension (128). Currently available anti-hypertension medications such as Serpasil are reported to contain alkaloids like reserpine, rescinnamine, and serpentine which originate from plants (128). To date, several phenolic compounds with antioxidant, anti-inflammatory, and potential anti-hypertensive effects have been investigated to establish their effectiveness in managing PE (129). Notably, baicalin, a flavonoid produced in underground parts of *Scutellaria baicalensis* (130), has demonstrated anti-inflammatory, cardioprotective, antiplatelet, anticoagulant, profibrinolytic, and anti-hypertensive effects (131-133). *In vitro* and *ex vivo* studies that have evaluated the activity of baicalin confirm its anti-inflammatory and myorelaxant properties, inhibition of renin activity, attenuation of chronic hypoxia-induced pulmonary hypertension, and promotion of embryo adhesion and implantation after use (134).

Another widely investigated polyphenol is curcumin which is found in turmeric rhizomes (*Curcuma longa* L., *Zingiberaceae*) and is reported to positively impact the management and treatment of pregnancy-related disorders viz., PE, as well as foetal growth disorders (129). A recent animal study has demonstrated the ability of curcumin to improve pre-eclampsia-like phenotype by inhibiting the Toll-like receptor 4 signaling pathway in lipopolysaccharide (LPS)-induced in rats (135). Likewise, curcumin inhibits the expression of proinflammatory factors and macrophage infiltration in the placenta and ameliorates LPS-induced adverse pregnancy outcomes in mice (136).

Punicalagin, another example of a natural product, is an ellagitannin found in *Punica granatum* fruits and is the most abundant polyphenolic found in pomegranate juice (137). Earlier reports demonstrate that the long-term use of *P. granatum* juice reduces blood pressure and lipid peroxidation in patients with carotid artery stenosis (138). *In vitro* and *in vivo* studies demonstrate that pomegranate juice, which is abundant in the polyphenol punicalagin, reduces oxidative stress and attenuated spontaneous and induced apoptosis in villous explants and cultured human trophoblast (139).

Quercetin, a flavonol, commonly available, easily accessible, and abundant in plants, has shown anti-hypertensive effects *in vitro*, *in vivo*, and clinical trials, promoting embryonic,

foetal, and placental development (140). A study demonstrated that quercetin supplementation during pregnancy effectively reduced blood pressure and suppressed the production of tumour necrosis factor-alpha and interleukin-6 (IL-6) in the placenta of LPS-induced pre-eclamptic rats indicating its potential as a preventative and therapeutic molecule of PE (141). Furthermore, quercetin decreased systolic blood pressure, proteinuria, malonyldialdehyde, and inflammatory cytokines levels in L-NAME rats. In the same model, quercetin prevented the expression of VEGF and sFlt-1 mRNA and increased the survival rate and weight of pups (142).

Resveratrol is also naturally produced by the Ericaceae, Leguminosae, Liliaceae, Moraceae, Myrtaceae, Pinaceae, Polygonaceae, Rosaceae, and Vitaceae plant families (143). Investigations have highlighted the anti-hypertensive effects (i.e., the antioxidant and anti-inflammatory activity) and the mechanisms of action of resveratrol, via its effect in reducing IL-6 and interleukin 1 (IL-1) expression, as well as enhancing endothelial NO production (144-146), improving markers of endothelial cells (147), and inhibiting vascular endothelial growth. Interestingly, Silibinin, a flavonolignan found in the fruits and seeds of *Silybum marianum* (L.) has recently been shown to be effective in lowering systolic pressure by upregulating chemokine receptor type 4 (CXCR4) in bone marrow, downregulating CXCR4 in pulmonary arteries and IL-1 production (148, 149). The data highlights their potential use in managing HDPs and suggests that naturally occurring compounds of plant origin and plant extracts are promising investigative areas to manage PE. Despite the availability of data regarding various plant extracts and phytochemicals with anti-inflammatory and anti-hypertensive properties, future research exploring its effectiveness in reducing the symptoms of PE and managing the disease in animal models and clinical trials is warranted.

2.8. Animal models for the study of pre-eclampsia

As the prevalence of PE continues to rise globally, extensive efforts are being made to fully elucidate the pathogenic mechanisms as well as create safe therapeutics for both mother and foetus. In order to evaluate the usefulness of medicinal plant extracts and phytochemicals, a suitable pre-clinical model is required. Pre-clinical models of PE are characterized by the development of hypertension during pregnancy, together with proteinuria, renal pathology, impaired remodeling of blood vessels supplying the uterus, placental dysfunction, and intrauterine growth restriction (150). Various animal models have been suggested such as the

reduced uterine perfusion pressure (RUPP) model, which involves induction of placental ischemia by reducing uterine blood flow by the placement of clips on the uterine arteries and abdominal aorta, that results in a pre-eclamptic state (151). Another interesting model is the sFlt-1 rodent model, which induces hypertension, proteinuria, and glomerular endotheliosis, the classic lesion of PE in pregnancy (17). Likewise, the BPH/5 mouse model has been widely studied due to the spontaneous development of a superimposed PE-like syndrome, which manifests with hypertension, proteinuria, and reduced foetal viability (152). In early pregnancy, BPH/5 mice exhibit placental dysfunction resulting in compromised maternal–foetal circulation and a progressive inflammatory response at the utero–placental interface (152). However, these models are limited in that the RUPP and sFlt-1 models are unable to reproduce liver dysfunction and intrauterine growth restriction associated with PE development (17, 151), while the BPH/5 model indicates pre-existing hypertension in non-pregnant mice (152). Therefore, alternative animal models must be available to further develop the study of PE pathogenesis.

Recently, AVP, a vasoconstrictor and antidiuretic hormone demonstrated the characteristic features of PE in a mouse model (153). The infusion of AVP into pregnant C57BL/6J mice resulted in hypertension, renal glomerular endotheliosis, intrauterine growth restriction, decreased placental growth factor (PGF), altered placental morphology, placental oxidative stress, and placental gene expression consistent with human PE (154). In non-pregnancy, AVP controls angiogenesis in the hypothalamus (155) and exerts physiological effects via V1a receptors, thus stimulating vasoconstriction (156). Moreover, it promotes water reabsorption in the kidney via V2 receptors (157), thus regulating blood pressure and blood volume (153, 156). These findings support the practicality of this model in the study of PE. Moreover, a recent study conducted in our laboratory provided a thorough physiological characterization of the AVP-induced (150 ng/hr) model in rats (158). The data obtained confirm the usefulness of the rat model in the study of PE, since AVP-induced vasoconstriction increases peripheral resistance and successfully mimics the pathological changes associated with PE development in humans (158).

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SECTION B: PAPER 1

Title: South African medicinal plants displaying angiotensin-converting enzyme inhibition: potential use in the Management of pre-eclampsia

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Review Article

South African medicinal plants displaying angiotensin-converting enzyme inhibition: Potential use in the management of preeclampsia



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ABSTRACT

In resource-limited settings, such as South Africa, hypertensive disorders of pregnancy such as preeclampsia, is the most common direct cause of maternal deaths. Current management strategies of preeclampsia primarily involve the use of pharmaceutical drugs, which are frequently associated with undesirable side-effects. Moreover, these drugs are often not easily accessible due to financial and economic constraints. Consequently, many patients rely on traditional medicine obtained from medicinal plants to manage health-related conditions.

Angiotensin-converting enzyme inhibitors are widely used drugs for the management of preeclampsia. This narrative review aims to highlight the use of indigenous medicinal plants from South Africa with Angiotensin-converting enzyme inhibitory activity whilst also evaluating their potential use in the treatment of hypertension in pregnancy. This information will influence traditional healers and sango-mas in their patient management. Furthermore, the antihypertensive potential of these plants will be unraveled thus facilitating the development of new naturally occurring pharmaceutical products to reduce maternal and neonatal mortality and morbidity.

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1. Introduction

Globally, hypertensive disorders of pregnancy (HDP) such as chronic hypertension, gestational hypertension, preeclampsia, severe preeclampsia, eclampsia and the HELLP syndrome (haemolysis, elevated liver enzymes and low platelet levels) are a major cause of maternal and perinatal morbidity and mortality. In 2019, Rana et al. (2019) reported that preeclampsia (PE) accounts for >70,000 maternal and >500,000 foetal deaths each year [1]. PE affects up to 8% of pregnancies worldwide [2], with a higher prevalence in low and middle-income countries (LMICs) [3]. Complications associated with PE are common in resource-limited

settings such as South Africa (SA) [4]. Despite a decline in the tri-ennium, HDP remains the second cause of maternal deaths, which emanate from patients attending antenatal clinics during the late stages of pregnancy, poor antenatal care such as failure to detect women at risk of PE, and inadequate emergency transport services to transfer women with complications associated with this disorder timeously [4].

Preeclampsia, a pregnancy-specific disorder, is defined as new-onset hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg) coupled with one or more of the following conditions: with/without proteinuria (urinary protein ≥ 300 mg per 24 h), maternal organ dysfunction, liver, and renal injury or foetal growth restriction; such clinical characteristics are usually detected at or after 20 weeks of gestation [5]. Clinical evidence suggests that hypertension is the most severe symptom influencing maternal and neonatal health in PE [6]. Whilst the exact aetiology of PE remains unknown, its pathogenesis is most likely

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dependent on the complex interaction among increased angiotensin II activity, endothelial dysfunction, neurovascular anomalies, and excessive vasoconstriction [7]. Therefore, the management of hypertension in PE involves pharmaceutical agents that target specific physiological mechanisms involved in blood pressure regulation, such as the renin-angiotensin-aldosterone system (RAAS) [8]. To-date, angiotensin-converting enzyme inhibitors (ACEI's) have proven to be valuable in the management of hypertensive disorders. ACEI's interfere with RAAS by inhibiting angiotensin II production and thereby stimulating blood vessel dilation that causes a decrease in blood pressure (Fig. 1) [9]. Notably, this inhibitory effect increases sodium and urine excretion, reduces resistance in renal blood vessels, increases venous capacity, whilst decreasing cardiac output [9].

2. Issues related to the current pharmaceutical management of hypertensive disorders during pregnancy

In addition to synthetic antihypertensive agents such as diuretics, beta-blockers, calcium channel blockers, ACEI's are recommended for the management of hypertension in pregnancy (Table 1). Nonetheless, the use of ACEI's is costly, thus inaccessible to patients from lower socioeconomic backgrounds [10]. Other limitations include a reduced efficacy with prolonged use, various adverse side effects, and teratogenic effects if used during the last two trimesters of pregnancy (Table 1) [5,10,11].

Clinically in South Africa, ACEI's and beta-blockers are the most commonly prescribed drugs in the management of HDP, especially PE [23]. Despite the frequent prescribing of ACEI's for the treatment of HDP, evidence suggests that synthetic ACEI's are contraindicated during the second and third trimesters as a result of suspected fetopathy [24]. However, data pertaining to the consequences observed during first-trimester exposure in pregnant women with chronic hypertension is poorly described, resulting in conflicting opinions regarding their safety [24–28]. For example, Cooper et al. (2006) suggests that the use of ACEI's may be associated with possible teratogenicity, since an increased risk of foetal cardiac valve and central nervous system defects was shown after first trimester ACEI exposure [26] in contrast to no reported teratogenic risk by others [29,30]. Likewise, a systematic analysis of published cases involving intrauterine exposure to ACEI's, highlights that most complications are less frequent in first trimester exposure compared to exposure during the second and third trimesters or throughout gestation [26,31,32]. This may be attributed to the possibility that Angiotensin II is responsible for foetal kidney development towards the end of pregnancy rather than early foetal development [32]. Moreover, prenatal renal development is dependent on a fully functional RAAS, hence it is possible that abnormalities arising after *in-utero* ACEI exposure may be due to the drug itself or underlying maternal ailments [31]. Antenatal screening of underlying complications prior to administration of ACEI's should be a prerequisite in pregnant women predisposed to

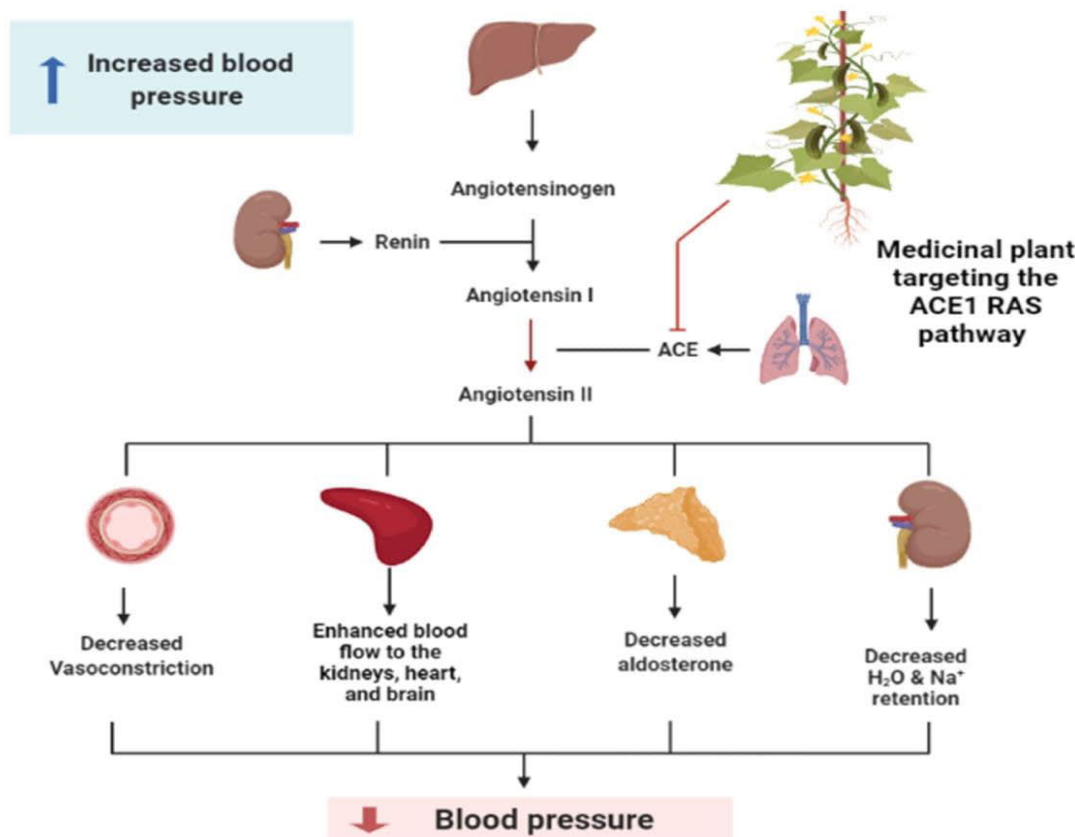


Fig. 1. Diagrammatic representation of the effect of medicinal plants on the angiotensin-converting enzyme I renin-angiotensin system (ACE1 RAS) pathway. Renin is produced by the kidneys in response to low blood volume, low sodium, or high potassium levels. Renin's primary substrate is angiotensinogen, which is produced in the liver. Renin catalyzes the cleavage of circulating angiotensinogen, resulting in angiotensin I. Angiotensin II stimulates the secretion of aldosterone and is involved in sodium retention. The retention of water and sodium causes an increase in blood volume and thus blood pressure. ACEI's such as medicinal plants inhibit the action of ACE, reducing the conversion of angiotensin I to angiotensin II. Muscle contraction around blood vessels is reduced, successfully dilating vessels, and lowering blood pressure. Aldosterone levels drop, as does water/sodium reabsorption, lowering blood pressure. Image created with [BioRender.com](https://www.biorender.com).

Table 1
Antihypertensive drugs for hypertension treatment during pregnancy.

Class of drugs	Candidate example	Side effects	References
Central α -agonist α - β -blocker	Methyldopa Labetalol	Sedation and impaired sleep patterns Fatigue and bronchospasm Fetal bradycardia Neonatal hypoglycemia Decreased uteroplacental flow	[12,13] [14–16]
Calcium channel blockers Diuretics	Nifedipine Furosemide	Circulatory collapse and neuromuscular blockade Vascular volume contraction causes further elevations of blood pressure in PE women	[17] [18]
Vasodilator	Hydralazine	Hypotension Oliguria Fetal distress Lupus-like syndrome Peripheral neuropathy	[19]
ACEI's and angiotensin receptor blockers	Captopril/Atenolol	Renal dysgenesis Pulmonary hypoplasia Neonatal anuric renal failure Fetal death	[20–22]

hypertension. Of note, babies delivered by pregnant women managed with captopril, a drug with a short elimination half-life, throughout pregnancy or towards the end of the pregnancy, revealed no neonatal complications in 95% of these babies [31]. Thus, during pregnancy, ACEIs should be discontinued early in the first trimester to prevent potential harmful effects associated with late pregnancy exposure [25]. Based on the conflicting evidence and side effects associated with the use of synthetic antihypertensive drugs, there is an urgent need for safer, more effective, and less expensive treatment alternatives with minimal or no side effects for the management of HDP. Modern medicine includes the use of several drugs that are derived from medicinal plants [33]. Medicinal plants are accessible in low-income environments [34] and represent a valuable source in the development of new therapeutic compounds.

2.1. Medicinal plants for the treatment of pregnancy-related hypertension

Phytotherapy is a global tradition that involves the use of plants to preserve the general well-being of an individual. Herbal therapy is utilized by 60% of the population of high-income countries and 80% of LMICs, including SA [34]. Over the last decade, there has been an exponential increase in the use of herbal alternative therapies across the world [35]. South Africa is home to 9% of the world's higher plants [36] and over 3000 of these plants are currently utilized in the treatment and management of numerous illnesses by several traditional healers [37]. It is worth noting that over 60–80% of the SA population from rural areas, make use of medicinal herbs for their primary healthcare needs [34]. Many rural communities prefer to use medicinal plants and their products over synthetic commercial drugs for reasons such as ease of accessibility and economic affordability [38]. Notwithstanding, traditional medicines are more acceptable from a cultural and spiritual standpoint, hence most ethnic groups are dependent on their healing capacity as their primary source of health care [39].

In SA, more than 100 plant species have been documented to treat hypertension traditionally [34]. However, only thirty-five are endemic to South Africa and evaluated for their *in vitro* ACE inhibitory activity [40,41]. Of the thirty-five, only twelve displayed strong ACE inhibitory potential *in vitro* (greater than 50% ACE inhibition) (Fig. 2). Despite their scientific value and benefits against a variety of cons/infections, the mechanism of action of only a few plants has been investigated in pre-clinical trials for hypotensive and antihypertensive efficacy. This narrative review highlights

medicinal plants with ACE inhibitory activity and their potential use in the treatment of hypertension associated with preeclampsia, in an attempt to reduce maternal and neonatal mortality and morbidity in low resource environments such as SA.

2.2. Medicinal plants targeting the ACE1 RAS pathway for the potential management of preeclampsia

Medicinal plants (together with their common names, location, traditional uses, and bioactive compounds) currently in use, that potentially lower blood pressure by modifying the ACE 1 RAS pathway is summarized in Table 2. Plants that are considered to have potential antihypertensive properties are required to inhibit the ACE enzyme (and the subsequent conversion of angiotensin I to angiotensin II) by more than 50%. These medicinal plants are widespread throughout SA. Different morphological parts of the plant are used for treatment, with the leaves mainly being used, which is in line with accepted protocols for plant conservation and sustainable use [34]. Most plant extracts isolated use polar solvents such as water, methanol, and ethanol and have high ACE inhibitory activity.

Phytochemical screening of 12 selected plants indicates secondary metabolites such as flavonoids, saponins, and alkaloids (Fig. 3). Additionally, the detection of saponins, tannins, flavonoids, and alkaloids in the plants that demonstrated antihypertensive effects proposes a link between these classes of compounds with ACE inhibition.

2.2.1. *Adenopodia spicata*

The *in vitro* inhibition of ACE by *Adenopodia spicata* aqueous and ethanolic leaf extracts [40] were 97% and 72%, respectively but were not significant (8%) when using root extracts [40]. The phytochemical analysis confirmed flavonoids and saponins as the plant's main bioactive components [54]. While saponins isolated from *A. spicata* has not been researched for their antihypertensive effect, oral administration for five days of saponin isolated from the leaves of *Camellia sinensis* to spontaneously hypertensive rats showed a time-dependent decrease in blood pressure and mean blood pressure [55]. A single administration of saponin also showed a long-lasting hypotensive effect in these rats [55]. Likewise, partially purified soybean saponin also significantly reduced blood pressure in spontaneously hypertensive rats [56]. This data suggests that the saponin found in *A. spicata* may have antihypertensive potential, however, clinical trials are required to verify its therapeutic potential.

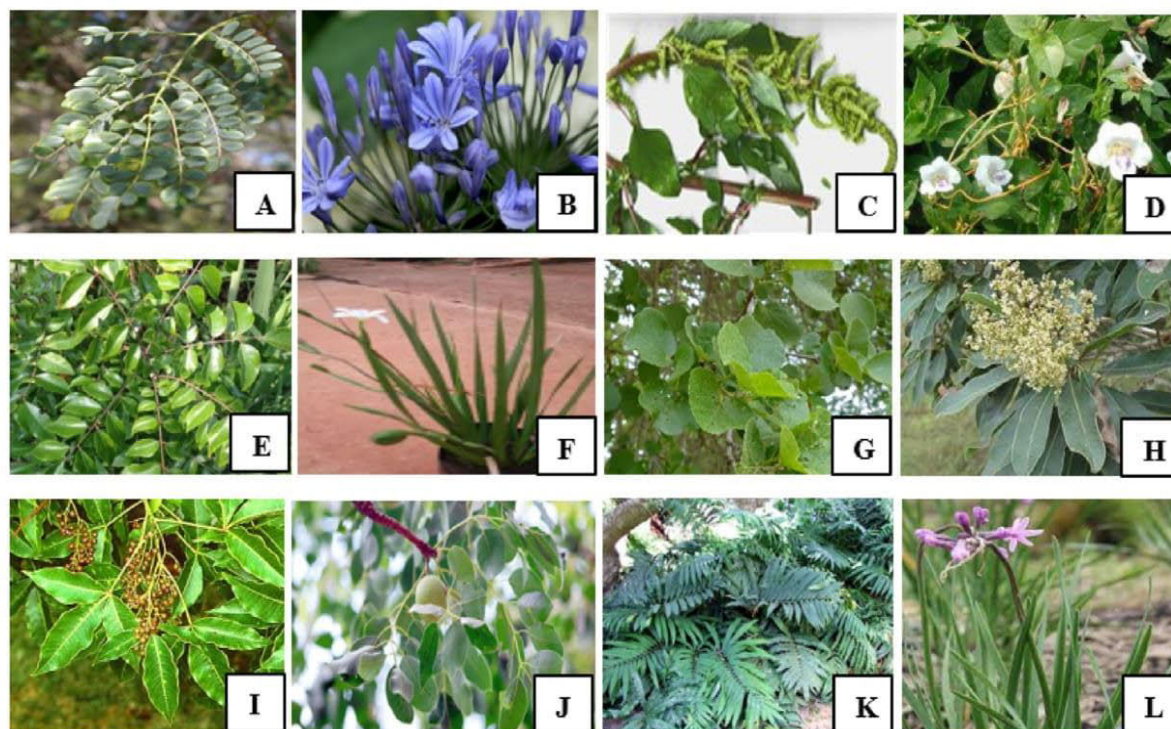


Fig. 2. South African medicinal plants with promising ACE inhibitory activity and their traditional uses. **A:** *Adenopodia spicata* (Chest or breast pain, syphilis, hypertension) **B:** *Agapanthus africanus* (Chest pains, coughs, heart disease) **C:** *Amaranthus dubius* (Kidney problems, anemia, fever, hemorrhage, stomach problems, hypertension) **D:** *Asystasia gangetica* (Asthma) **E:** *Clausena anisata* (Heart disease, tapeworms, fever, liver disease) **F:** *Dietes iridioides* (Dysentery, hypertension) **G:** *Dombeya rotundifolia* (Heart problems, ulcers, stomach problems, fever, nausea, diarrhea) **H:** *Protorhus longifolia* (HBP, heartburn, internal bleeding, diarrhea, dysentery) **I:** *Rhus chirindensis* (Measles, cough, chest pain, syphilis, convulsions, epilepsy, HBP) **J:** *Sclerocarya birrea* (Dysentery, diarrhea, rheumatism, malaria, hemorrhoids) **K:** *Stangeria eriopus* (Headaches, internal parasites, HTN) **L:** *Tulbaghia violacea* (Sinus conditions, headaches, cough, colds, asthma, tuberculosis, intestinal worms and hypertension).

2.2.2. *Asystasia gangetica*

The *in vitro* inhibition of ACE by *Asystasia gangetica* aqueous and methanolic leaf extracts were 20% and 51%, respectively [41]. Furthermore, an *in-vivo* study on the effects of the aqueous leaf extract of *A. gangetica* (200 mg/kg) on blood pressure and heart rate of spontaneously hypertensive rats [57] significantly reduces the systolic, diastolic, and mean arterial blood pressure [57]. This reduction in systolic, diastolic, and mean arterial blood pressure produced by co-infusion with angiotensin I can be attributed to *A. gangetica* inhibiting the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor [58].

2.2.3. *Clausena anisata*

Clausena anisata is a medicinal plant indigenous to Southern Africa [59]. The *in vitro* inhibition of ACE by *C. anisata* aqueous [40] and ethanolic leaf extracts were 54% and 1%, respectively [40]. An *in-vivo* study that administered bolus injections of the aqueous leaf extract of the plant (400 mg/kg/bw) to spontaneously hypertensive rats significantly reduced aortic blood pressure [59]. The report further demonstrated that the same amount of extract added daily to the drinking water of spontaneously hypertensive rats significantly decreases systolic, diastolic, and mean arterial blood pressure after 40 days of treatment [59]. The bioactive phytochemicals in the plant are carbazole alkaloids and coumarins [40,60], in which coumarins (Fig. 3) demonstrates antihypertensive properties [61]. *C. anisata* extracts were shown to significantly reduce the blood pressure of hypertensive rats, most likely by reducing the angiotensin II levels, which act via the ACE inhibitory mechanism [40]. This report delivers an essential basis for further studies into the isolation and characterization of active biomolecules that might be responsible for lowering blood pressure.

2.2.4. *Dietes iridioides*

Infusions of *Dietes iridioides* made from the inner part of its rhizomes are taken orally or in enemas, are utilized in childbirth and to treat hypertension [62]. The *in vitro*, antihypertensive impact of the leaf extracts of *D. iridioides* show 80% and 7% ACE inhibition using water and ethanol, respectively [40] in comparison to their roots which have low inhibition (13%) [40]. A study conducted on the cardiovascular effects of the leaf extract of *D. iridioides* (400 mg/kg/bw) in spontaneously hypertensive rats demonstrated significant decline of short- and long-term blood pressure (systolic, diastolic and mean arterial pressure parameters) within 20 min for 20 days, using bolus injections of the plant [63]. In addition, following the administration of the plant extract to the rats, an increase in plasma nitric oxide levels was noted indicating that the vasodilatory nitric oxide may be responsible for the decrease in aortic blood pressure [64]. Furthermore, a study that compared the blood pressure effects of *D. iridioides* with a combination of *D. iridioides* and perindopril (a known angiotensin-converting enzyme inhibitor) demonstrated a remarkable decrease in aortic blood pressure compared to both treatments alone [63].

2.2.5. *Sclerocarya birrea*

An *in vitro* study by Ojewole (2006) found that an aqueous extract of *Sclerocarya birrea* stem bark induced concentration-dependent relaxations of endothelium-intact rat isolated aortic rings precontracted with noradrenaline. Bolus intravenous administrations of the stem-bark extract (25–400 mg/kg) significantly decreases systemic arterial blood pressure and heart rate in anesthetized normotensive and hypertensive Dahl salt-sensitive rats [65]. Acute intravenous administration of *S. birrea* crude stem bark extract (120 mg/kg) to non-diabetic and streptozotocin-

Table 2
South African medicinal plants that have ACE inhibitory potential.

Plant Species (Family)	English Name/ (Traditional Name)	Traditional uses	Location	Phytochemicals	References
<i>Adenopodia spicata</i> (Fabaceae)	Spiny splinter bean (Ubobo)	Bark - chest or breast pain, syphilis, hypertension	Southern Africa	Flavonoids, Saponins	[40]
<i>Agapanthus africanus</i> (Amaryllidaceae)	African lily (Ubani)	Leaves and roots - chest pains, coughs, heart disease, ease labor	South Africa	Flavonoids, sitosterol, yuccagenin, agapanthagenin, spirostan sapogenins	[40]
<i>Amaranthus dubius</i> (Amaranthaceae)	Wild spinach (Imbuya)	Leaves - kidney problems, anemia, fever, hemorrhage, stomach problems, hypertension	Found worldwide	Flavonoids, Niacin, thiamine, riboflavin, ascorbic acid, hydrocyanic acid, oxalic acid	[41,42]
<i>Asystasia gangetica</i> (Acanthaceae)	Creeping foxglove (Isihobo)	Leaves – asthma	Tropics	Flavonoids, Alkaloids, terpenes, phenols, salidroside, apigenin, ajugol, megastigmaneglucoside, benzyl-β-D- glucopyranoside, cardiac glycosides, tannins	[41,43]
<i>Clausena anisata</i> (Rutaceae)	Horsewood (Umnukambhiba)	Leaves and roots - heart disease, tapeworms, fever, liver disease	Africa	Not reported	[40,44]
<i>Dietes iridioides</i> (Iridaceae)	African iris (Isishuphe somfula)	Leaves, roots, and rhizomes - dysentery, hypertension	Sub-Saharan Africa	Flavonoids	[40,45]
<i>Dombeya rotundifolia</i> (Malvaceae)	Wild pear (iNhlizinyonkhulu)	Leaves and roots - heart problems, ulcers, stomach problems, fever, nausea, diarrhea	Southern Africa and northwards to central and eastern tropical Africa	Saponins, tannins and cardiac glycosides	[40,46]
<i>Protorhus longifolia</i> (Anacardiaceae)	Red beech (Uzintlwa)	Bark and leaves - HBP, heartburn, internal bleeding, diarrhea, dysentery	South Africa, Swaziland	Flavonoids, glycosides and sterols	[40,47]
<i>Rhus chirindensis</i> (Anacardiaceae)	Red currant (Umhlabamvudu)	All plant parts - measles, cough, chest pain, syphilis, convulsions, epilepsy, HBP	KwaZulu/Natal, Swaziland, Zimbabwe, and Mozambique	Flavonoids	[40,48]
<i>Sclerocarya birrea</i> (Anacardiaceae)	Marula (Ukanyi)	Bark, leaves, and stems - dysentery, diarrhea, rheumatism, malaria, hemorrhoids.	North-eastern South Africa and parts of eastern Botswana.	Polyphenols, tannins, flavonoids, alkaloids, anthocyanins, and saponosides coumarins, triterpenoids, and phytosterols (β-sitosterol) Quercetin, kaemp-ferol, gallic acid, (-)-epicatechin 3-O-galloyl ester, (-)-epigallocatechin 3-O- galloyl ester	[40,49]
<i>Stangeria eriopus</i> (Zamiaceae)	Natal Grass Cycad (Umfigwani)	Root and leaves - headaches, internal parasites, HTN.	East coast of South Africa and southern Mozambique	Alkaloids, amino acids, biflavones, fatty acids, glycosides, polyphenols, saponins, and tannins	[40,50]
<i>Tulbaghia violacea</i> (Alliaceae)	Garlic (isihaga)	Rhizome, bulb, leaves and roots- sinus conditions, headaches, cough, colds, asthma, tuberculosis, intestinal worms and hypertension	Eastern Cape, Limpopo and KwaZulu-Natal	Bioflavonoids, steroidal saponins	[51–53]

treated diabetic rats resulted in momentary vasodepressive effects, with maximal activity occurring within 60 min of the extract's infusion. Long-term administration of the plant's stem-bark extract (120 mg/kg for 5 weeks) resulted in a significant reduction in mean arterial blood pressure. Compared to the control group, blood pressure was lower over the course of the 5-week study [66]. Furthermore, in a study conducted by Masoko et al. (2008), dichloromethane, hexane and acetone *S. birrea* stem, bark and leaves displayed strong antioxidant activity [67]. Polyphenols such as galloylate catechins contributed significantly to the antioxidant activity of *S. birrea* [68].

2.2.6. *Tulbaghia violacea*

The ACE inhibitory potential of *Tulbaghia violacea* was demonstrated *in vitro* using aqueous (68%) and methanolic (71%) leaf

extracts [40,41,69]. The aqueous extracts of the leaf and bark also exhibited an ACE inhibitory potential of 72% and 49%, respectively, whilst the ethanolic leaf extracts inhibited the activity of ACE by 61% [40]. Analyses of the methanolic leaf extract tested at varying concentrations accentuated the reduction in systolic, diastolic, and mean arterial pressure of normotensive and spontaneously hypertensive rats in a dose-dependent manner, underpinning its antihypertensive impact. The reduction in blood pressure may be stimulated by the plant's actions on the ACE and β-adrenoceptors. Furthermore, *T. violacea* decreased systolic blood pressure in Dahl salt-sensitive rats by reducing renal angiotensin II type 1 receptor gene expression [69]. Antioxidant studies of the extracts reveal potent antioxidant activity with low IC₅₀ values [53,70]. In addition, a two-week co-treatment with the *T. violacea* extracts significantly decreased elevated thiobarbituric reacting substance (TBARS) and

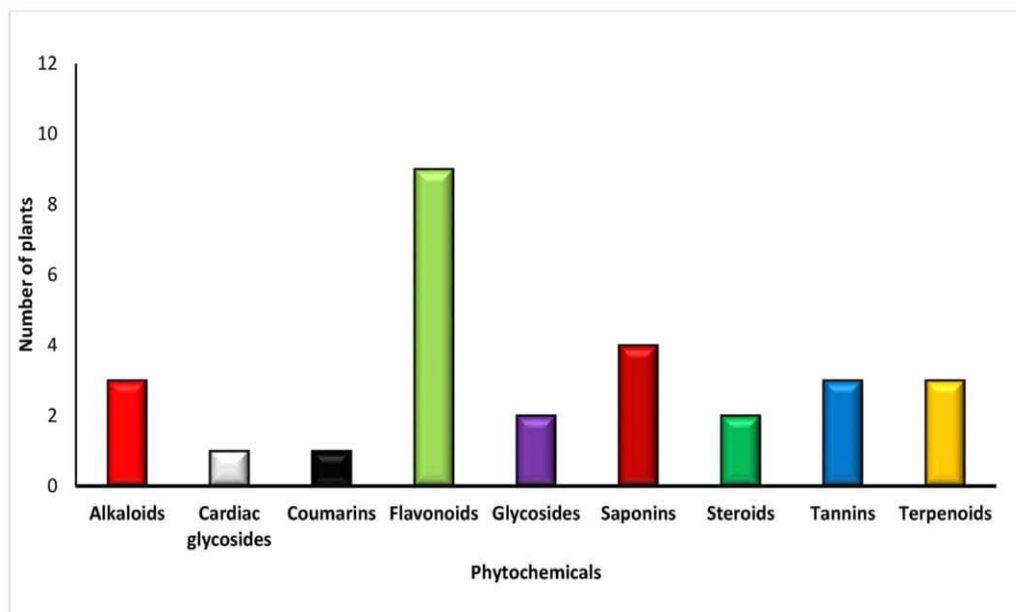


Fig. 3. Distribution of reported secondary metabolites in the plants displaying ACE inhibitory potential.

reversed endothelial dysfunction and tissue antioxidant enzyme activity to near normal concentration [71]. The activity of serum markers of liver and kidney damage in extract-treated groups were significantly reduced, confirming this protective effect [71]. Treatment with the extract also decreased liver TBARS levels, improved liver superoxide dismutase, catalase, and glutathione peroxidase, and increased plasma nitric oxide concentrations in rats, supporting the antioxidant and hepatoprotective effects [72]. Additionally, the *in vitro* antioxidant activities of the plant extract validates its

use in preventing oxidative stress and, thereby, concomitant disorders such as hypertension [53].

2.3. Concerns regarding traditional medicines

The widespread accessibility and use of herbal medicines, potential herbal toxicity and herb–drug interactions are major global concerns; particularly the lack of scientific evidence with regards to efficacy and/or safety is worrying. Medicinal plants comprise a

Table 3
Toxicity studies of some South African medicinal plants.

Plant	Acute/sub-chronic toxicity	Model of experimentation	Parts of plant/solvent used	Result	References
<i>Asystasia gangetica</i>	Acute toxicity	A single dose of 1000, 2000 and 5000 mg/kg of the extract were administered orally to male and female Wistar rats	Whole plant/methanolic extract	No mortality reported nor was there any sign of toxicity after 24 h and for 14 days thereafter.	[74]
<i>Clausena anisata</i>	Acute toxicity	A single dose of between 500 and 5000 mg/kg body weight was administered orally to male Swiss mice	Leaves/hexane extract	No mortality observed within 48 h. Physical signs observed (Decreased motor activity, respiration and feeding, closure of eyes)	[75]
			Leaves/Chloroform extract	Doses of 5000 and 2811 mg/kg produced 60% and 40% mortality, respectively, within 48 h. Oral LD ₅₀ of the extract was calculated to be 4166.7 mg/kg.	[75]
<i>Sclerocarya birrea</i>	Acute toxicity	A single dose of 3000 mg/kg body weight was administered to male and female albino rats.	Kernel/aqueous extract	No sign of toxicity or mortality observed within 48 h.	[76]
	Sub-chronic toxicity	Animals were orally administered with doses of 1000, 2000, 3000 and 4000 mg/kg body weight of the extract once daily for 28 days.		Doses of 3000 and 4000 mg/kg/day revealed liver and kidney abnormalities	[76]
<i>Tulbaghia violacea</i>	Acute toxicity	A single dose of 5/kg body weight was administered orally to male and female Wistar rats	Rhizomes/methanolic extract	No mortality observed and no indication of toxicity, behavioural or physiological changes.	[77]
	Sub-chronic toxicity	Animals were orally administered with doses of 125, 250 and 500 mg/kg daily for 28 days		No mortality observed and no signs of toxicity reported	[77]

complex mixture of approximately 400 or more chemicals in comparison to synthetic drugs which are typically made up of a single chemical [73]. It is relatively simple to determine the activity and side effects of a single chemical, however, it is increasingly difficult to record the composite interactions and synergies occurring amongst the several chemicals found in a plant, or crude plant extract that is traditionally used. Toxicological issues linked with the use of traditional medicines are associated with serious adverse events including cardiovascular issues, liver toxicity or malfunction, hematologic, renal toxicity, and fatality (Table 3). The low frequency of adverse reports associated with traditional medicine in developing countries could be because consumers commonly esteem them as safe and thus assume their symptoms are unrelated to their use.

Therefore, it is imperative that more evidence-based studies demonstrating the efficacy of traditional medicine is conducted. Furthermore, medicinal plants should be phytochemically characterized to identify their bioactive compounds since other compounds present in crude extracts may cause unfavorable side effects. Albeit the phytochemical analysis should be complemented by studies on the mechanism of action and the toxicological profile of the medicinal plants. Additionally, there is a paucity of data on the toxicity of medicinal plants in SA during pregnancy. This warrants further toxicology studies that profile the potential use of medicinal plants and pre-clinical toxicological research during pregnancy. Since PE requires close monitoring and management across the gestational period, there is a need for chronic toxicity studies.

3. Conclusion and future prospective

Phytotherapy continues to create broader awareness and publicity due to its therapeutic properties and negligible side effects. We provide a summary of the effects of South African medicinal plants in the ACE1 RAS pathway, as a potential treatment of HDP, such as PE. *In vitro* screening of medicinal plants requires the support of *in vivo* studies that show clear evidence of its effectiveness to progress to the pre-clinical trial stage. Systematic pharmacokinetic and pharmacodynamic *in vivo* investigations are also required to evaluate the antihypertensive potential of documented medicinal plants for managing HDP. Additionally, research-based evidence on whether ACEI rich herbs are safer than synthetic ACEI's is lacking. Hence, large scale studies are required to evaluate the safety of ACEI rich herbs compared to synthetic ACEIs in pregnancy.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: RR, SB, RM and NG; methodology and study design, SB, RM, NG; all authors contributed to the formal analysis and investigation; original draft preparation, RR; Review and editing, SB, RM, JM, TN, and NG. All authors have read and agreed to the final version of the manuscript.

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CHAPTER 3

PAPER 2

Title: Antioxidant and angiotensin-converting enzyme inhibitory potential of South African traditional medicinal plants and plant-derived compounds

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Antioxidant and angiotensin-converting enzyme inhibitory potential of South African traditional medicinal plants and plant-derived compounds

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ABSTRACT

Plants used in African traditional medicine to manage hypertension are potential angiotensin-converting enzyme (ACE) inhibitors. We, therefore, aimed to determine the antioxidant and anti-hypertensive properties of the methanol extracts of six plants, namely, *Artemisia afra* Jacq. ex Willd., *Clausena anisata*, *Dombeya rotundifolia*, *Sclerocarya birrea*, *Rhus chirindensis*, and *Warbugia salutaris* and selected compounds, viz., 3 β -taraxerol, β -amyrin, oleanolic acid, hesperidin, nicotinamide, and quercetin. The antioxidant and anti-hypertensive activities were determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, the nitric oxide (NO) scavenging assay and the ACE inhibition assay respectively. *D. rotundifolia* (IC₅₀: 63.02 \pm 0.5 μ g/mL), 3 β -taraxerol (IC₅₀: <1 μ g/mL), β -amyrin (IC₅₀: 105.04 \pm 1.36 μ g/mL), oleanolic acid (IC₅₀: 32.20 \pm 0.20 μ g/mL) and quercetin (IC₅₀: <1 μ g/mL) displayed strong DPPH free radical scavenging activity ranging from 80 to 90%. Additionally, *D. rotundifolia* demonstrated 72% inhibition against NO displaying the lowest IC₅₀ of 63.45 \pm 2.9 μ g/mL. A 75–87% inhibition was noted for 3 β -taraxerol (IC₅₀: <1 μ g/mL), β -amyrin (IC₅₀: <1 μ g/mL), oleanolic acid (IC₅₀: <1 μ g/mL) and quercetin (IC₅₀: 125.02 \pm 1.77 μ g/mL), similar to ascorbic acid (standard). A significant ACE inhibitory activity of 92% with the lowest IC₅₀ (110.16 \pm 0.12 μ g/mL) was demonstrated for *D. rotundifolia* followed by *R. chirindensis* (79%), *S. birrea* (67%), *A. afra* (54%) and *C. anisata* (54%). Our findings suggest that these medicinal plants and bioactive compounds have great antihypertensive potential via inhibitory effects on ACE in the renin-angiotensin-aldosterone system pathway.

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1. Introduction

Herbal remedies are affordable and easily accessible in developing countries and have minimal side effects compared to synthetic drugs. This has contributed to the rapidly rising use of phytotherapy in developing countries, with almost 80% of the population depending on traditional medicine for their principal healthcare requirements (Ekor, 2014). In South Africa, more than 100 medicinal plants are used by traditional healers for managing hypertension (Balogun and Ashafa, 2019), with many of these plants being investigated for potential leads in drug development (Sasidharan et al., 2011). It is predicted that by 2025, almost 1.56 billion people worldwide will

suffer from hypertension (Kearney et al., 2005), which if left untreated, may cause end-organ damage (Aggarwal and Khan, 2006).

The relationship between the development of hypertension, the increased activity of reactive oxygen species (ROS), and decreased antioxidant capacity has been demonstrated in human hypertension (Tain and Hsu, 2022) and many experimental models of hypertension (Addabbo et al., 2009; Harrison and Gongora, 2009; Vaziri, 2004). This leads to an oxidant and antioxidant imbalance in superoxide and nitric oxide (NO) production resulting in reduced vasodilation, which significantly contributes to increased vascular resistance and the subsequent development of hypertension (Webb and Werring, 2022).

Current hypertension management protocols include pharmacological agents that target the renin-angiotensin-aldosterone system (RAAS) for blood pressure regulation (Brown and Garovic, 2011). Therapeutic agents that alter RAAS activity include angiotensin-converting enzyme inhibitors (ACEIs), AT1-receptor blockers (ARBs), and

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aldosterone-receptor antagonists (ARAs) (Zaman et al., 2002). The ACEIs selectively inhibit angiotensin II production (a potent vasoconstrictor) and reduces circulating Angiotensin II levels, thereby reducing blood pressure and the risk of hypertension complications (Colafella et al., 2019). This inhibitory effect increases sodium and urine excretion, stimulates vasodilation of renal vasculature, reduces vascular resistance, increases venous capacity, and reduces cardiac output (Colafella et al., 2019). However, chronic use of synthetic ACEIs, such as captopril and enalapril elicit adverse drug effects, such as dry cough, skin rashes, and allergic reactions (Herman et al., 2017; Steckelings et al., 2001). Such reports have stimulated the search for naturally available antioxidants and ACEIs with reduced negative effects.

Since South Africa is home to a large and diverse variety of indigenous plants, with an unexploited reservoir of potentially active biomolecules with antihypertensive effects, this study aimed to screen selected medicinal plants and their major secondary metabolites for their antioxidant and ACE inhibitory activities.

2. Material and methods

2.1. Materials

The plant species viz., *A. afra*, *C. anisata*, *D. rotundifolia*, *S. birrea*, *R. chirindensis*, and *W. salutaris*, most commonly in use, were purchased from the Silverglen Nature Reserve in Chatsworth, Durban, South Africa. 3 β -taraxerol (95.0%; Mw: 426.72 g/mol), β -amyryn (98.5%; Mw: 426.72 g/mol), oleanolic acid (97%; Mw: 456.70 g/mol), hesperidin (80%; Mw: 610.56 g/mol), nicotinamide (99.5%; Mw: 122.12 g/mol) and quercetin (95%; Mw: 302.24 g/mol) standards were purchased from Merck, Germany.

2.2. Methods

2.2.1. Preparation of extracts

Plant leaves for each species were thoroughly washed under tap water, air dried at room temperature (approximately 25 °C) for three weeks and then crushed using a mill. Ground plant material (~2 kg) was extracted with methanol (MeOH) by continuous shaking on an orbital shaker at room temperature for 48 h. The extract was filtered (Whatman No. 42 filter paper) and the solvent was evaporated at 64 °C on the rotary evaporator. The dried extracts were stored at 4 °C for further analysis.

2.2.2. Antioxidant activity

2.2.2.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) analysis. The scavenging activity of the plant extracts and biomolecules on the stable radical DPPH was determined according to the previously published method with minor modifications (Chidambara Murthy et al., 2002). At varying concentrations, 150 μ L of MeOH plant extracts (50, 100, 150, 200, and 250 μ g/mL) or compounds (15, 30, 60, 120, 240 μ g/mL) were mixed with 2.85 mL of a methanolic solution of DPPH (0.1 mM). An equal amount of MeOH and DPPH with no extract was used as the negative control, and ascorbic acid served as the positive control. The reaction was incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm against MeOH as a blank using a UV–Vis spectrophotometer (SPECTROstarnano BMG LABTECH, Germany). All tests were performed in triplicate. The free radical scavenging activity (%) was expressed and calculated as follows:

$$\% \text{Scavenging activity (DPPH)} = \frac{A_c - A_s}{A_c} \times 100$$

where A_s = absorbance of sample and A_c = absorbance of control.

2.2.2.2. Nitric oxide (NO) scavenging method. The scavenging activity of the plant extracts and compounds against NO was determined as previously published with some minor modifications (Govindarajan et al., 2005). A mixture of 0.5 mL of a 10 mM sodium nitroprusside solution in phosphate-buffered saline was added to 1 mL of the extracts at different concentrations (50, 100, 150, 200, and 250 μ g/mL) or compounds (15, 30, 60, 120, 240 μ g/mL). The extract and compound mixtures were then incubated at 25 °C for 180 min. Thereafter, the incubated mixture (1.5 mL) was diluted with a solution of 1.5 mL Griess reagent (made up of 1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). The absorbance of the resulting chromophore was measured at 546 nm using a UV–Vis spectrophotometer (SPECTROstarnano BMG LABTECH, Germany). Negative control samples without the extracts but with an equal volume of buffer were prepared similarly to test samples. Ascorbic acid was used as the positive control. The radical scavenging activity was expressed as a percentage as follows:

$$\% \text{Scavenging activity (NO)} = \frac{A_c - A_s}{A_c} \times 100$$

where A_s = absorbance of sample and A_c = absorbance of control.

2.2.3. Angiotensin-converting enzyme (ACE) inhibition assay

The ACE inhibition assay was conducted according to a previously published protocol with minor modifications (Li et al., 2005). Methanolic plant extracts (20 μ L) at varying concentrations (50, 100, 150, 200 and 250 μ g/mL) and compounds (15, 30, 60, 120, 240 μ g/mL) were suspended in sodium borate buffer, 50 μ L of 5 mM hippuryl-histidyl-leucine (HHL) (in 0.1 M sodium borate buffer) and 0.3 M sodium chloride (pH 8.3). This mixture was then pre-incubated at 37 °C for 30 min, followed by adding 10 μ L (1 U/mL) ACE solution to initiate the reaction by incubating at 37 °C for 30 min. Subsequently, 100 μ L 1 M HCl was added to stop the reaction, and the absorbance was read at 492 nm. A buffer replaced the enzyme solution for the sample blank, whereas a sample control buffer replaced the sample. The inhibition percentage (%) was calculated as follows:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c - \text{Absorbance (blank)}} \times 100$$

where A_s = absorbance of sample and A_c = absorbance of control.

2.3. Statistical analysis

All data were analyzed using IBM SPSS Statistics version 27 software for Windows and are presented as mean \pm SD. The one-way ANOVA and Tukey's HSD-multiple range *post hoc* tests were used to determine the significant difference between groups. A $p < 0.05$ was considered statistically significant.

3. Results

Fig. 1A and B show the DPPH radical scavenging activities of the plant extracts and compounds, respectively. At 100–200 μ g/mL, all extracts displayed a significantly lower activity ($p < 0.05$) than ascorbic acid. At 250 μ g/mL, the extract from *D. rotundifolia* demonstrated significantly higher activity ($p < 0.05$) than the other extracts, in comparison to ascorbic acid (87%). Quercetin and 3 β -taraxerol displayed potent activity at all concentrations tested, with IC_{50} values < 1 μ g/mL. Oleanolic acid and β -amyryn demonstrated dose-dependent scavenging activity with the IC_{50} value of oleanolic acid (32.20 ± 0.20 μ g/mL) being similar to ascorbic acid (32.95 ± 0.26 μ g/mL). At 240 μ g/mL, the triterpenes and flavonol presented similar radical scavenging activity to ascorbic acid.

The NO scavenging activities of the plant extracts and the compounds are shown in Fig. 2A and B respectively. Overall, all extracts were able to effectively inhibit NO in a dose-dependent manner. At 250 μ g/mL, all extracts except *S. birrea* showed similar activity to ascorbic acid, with the IC_{50} of *D. rotundifolia* (63.45 ± 2.9 μ g/mL)

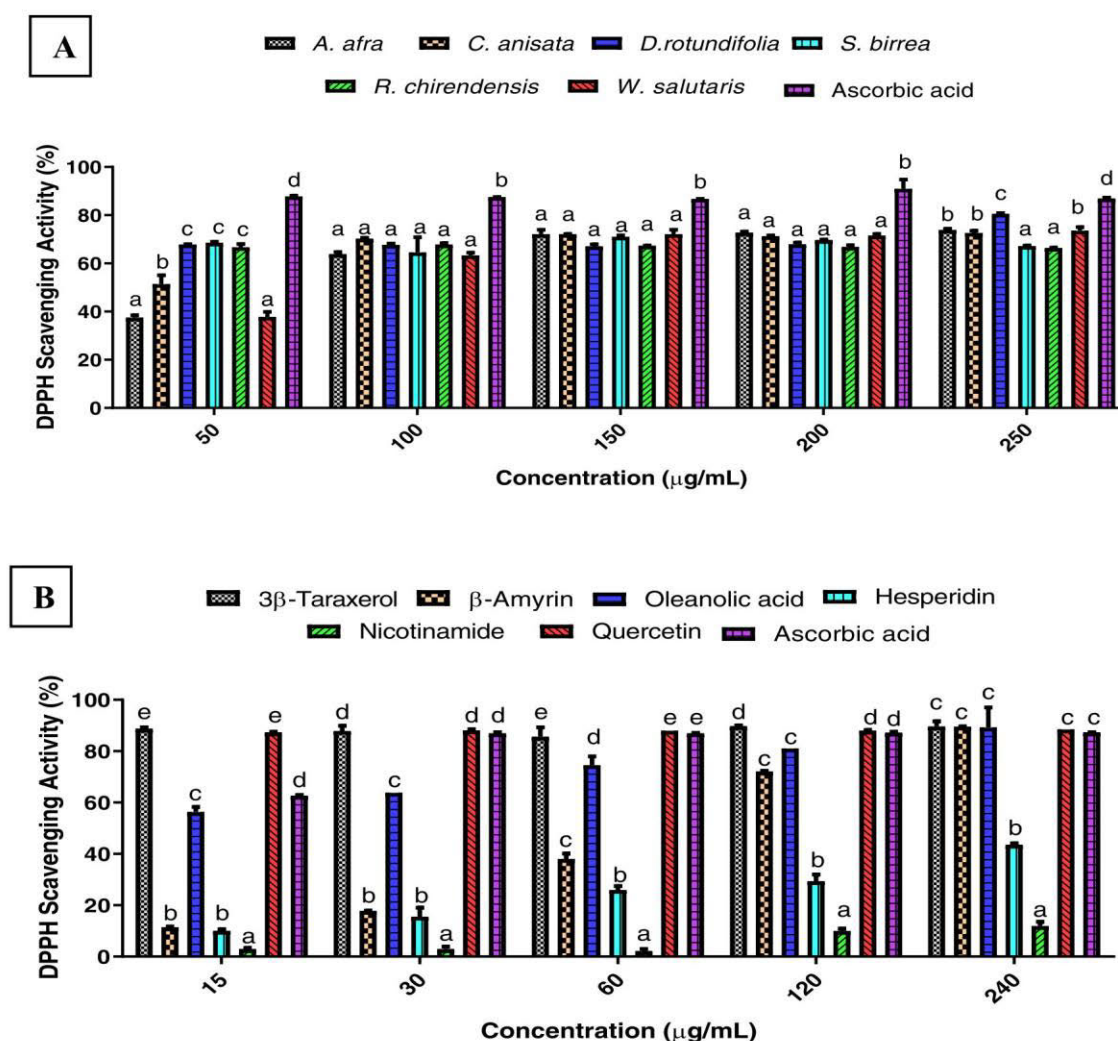


Fig. 1. DPPH scavenging activity of plant extracts (A) and biomolecules (B). Data are presented as mean \pm SD, ($n = 3$). ^{a–d} Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range posthoc test, IBM SPSS for Windows).

being relatively lower in comparison to the other extracts. Quercetin, 3 β -taraxerol, β -amyirin, and oleanolic acid inhibited NO effectively, with quercetin showing a more potent inhibition (87%) than ascorbic acid (81%) at 240 $\mu\text{g/mL}$.

The ACE inhibitory activity of the plant extracts and compounds using the ACE inhibition assay is shown in Fig. 3A and B, respectively. A significant ACE inhibitory activity of 92% was demonstrated for *D. rotundifolia*, followed by *R. chirindensis* (79%), *S. birrea* (67%), *A. afra* (54%) and *C. anisata* (54%). The highest ACE inhibition of 92% was observed at 250 $\mu\text{g/mL}$ in *D. rotundifolia*, similar to captopril (Fig. 3A).

According to the heat map and IC_{50} values shown in Fig. 4, the extract of *D. rotundifolia* exhibited the lowest IC_{50} value ($110.16 \pm 0.12 \mu\text{g/mL}$), followed by *R. chirindensis* ($129.49 \pm 2.08 \mu\text{g/mL}$), and *S. birrea* ($160.79 \pm 0.03 \mu\text{g/mL}$). With regard to the compounds, hesperidin and nicotinamide exhibited ACE inhibitions of 71% and 67%, respectively, at a concentration of 240 $\mu\text{g/mL}$ (Fig. 3B). Moreover, hesperidin exhibited the lowest IC_{50} of $201.13 \pm 1.56 \mu\text{g/mL}$ among the compounds tested, followed by nicotinamide (IC_{50} : $239.04 \pm 0.89 \mu\text{g/mL}$) (Fig. 4).

4. Discussion

Oxidative stress is believed to be a key factor in the pathogenesis of hypertension (Montezano and Touyz, 2012; Rodrigo et al., 2011).

NO released by the endothelium causes vascular relaxation (Rafieian-Kopaei et al., 2013); however, it is rapidly degraded by the oxygen-derived free radical superoxide anion, which is a vasoconstrictor that influences NO biosynthesis and bioavailability (Baradaran et al., 2014). In hypertensive patients, angiotensin II increases recurrently, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated, which causes a rise in ROS. Increased vasorelaxation and reduction of endogenous production of ROS have been shown to improve endothelial function and, as a result, hypertension (Perez-Vizcaino et al., 2006). Due to the fundamental role that oxidative stress plays in the etiology of human hypertension, it is more advantageous for an antihypertensive drug to have an antioxidant effect (Griendling et al., 1994).

The present study evaluated the antioxidant activity of six plant extracts and six plant-derived compounds using the DPPH free radical assay (Koleva et al., 2002) and the NO free radical scavenging method (Govindarajan et al., 2005). DPPH is a free radical that can accept an electron or hydrogen radical to become a diamagnetic molecule (Patel Rajesh and Patel Natvar, 2011) and is used as a substrate to evaluate the free radical scavenging activity of antioxidative agents in vitro. Our findings demonstrate free radical scavenging activity for all plant extracts, as seen in Fig. 1A. This indicates that polar compounds or those that have an affinity for methanol or ethanol have antioxidant abilities (Amorati and Valgimigli, 2015). *D.*

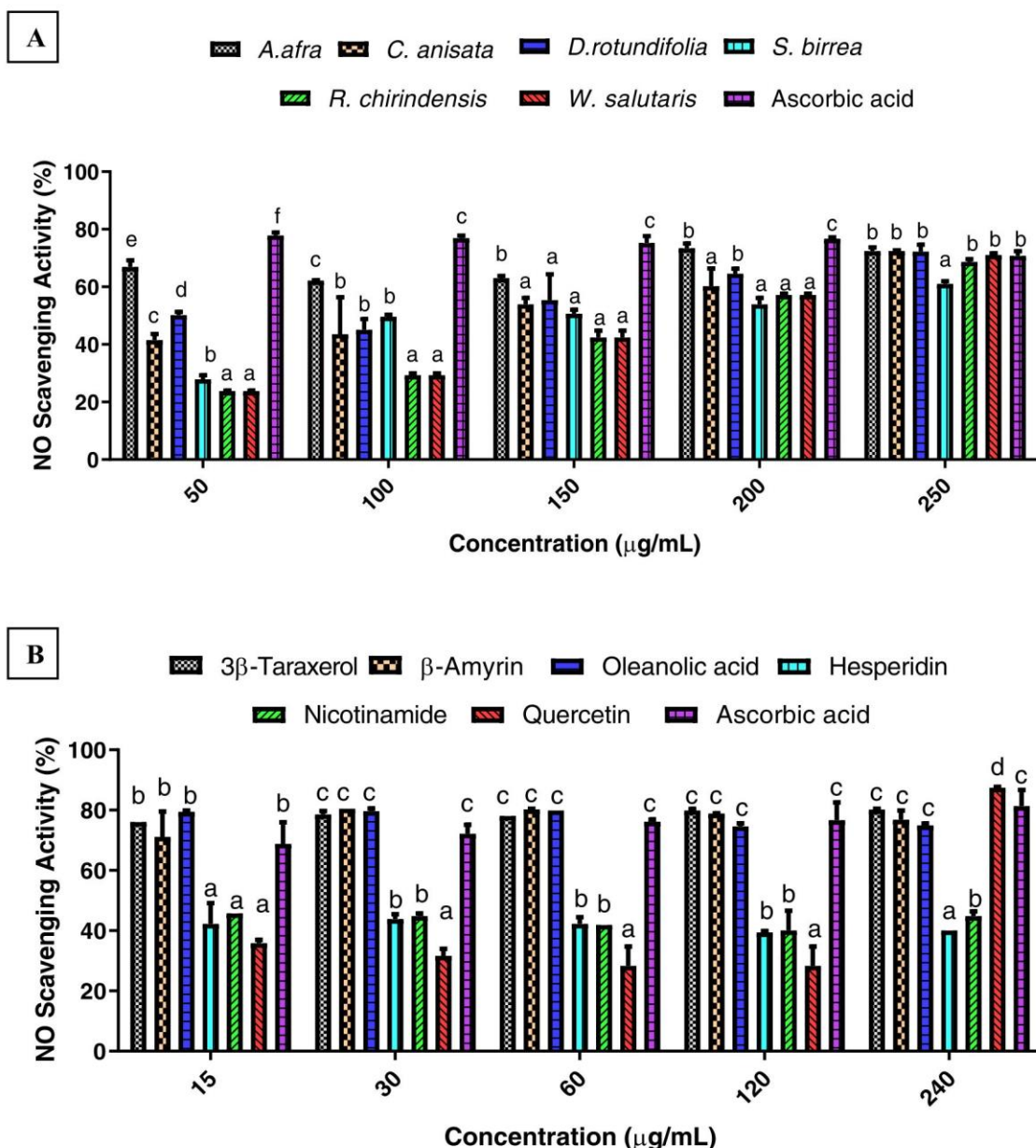


Fig. 2. NO scavenging activity of plant extracts (A) and biomolecules (B). Data are presented as mean \pm SD ($n = 3$). ^{a–f}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range posthoc test, IBM SPSS for Windows).

rotundifolia showed the most vigorous activity (81%) with the lowest IC_{50} value ($63.02 \pm 0.5 \mu\text{g/mL}$), followed by *A. afra* (74%; $65.24 \pm 0.5 \mu\text{g/mL}$). Our results are supported by Kudumela and Masoko (Kudumela and Masoko, 2018), who showed similar activity against DPPH by the aqueous leaf extracts of *D. rotundifolia*.

Multiple classes of phytochemicals, including cardiac glycosides, flavonoids, phenolics, saponins, steroids, tannins, and terpenoids, have been isolated from the leaves of *D. rotundifolia* (Amoo et al., 2012; Kudumela and Masoko, 2018; Mkhize et al., 2018; Ndwigah et al., 2005; Reid et al., 2005). Oleanolic acid, β -taraxerol and β -amyrin have been isolated as major secondary metabolites from *D. rotundifolia* in our laboratory (Shelembe, 2019). These triterpenes were shown to possess intense free radical scavenging activity of 80–90% in this study, which could account for the plant's potent antioxidant power. A study by Jamila et al. (2015) reported that taraxerol isolated from *Garcinia hombroniana* was more potent than Trolox and equipotent to gallic acid in DPPH radical scavenging activity (Chen et al., 1995;

Jamila et al., 2015). The similarities in the chemical structures of the triterpenes, particularly the presence of the hydroxy group at position 3 may explain their powerful antioxidative capabilities.

Low concentrations of bioregulatory NO molecules are sufficient to exert various physiological effects, including blood pressure regulation, neural signal transduction, platelet function, and antimicrobial and antitumor activities (Schinella et al., 2002). Extracts and bioactive compounds from plants may exert counteractive effects on NO formation and possibly prevent the negative influences that elevated NO levels exert (Schinella et al., 2002). In the present study, a significant decrease in the NO radical was observed for all plants and compounds, which may be associated with their scavenging ability. *D. rotundifolia* (72%) and *W. salutaris* (71%), a genus known to be rich in flavonoids such as quercetin, strongly inhibited NO production demonstrating activity comparable to ascorbic acid. To our knowledge, this is the first report on the NO scavenging activities of these medicinal plants.

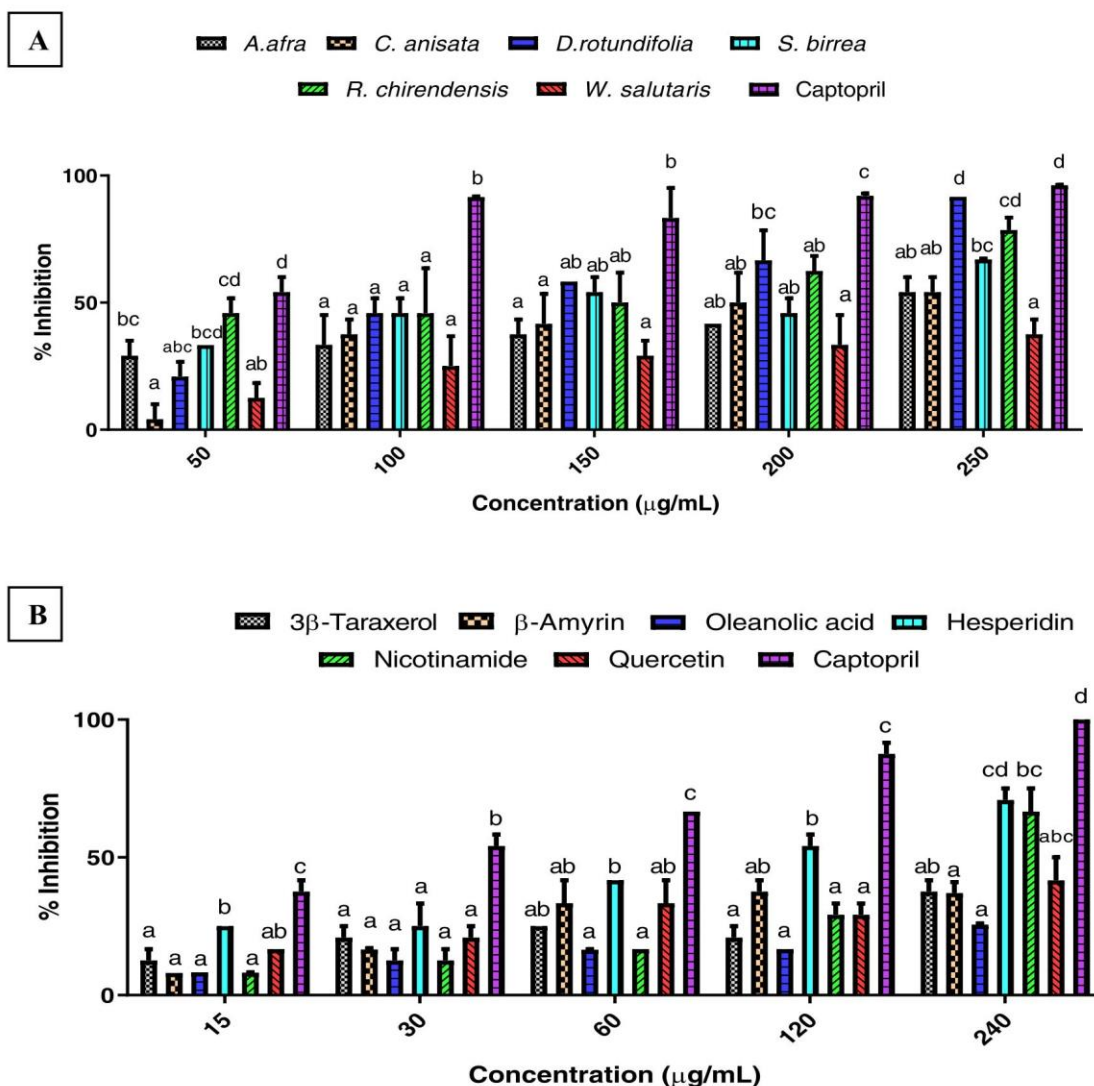


Fig. 3. ACE inhibitory activity of plant extracts (A) and bioactive compounds (B). Data are presented as mean ± SD. (n = 3). ^{a–f} Values with different letters above the bars for a given concentration are significantly different from each other (p < 0.05, Tukey's HSD-multiple range posthoc test, IBM SPSS for Window).

Studies have demonstrated that natural products such as vitamin C, β-carotene, epigallocatechin, carnosol, curcuminoids, pycnogenol, coumarins, and phenylethanoids alleviate the adverse effects of NO (Arroyo et al., 1992; Chan et al., 1995; Fraga et al., 1991; Miyake et al., 1999; Rao, 1997; Virgili et al., 1998; Xiong et al., 2000). Our findings show that the natural products, 3β-taraxerol, β-amyirin, oleanolic acid, and quercetin significantly inhibit NO production, which may help reduce the adverse physiological effects introduced by excess NO production. These findings are significant as therapies targeting free radicals by reducing reactive nitrogen species or ROS generation may help minimize vascular injury thus preventing or regressing hypertensive end-organ damage.

Synthetic ACE inhibitors are widely available for use as antihypertensive drugs; however, many have been linked to various undesirable side effects (Kumar et al., 2010). Natural products are being more widely explored as possible antihypertensive agents, based on their ACE inhibitory potential. Plants considered to possess potential antihypertensive properties are required to inhibit the ACE enzyme by more than 50% (Duncan et al., 1999; Ramesar et al., 2008). Our data demonstrated a >50% ACE inhibition activity in five methanol plant extracts (*A. afra*, *C. anisata*, *D. rotundifolia*, *R. chirindensis* and *S.*

birrea), hesperidin, and nicotinamide. The highest ACE inhibition of 92% was observed in *D. rotundifolia*, similar to the standard captopril. Interestingly, Duncan et al. (Duncan et al., 1999) evaluated the anti-hypertensive activities of aqueous and ethanolic leaf extracts of *R. chirindensis*, *D. rotundifolia*, *S. birrea* and *C. anisata* using a similar assay we examined, and reported an ACE inhibitory activity of 85%, 83%, 68%, and 54%, respectively (Duncan et al., 1999). Moreover, known South African medicinal plants with the potential to lower blood pressure by lowering the ACE inhibition activity in the RAAS pathway were recently reviewed (Reddy et al., 2022). Interestingly, *C. anisata* was reported to lower blood pressure at a dose of 400 mg/kg in a hypertensive rat model in a 40-d experimental study, suggesting its antihypertensive effects (Lechaba et al., 2016). In addition, in a survey conducted by Ojewole et al., 2006, bolus intravenous administrations of *S. birrea* stem-bark extract (25–400 mg/kg) resulted in significant (p < 0.05–0.001) reductions in systemic arterial blood pressures and heart rates of anaesthetized normotensive and hypertensive Dahl salt-sensitive rats (Ojewole, 2006). Furthermore, several phenolic compounds have been reported to effectively reduce ACE activity, including flavonoids (Ojeda et al., 2010), flavanols (Ottaviani et al., 2006), flavonols (Oh et al., 2004), anthocyanins (Kwon et al.,

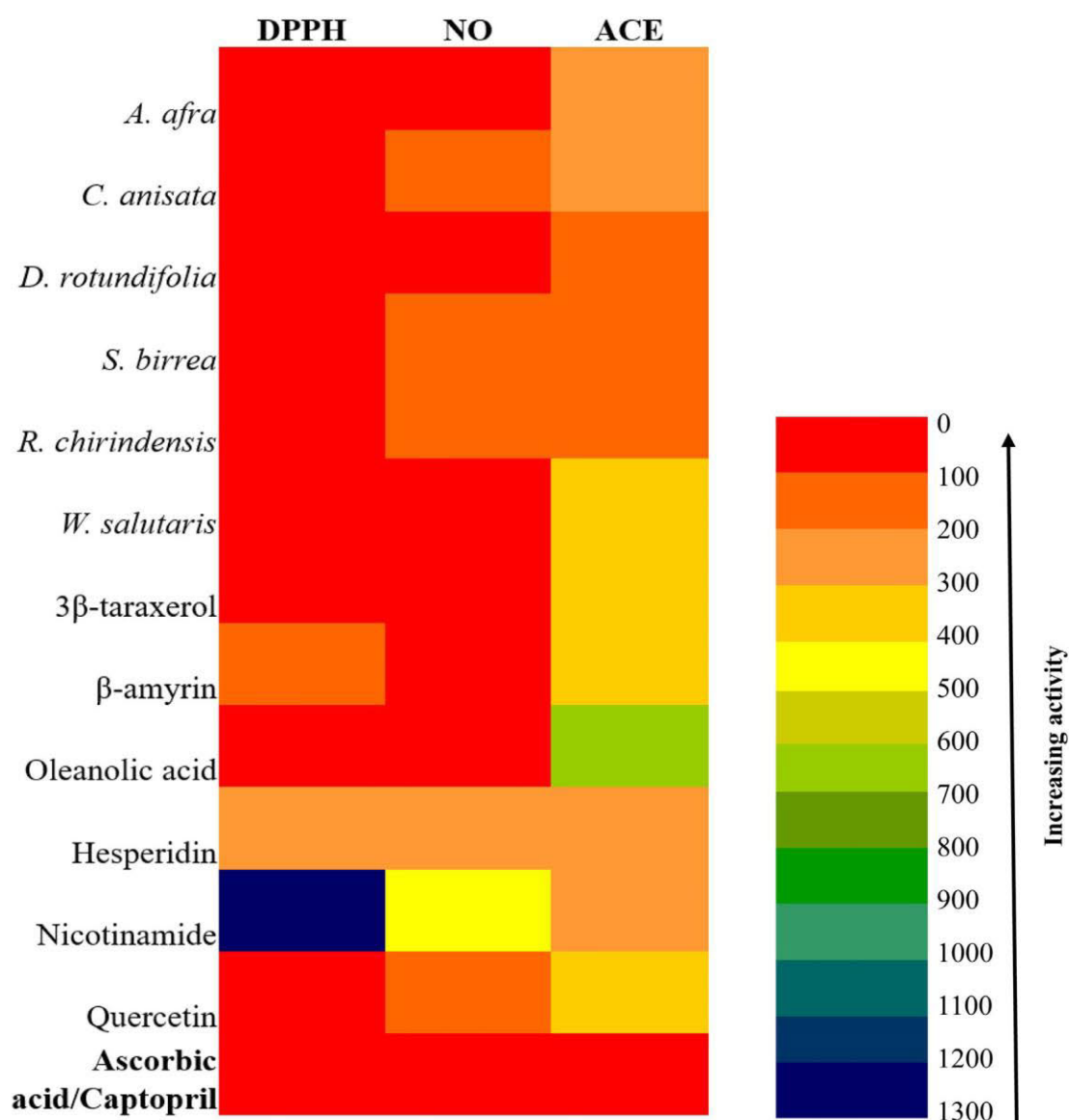


Fig. 4. Heat map of plant extracts, bioactive compounds, and their antioxidant and ACE inhibitory activity when compared to standards (ascorbic acid and captopril, respectively). Color scale bar displays IC_{50} values ranging from (0–1300 $\mu\text{g/mL}$). Low IC_{50} values (red color bar) represent better activity.

2010), isoflavones (Montenegro et al., 2009), and flavones (Loizzo et al., 2007). These secondary metabolites commonly found in plants may be responsible for the inhibition of ACE in the five active extracts investigated in our study. Our findings may pave the way for new ACE inhibitory alternatives with improved drug profiles and potentially fewer side effects. Moreover, these functionalities are potentially useful to manage hypertension-induced vascular complications. Future toxicological and pharmacological studies are required on a case-by-case basis.

5. Conclusion

The potential value of natural medicines in the treatment of hypertension has been rediscovered due to the undesirable side effects of current small-molecule drug modalities. Our findings revealed that the medicinal plant, *D. rotundifolia*, demonstrated significant ACE inhibitory and antioxidant potential due to the synergies between its triterpenes 3β-taraxerol, β-amyrin, and oleanolic acid relative to the other plant extracts. These findings suggest its potential to ameliorate oxidative stress-associated disorders, such as

hypertension. Nevertheless, more detailed evaluations aimed at assessing several parts of the species used as traditional medicines and evaluating their toxicological properties are needed. In vivo trials are required to evaluate the efficacy of crude extracts of *D. rotundifolia* or compounds isolated from the species in the hope of developing more potent and safer antihypertensive drugs for use in developing countries.

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Declaration of Competing Interest

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

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CHAPTER 4

PAPER 3

Title: Mating Success of Timed Pregnancies in Sprague Dawley Rats: Considerations for Execution

Authors: Rebecca Reddy, Virushka Pillay, Sooraj Baijnath, Sanil D. Singh, Sapna Ramdin, Thajasvarie Naicker and Nalini Govender

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Technical Note

Mating success of timed pregnancies in Sprague Dawley rats: Considerations for execution

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ABSTRACT

This study compares three different mating techniques in Sprague-Dawley rats, using the pregnancy rate as the main indicator of success. It provides recommendations for timed-pregnancy experiments to achieve an appropriate sample size for the study of human pregnancy disorders. The implementation of a preconditioning phase, determination of the estrous cycle, the use of two mating strategies (Lee-Boot and Whitten effect), female: male mating ratios, and cohabitation duration should be considered as they improve the mating success rate.

1. Introduction

Rodent models have become an indispensable tool in our understanding of human pregnancy and pregnancy-related disorders due to their placental architecture. The similarities that exist between human and rat placental development such as haemochorial placentation, deep myometrial trophoblast invasion, and spiral artery remodelling make the rat model ideal for studying human pregnancy disorders [1]. However, establishing an accurate gestational day 0 in experimental animals, with a sufficient sample size for statistical correlation with human pregnancy timelines, remains a challenge for scientists [2]. Non-invasive timed-mating involves a laborious wasteful use of animals where approximately five proven females with proven males are utilized to obtain at least one successful conception for each mating cluster [2]. This is in opposition to the 3Rs of animal research (i.e. replacement, reduction, and refinement), guiding the humane use of animals in research [3], which now also incorporates a 4th R (i.e. animal rehabilitation) to direct laboratory use of animals [4].

The Lee-Boot and Whitten effects are two phenomena that influence reproductive processes in mice. Stimuli from the social environment are important regulators of reproductive events in mammals [5], and are reported to disrupt pregnancy as well as influence the frequency and composition of oestrous cycle in mice [6]. The Whitten effect induces and synchronizes the oestrus cycles among unisexual grouped females in

the presence of a male [7,8]. In mice, a pregnancy-blocking chemosignal found in the males urine causes pre-implantation pregnancy failure in newly mated female mice, however, the females are able to recognize the chemosignal with whom they mated, preventing him from aborting his offspring [9]. In contrast, the Lee-Boot phenomenon suppresses or prolongs the oestrous cycles of mature female rodents when housed in groups and isolated from males [10]. This effect is due to the effects of an estrogen-dependent pheromone which is released via the urine and acts on the vomeronasal organ of recipients [11,12]. The pheromone lowers the concentration of luteinizing hormone and elevates prolactin levels, synchronizing or stopping the recipient's cycle [13].

Whilst the Lee-Boot and Whitten model typically promotes mating induction in mice [14,15], limited studies are available in rats to demonstrate the efficacy of these mating phenomena [16,17]. The disadvantage of the mouse model is their small size requires fine surgical skills; whilst larger litter sizes are fraught with errors when tracking individual foetal measurements [18,19]. Rats are preferable due to the ease of handling [20]. Moreover, their vaginal fissure is visible for vaginal swabbing [20], whereas in mice, swabbing causes inflammation which obscures cytological assessments over time [21]. Additionally, due to the similarities in rat and human placental development, the rat model has provided clarity in the understanding of trophoblast-directed spiral artery remodelling [1] which supports its extrapolation for use in understanding vascular modifications [22] and cardiac output [23].

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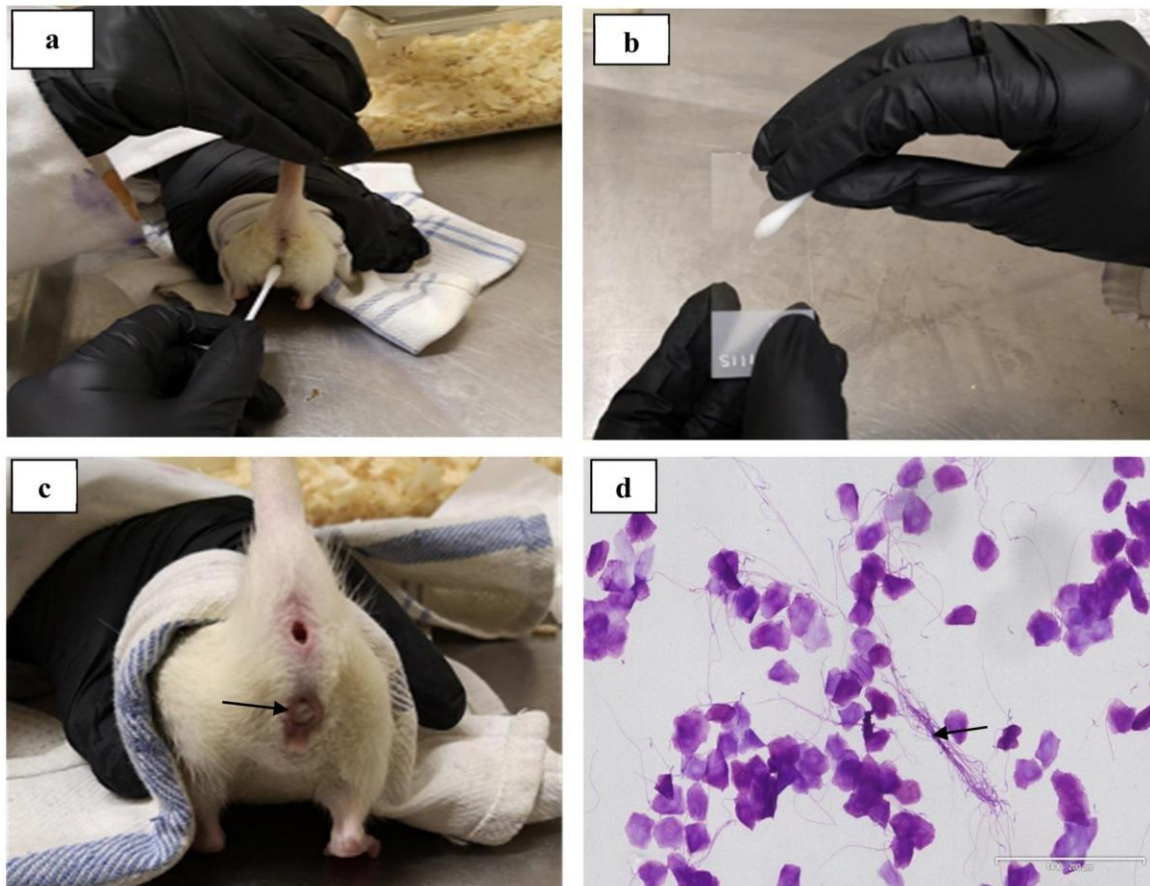


Fig. 1. Vaginal lavage collection (a); smear preparation (b); vaginal plug (black arrow) confirming mating (c) and sperm (arrow) in the vaginal smear (d).

Moreover, the short rat oestrous cycle which lasts approximately 4–5 days [24], makes rats an ideal reproductive model to study variations in the estrous cycle [25]. This involves cytological changes in epithelial cells, cornified cells, and leukocytes within vaginal smears [26,27]. The cellular details of vaginal smears are recognized for their accuracy in characterizing the oestrous cycle, thus the ability to correctly define cellular variants is essential [28]. However, despite the ability to effortlessly manipulate rodent cycles, routine anxieties experienced in animal units to achieve a sufficient pregnant sample cohort of sufficient size is a challenge.

We aimed to establish guidelines for consideration when conducting timed-pregnancy experiments in the Sprague-Dawley rat model that facilitates a high success rate by evaluating various approaches to mating. Being able to improve the success rate of these experiments will positively impact the study of disease progression and drug trials of pregnancy disorders so that the clinical profiles can be fully established throughout the gestational period (i.e., trimesters 1, 2, and 3).

2. Methods

2.1. Ethical considerations

All experimental practices were approved by the Institutional Animal Research Ethics Committee (AREC/046/017 and AREC/015/020D). Sixty young adult female Sprague Dawley rats aged 10–12 weeks (weighing 180–240 g) were obtained from the Biomedical Resource Unit (BRU). All animals were housed in polycarbonate cages under standard laboratory conditions (temperature: 22–24 °C), humidity (60 %), and illumination (12 h light/dark cycles) with ad libitum access to standard rat chow and normal drinking water.

2.2. Project design

Three different mating methods were investigated to establish the most suitable method for future studies. The Lee-Boot effect was induced in all 3 approaches by grouping female Sprague Dawley rats ($n = 6$) in the absence of males and housed in 10 large polycarbonate cages for 4 days. Vaginal smears were performed daily to determine the transitioning from proestrus to the estrous stage of the cycle and to determine the optimum period for the introduction of the male for mating. The mating methods varied in terms of the female: male rat ratios and the number of nights animals were housed together.

2.2.1. Method 1

Fourteen female Sprague Dawley rats were housed in groups of 4–5 in polycarbonate cages in close proximity to seven male Sprague Dawley rats for 4 days. Male urine-laden bedding was scattered daily into the female cages. Mating was conducted on the 5th day, based on a 2 female:1 male ratio for 24 h.

2.2.2. Method 2

Sixteen female Sprague Dawley rats were weight-matched into pairs where male Sprague Dawley rats were housed in close proximity to the female cages for 4 days. Male urine-laden bedding was dispersed daily into the female cages. Mating was conducted on the 5th day and was based on a 2 female:1 male ratio for 72 h.

2.2.3. Method 3

Thirty female and male Sprague Dawley rats were housed in polycarbonate cages in close proximity to each other for 4 days. Male urine-laden bedding was distributed on a daily basis into the female cages.

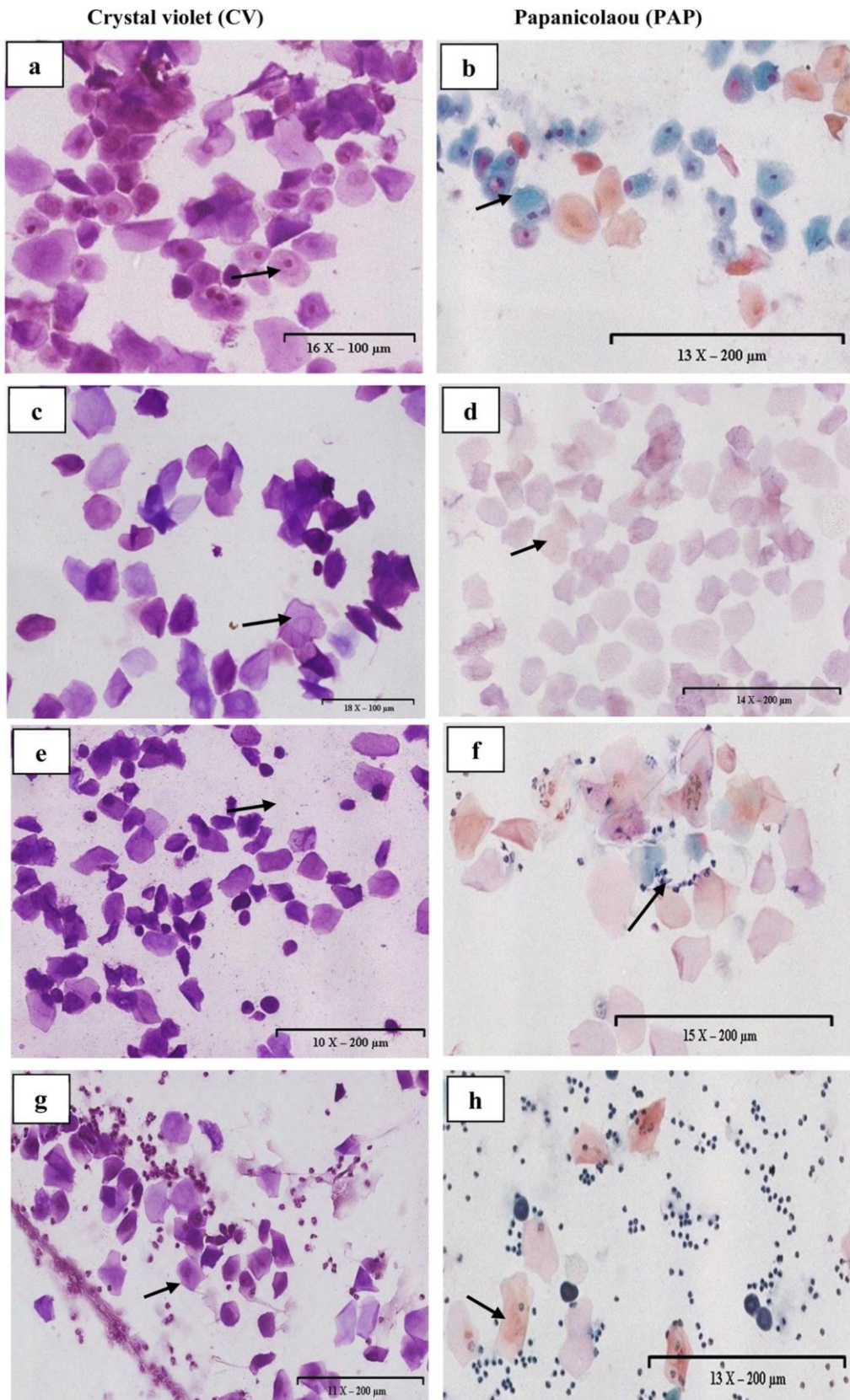


Fig. 2. Differentiation of the Estrous cycle using the crystal violet and Papanicolaou stain. Proestrus stage showing nucleated epithelial cells, anucleated cornified squamous epithelial cells (arrows; a, b); Estrus showing cornified squamous epithelial cells, arranged in sheets and clusters (arrows; c, d); Metestrus showing darkly pigmented polymorpho nuclei in leukocytes (arrows; e, f) and Diestrus stage showing cornified squamous epithelial cells and nucleated epithelial cells (arrows; g, h).

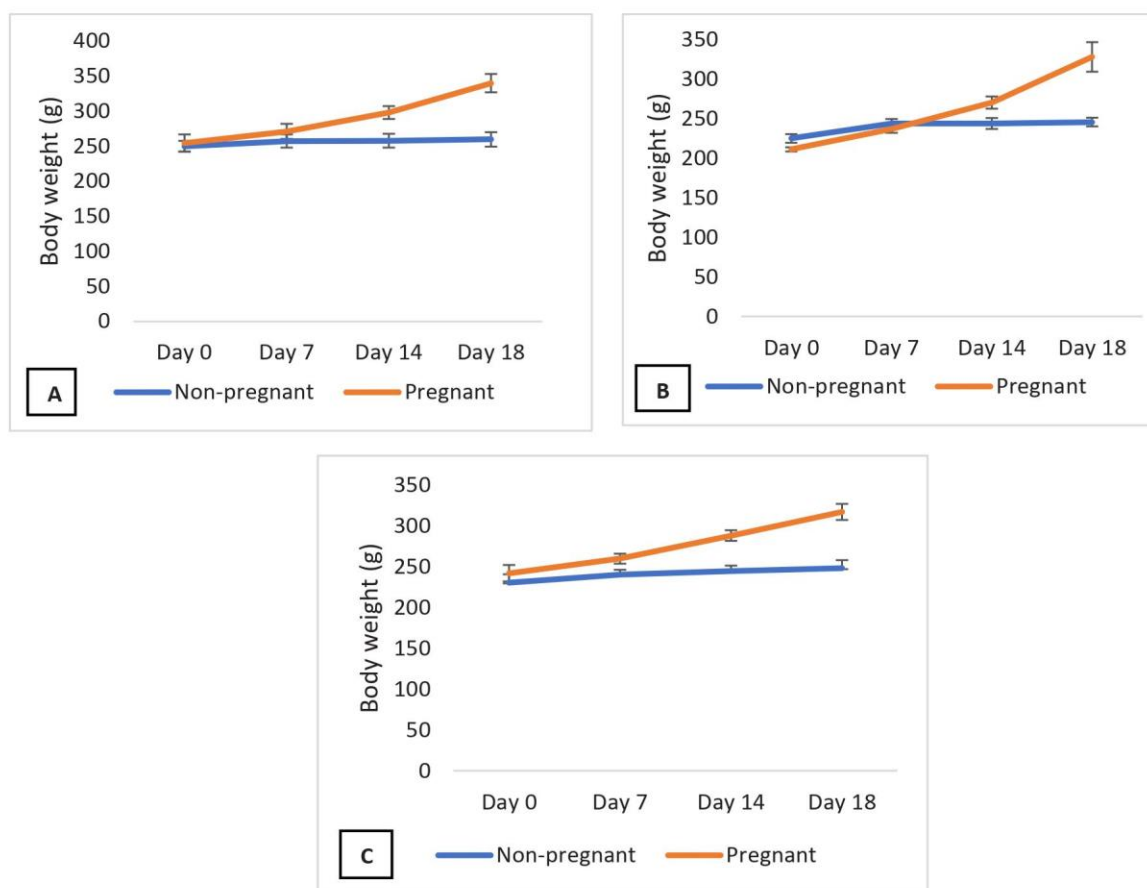


Fig. 3. Body weight measurements of pregnant and non-pregnant rats observed in (a) method 1; (b) method 2 and (c) method 3.

After the 4th day of male pheromone exposure, the 30 male rats were placed individually into polycarbonate cages. Females were introduced into the males' cages on the 5th day and this method was based on a 1female:1 male ratio for 48 h.

Post the selected timeframes for each method, the male rats were removed from the female cages and returned to the BRU.

2.3. Vaginal histology: staging the estrous cycle

Vaginal smears were performed using sterile cotton-tipped swabs dipped in saline, via gentle, quick, and shallow vaginal swabbing (Fig. 1a) and immediately prepared (Fig. 1b). Vaginal epithelial cells were stained using the Crystal violet (CV) and Papanicolaou (PAP) staining procedures and assessed using previously published criteria [29,30]. A visible vaginal plug (Fig. 1c) and the presence of sperm in the vaginal smear (Fig. 1d) were used as indicators for positive mating. Slides were viewed and photographed using a Leica Slide Scanner SCN 1000 (Leica, Germany) and a Zeiss photomicroscope.

3. Results and discussion

The execution of timed pregnancies is cumbersome and fraught with difficulties in achieving high pregnancy rates of the same gestational day 0. This attainment often leads to the wastage of many animals. We implemented both the Lee-Boot and Whitten effects to induce mating. The Lee-Boot effect, a phenomenon in which mature female mice grouped in the absence of males, can suppress or prolong their estrous cycle [16,17]. The presence of a male mouse induces and synchronizes the estrous cycle of unisexual female mice, in preparation for mating demonstrating the Whitten effect [7,8,16,17]. This phenomenon occurs

due to the exposure of the female to the male pheromones present in their urine [31]. However, in a previously unpublished study, we have observed that these phenomena are poorly replicated in rats with low mating success rates. Hence, we aimed to evaluate different approaches in combination with these phenomena to improve mating success.

We evaluated the cellular changes of the estrous cycle using vaginal swabs conducted once a day. Repeated vaginal swabbing has been reported to produce an inflammatory response and multiplication of leukocyte numbers [21]. Swabbing once a day may therefore prevent an activated inflammatory response thus preventing erroneous cytological estimation during the staging of the estrous cycle. The histological and characteristic cellular features of each estrous stage are shown (Fig. 2a–h). The proestrus stage consisted of stratified, non-cornified epithelia (Fig. 2a–b), whilst large, stratified squamous epithelia with visible cornified surface layers defined the estrous stage (Fig. 2c–d). The metestrus stage was defined by visible stratified epithelia with invasive leucocytes, extending partially to complete cornification (Fig. 2e–f) whereas the diestrus stage was characterized by stratified, non-cornified epithelia with basal proliferating cells (Fig. 2g–h).

The use of both the CV and PAP stains enhanced cellular comparisons of the estrous cycle and improved the morphologic visualization and identification of the various stages. Moreover, the use of both staining procedures validated the detection of the various cell types when classifying the stages of the estrous cycle, enhancing the optimum time selection for the introduction of the male for mating. However, the CV stain is devoid of any colour variations when transitioning in the different stages of the cycle, and the nuclear and cytoplasmic details are not clearly differentiated [32]. In contrast, the polychromatic PAP stain demonstrates crisp nuclear features with a translucent cytoplasm, indicative of squamous differentiation and uniformity in the staining

Table 1
Mating success rates of three methods investigated.

Method	Description (female: male ratio; number of hours mated)	Number of females mated	Number of female rats pregnant	Mating success rate (%)
Method 1	2:1 ratio, 24 h	14	5	36
Method 2	2:1 ratio, 72 h	16	8	50
Method 3	1:1 ratio, 48 h	30	24	80

arrangement. Moreover, it imparts a consistent staining pattern to both nuclear and cytoplasmic details and aids in preserving the smears [32]. Our findings accentuate the precision of the PAP stain in defining the cellular features of the rat estrous cycle compared to the CV method. The PAP staining may be preferable to the CV staining method, since the visible colour changes observed during transitioning of the estrous cycle enables clear visualization of the cellular features of the cytoplasm and nucleus.

The introduction of male urine-laden bedding into the female cages enabled prior exposure to male pheromones, familiarizing the females with males and making them more receptive during mating. Rats are reported to have a specialized vomeronasal system that facilitates the detection and transmission of pheromonal information via physiological mechanisms [14].

We observed noticeable elevations in the weight measurements of the female rats in pregnant groups 7 days post mating in comparison to the non-pregnant groups (Fig. 3). However, we were unable to accurately confirm pregnancy in the whole cohort until delivery, indicative that weight alone is not a suitable indicator for mating success. Albeit our data is contrary to a study conducted by Stramek et al. [2], who also highlight significant elevations in body weights post mating, as a positive indicator in differentiating pregnant from non-pregnant rats.

Mating was confirmed in our study by a visible vaginal/copulatory plug and the presence of sperm in a vaginal smear (Fig. 1c–d). The percentage mating success rate achieved for each method implemented in our study is shown in Table 1. The mating methods outlined varied in terms of the female: male rat ratios and the number of nights animals were housed together. Previous studies have also explored the option of refining timed pregnancies by investigating varying housing timeframes with 2:1 female to male ratios [2,33]. Mader et al. [33] investigated a 2:1 female: male ratio for 48 h mating method in two strains of genetically engineered mice, viz: homozygous soluble epoxide hydrolase knockout mice and L7-tau-green fluorescent protein mice and confirm a success rate of 35 % of 51 % respectively. We however demonstrate an 80 % success rate when animals were housed for 48 h, albeit with a 1:1 female: male ratio. More recently, Stramek et al. [2] reported a mating success rate of 89 % in Wistar rats using the 2:1 female to male ratio for 24 h [2], contradictory to our findings, which demonstrated a 36 % success rate using the same method, albeit in Sprague-Dawley rats. The low success rate observed in method 1 in our study may be attributed to the irregular and unpredictable ovarian cycles in Sprague-Dawley rats, which possibly compromised the tracking of cytological changes. Off note, Sprague-Dawley rats also exhibit more anxiety-like behaviors and reduced motor function compared with other rat strains [34,35], which may subsequently affect their cycles.

Furthermore, the high pregnancy success rate observed by Stramek et al. [2] may be correlated with the use of 54 rats [2] versus the 14 rats used in our study. Since our data for methods 1 and 2 viz., 2:1 ratio and varying housing timeframes, indicated lower mating success rates, we opted to conduct the experiment using a 1:1 ratio. Our findings show that the implementation of LB and Whitten effect mating phenomena, combined with a 1 female:1 male ratio, and a 48-h mating period are successful indicators for a high pregnancy success rate in Sprague-Dawley rats. Based on our findings, we provide practical

Table 2
Key steps followed and rationale for such practices in timed mating animal trials.

Key points	Rationale for such practice
Preconditioning	To allow animals to acclimatize to standard laboratory conditions and allows for easier animal handling, minimal signs of stress, sample collection, and monitoring for any abnormal behavior
Introduction of male bedding into female cages	This exposure reduced animal stress levels and permitted easier contact between females and males, enabling faster mating
Vaginal lavage collection (once per day)	To prevent errors in the cytological estimation of the staging of the estrous cycle due to predisposition to an inflammatory response.
Use of comparative stains	To improve the visualization and identification of cellular morphology of the different stages of the estrous cycle.
Use of the Lee-Boot and Whitten effect mating strategies	To optimize the timing of pregnancies in rats and induce mating.
Varying female: male rat ratios	To improve the pregnancy success rate. The 1female:1 male ratio produced an 80 % pregnancy success rate
Varying mating periods	To improve the pregnancy success rate. The 48-h mating period demonstrates an 80 % pregnancy success rate

considerations (Table 2) to assist the planning and execution of timed-pregnancy studies with a high pregnancy success rate, making such experiments less laborious and reducing animal wastage.

CRediT authorship contribution statement

NG and SB conceptualized and developed the study. RR, VP, and SR conducted the experimental study. All authors contributed to, reviewed, and edited the final manuscript.

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The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

Competing interests

The authors declare no conflict of interest.

Data Availability

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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CHAPTER 5

PAPER 4

Title: Hesperidin improves physiological outcomes in an arginine vasopressin rodent model of pre-eclampsia

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Abstract

Ethnopharmacological relevance: Hesperidin, a flavanone commonly found in citrus fruits and herbal formulations, has emerged as a potential new therapeutic agent for modulating several diseases. Since pre-eclampsia is a growing public health threat, it may negatively impact the economy and increase the disease burden of South Africa. Phytocompounds are easily accessible, demonstrate minimal side effects and may confer novel medicinal options as a treatment and preventive preference.

Aim of the study: We aimed to investigate the physiological, biochemical, and haematological outcomes of hesperidin in an arginine vasopressin (AVP)-induced rodent model of pre-eclampsia.

Materials and Methods: Sprague-Dawley rats were surgically implanted with mini-osmotic pumps to deliver AVP (200 ng/h) subcutaneously. Animals were treated with hesperidin at 200 mg/kg.b.w via oral gavage for 14 days. Systolic and diastolic blood pressures were measured on GD 7, 14, and 18 using a non-invasive tail cuff method and were euthanised on GD 21. Biochemical and haematological analyses was conducted by a pathology laboratory.

Results: The findings showed that hesperidin administration significantly decreased blood pressure and urinary protein levels in pregnant rats ($p < 0.001$). Placental and individual pup weight also increased significantly in the pregnant hesperidin-treated groups compared to AVP untreated groups ($p < 0.001$). Biochemical and haematological markers such as white blood cell count and lymphocyte levels differed significantly ($p < 0.05$) in AVP groups treated with and without hesperidin.

Conclusion: Our results suggests that hesperidin is an antihypertensive agent with modes of action associated with its diuretic and blood pressure lowering effects and reduction of proteinuria in AVP-induced pre-eclamptic rats. Moreover, our findings suggests that hesperidin improves physiological outcomes indicating its applicability in the treatment and management of pre-eclampsia.

1. Introduction

Epidemiological research demonstrates a correlation between a high dietary intake of plant-based polyphenols and a decrease in cardiovascular morbidity and mortality (Chiva-Blanch et al., 2013; Ivey et al., 2015; Peterson et al., 2012). Flavonoids, a group of plant polyphenolic phytochemicals, have been widely explored for their pharmacological impact (Jain and Parmar, 2011; Lee et al., 2010; Morand et al., 2011). Furthermore, being naturally occurring, flavonoids appear to be highly safe and without potential severe adverse effects, even during pregnancy (Garg et al., 2001).

One such flavonoid is hesperidin, which is an abundant and inexpensive by-product of citrus cultivation and is the major phytochemical component of oranges, lemons, and other citrus fruits (Garg et al., 2001). Hesperidin can account for up to 14% of the fresh weight of young, immature oranges (Barthe et al., 1988). Multiple studies have demonstrated the health-promoting and pharmacological effects of hesperidin in treating type 2 diabetes, cancer, cardiovascular disease, and neurological and psychiatric disorders. (Aruoma et al., 2012; Khan and Dangles, 2014; Syahputra et al., 2022). Furthermore, hesperidin has exhibited vasodilator, anti-thrombotic, anti-inflammatory, anti-lipemic, and antioxidant activities (Dobiaš et al., 2016; Ohtsuki et al., 2002; Sun et al., 2017; Yamamoto et al., 2013; Yamamoto et al., 2008), demonstrating its potential use for the treatment and management of a wide range of health conditions. In addition to these, studies have demonstrated its' significant anti-hypertensive and diuretic effects of hesperidin in rats (Dobiaš et al., 2016; Galati et al., 1996; Yamamoto et al., 2008).

Since hypertension in pregnancy, especially pre-eclampsia, is associated with substantial maternal and foetal morbidity and mortality, access to therapeutic drugs is essential for clinical management (Acharya, 2016). Globally, pre-eclampsia affects 8% of pregnancies (Fasanya et al., 2021), with a higher prevalence in low and middle-income countries (World Health Organization, 2019). This disorder is characterised by new-onset hypertension ($\geq 140/90$ mmHg), with/without proteinuria (urinary protein ≥ 300 mg per 24 h) (Magee et al., 2022) and has multifactorial pathogenesis (Germain et al., 2007; Rambaldi et al., 2019; Shirasuna et al., 2020; Var et al., 2003). Moreover, pre-eclampsia is characterised by altered renal function (Wiles et al., 2022) and HELLP (haemolysis, elevated liver functions, low platelet count) syndrome (Brown et al., 2018) as well as foetal growth restriction and stillbirth (Chappell et al., 2021).

Current management strategies aim to lower blood pressure and alleviate maternal and neonatal complications (Bell, 2010). Plant phenolic compounds exhibiting antioxidant, anti-inflammatory, and anti-hypertensive properties may be valuable cost-effective, easily accessible sources for novel pharmaceutical agents in treating pre-eclampsia in low and middle-income countries (Ożarowski et al., 2021).

Therefore, this study aimed to investigate the effect of hesperidin on physiological, biochemical, and haematological outcomes in an arginine vasopressin (AVP) rat model of pre-eclampsia and determine its applicability in the treatment and management of pre-eclampsia.

2. Materials and Methods

2.1. Chemicals

AVP (> 95%; Mw: 1084.23 g/mol) and hesperidin (80%; Mw: 610.56 g/mol) were purchased from Merck, Germany.

2.2. Animal welfare

Pregnant and non-pregnant Sprague-Dawley rats were procured from the Biomedical Resource Unit (BRU), Westville Campus, University of KwaZulu-Natal (UKZN), Durban, South Africa. The animals were maintained under the guidelines approved by the Animal Ethics Committee, UKZN (Protocol approval number: AREC/067/017D). Animals were housed in pairs in medium-sized polycarbonate cages, with a caged area of 646 cm² at the BRU under standard laboratory conditions of temperature (22 to 24°C), humidity (60%), and illumination (12 h light/dark cycles), with *ad libitum* access to standard rat chow and regular drinking water.

2.3. Experimental study

Twenty-four Sprague-Dawley rats were surgically implanted with ALZET mini-osmotic pumps (model 2004; Durect Corporation, Cupertino, CA) on gestational day (GD) 2 to deliver AVP (200 ng/h) subcutaneously. The study groups (n=6) were defined as follows:

- Group 1: Pregnant with AVP delivery and saline (PAVPS)
- Group 2: Pregnant with AVP delivery and hesperidin (PAVPH)
- Group 3: Pregnant with AVP delivery and captopril (PAVPC)
- Group 4: Pregnant with saline delivery (PS)
- Group 5: Pregnant with hesperidin delivery (PH)
- Group 6: Non-pregnant with hesperidin delivery (NH)
- Group 7: Non-pregnant with AVP delivery and hesperidin (NAVPH)

The mini osmotic pumps remained implanted until sacrifice. All rats received a dose of hesperidin or captopril (positive control) by oral gavage daily for 14 days from GD 7 to GD 20. The dosage of AVP and captopril was determined based on previous reports (Kanthlal et al., 2020; Ramdin et al., 2022).

Systolic and diastolic blood pressure were measured on GD 7, 14, and 18 using the MRBP tail-cuff BP monitor (IITC Life Sciences Inc., USA) by placing animals in a suitably sized restrainer. Normal blood pressure was defined as systolic ≤ 120 mmHg and diastolic ≤ 80

mmHg. Hypertension in rats was defined as systolic ≥ 140 mmHg and diastolic ≥ 90 mmHg. Animals were housed in metabolic cages (Techniplast, Italy) on GD 7, 14, and 18 to collect 24 h urine samples and measure urinary output. Animals were euthanised on GD 21 *via* isoflurane overdose (Safeline Pharmaceuticals, South Africa). Blood samples were collected *via* cardiac puncture and centrifuged for 15 min at 3500 rpm at 4°C. The number and weight of placentae and pups were recorded. Biochemical and haematological analysis was carried out by a pathology laboratory using rodent reference ranges.

2.4. *Statistical analysis*

All statistical analyses were carried out using Stata (Version 12). All data are parametric (SK test) and summarised as mean \pm SD. Tukey's *post hoc* test was used to compare the means between groups. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of hesperidin on systolic and diastolic blood pressure: Non-invasive blood pressure monitoring

Changes in systolic and diastolic blood pressures following the oral administration of saline (negative control), hesperidin, and captopril in all study groups are shown (Figure 1 A-B). In all the AVP experimental groups, an initial elevation in systolic and diastolic blood pressures (> 130 - 160 mmHg and > 90 - 120 mmHg) was observed, followed by a significant decrease in systolic and diastolic blood pressures ($p < 0.05$) after treatment. On GD 18, a significant reduction was noted in both systolic and diastolic blood pressure in hesperidin-treated animals (PAVPH: 127 ± 7 mmHg; NAVPH: 128 ± 10 mmHg) compared to the negative control group (PAVPS: 157 ± 6 mmHg).

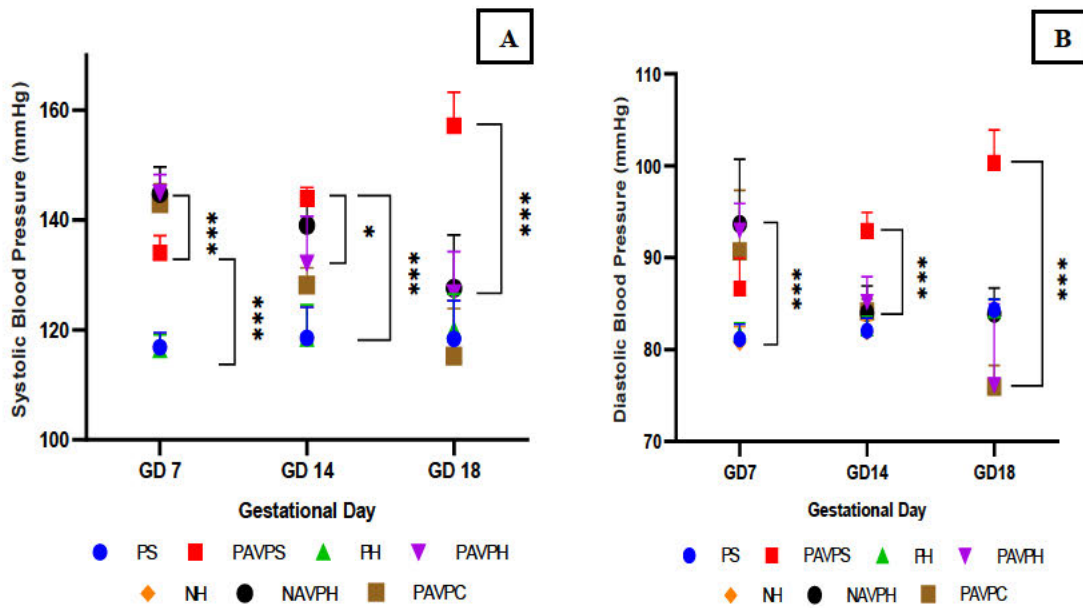


Figure 1: Changes in systolic blood pressure (A) and diastolic blood pressure (B) observed in experimental groups on GD 7, 14, and 18, respectively, following oral administration of saline, hesperidin, and captopril (mean \pm SD); * $p < 0.05$, *** $p < 0.001$.

KEY: Pregnant with saline delivery (PS), Pregnant with AVP delivery and saline (PAVPS), Pregnant with hesperidin delivery (PH), Pregnant with AVP delivery and hesperidin (PAVPH), Non-pregnant with hesperidin delivery (NH), Non-pregnant with AVP delivery and hesperidin (NAVPH), Pregnant with AVP delivery and captopril (PAVPC)

3.2. Effect of hesperidin on urine volume excretion

Our findings demonstrate a general decrease in urinary volume excretion in the PAVPS group (15 ± 4 ml; 11 ± 4 ml; 8 ± 1 ml) from GD 7 to GD 18 (Figure 2). In contrast, after treatment with hesperidin and captopril, an elevation was noted from GD 14 to GD 18 in the PAVPH, NAVPH, and PAVPC groups (Figure 2).

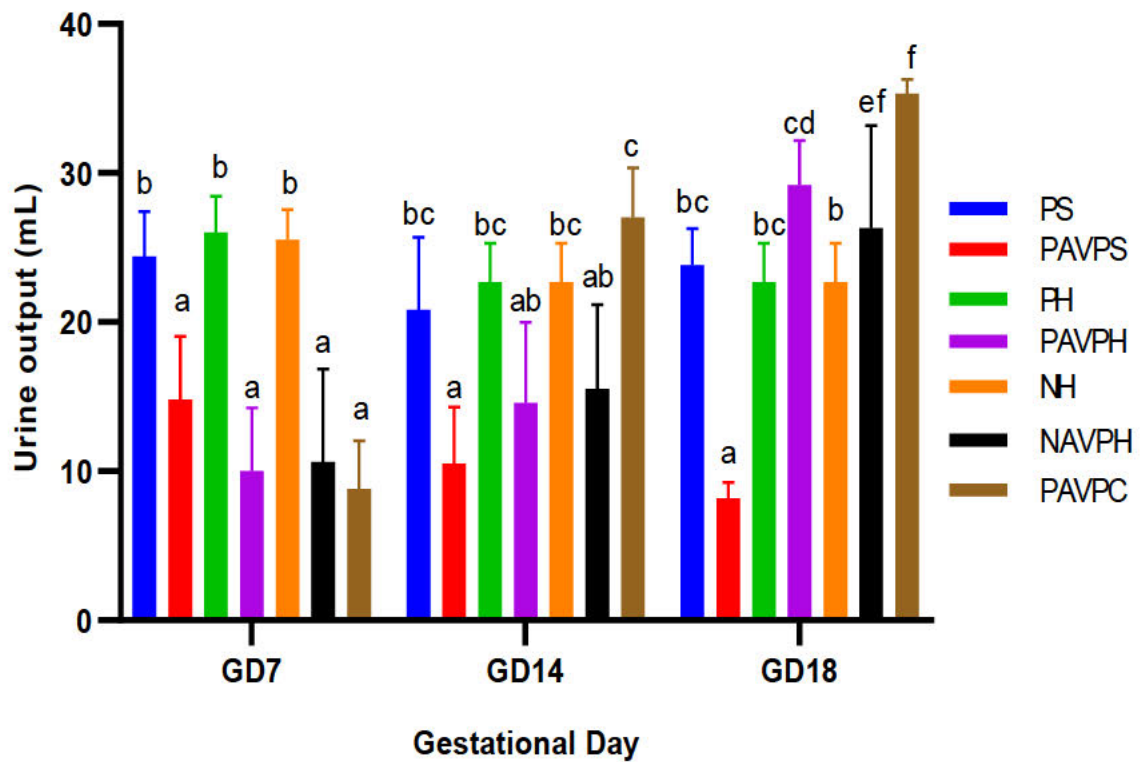
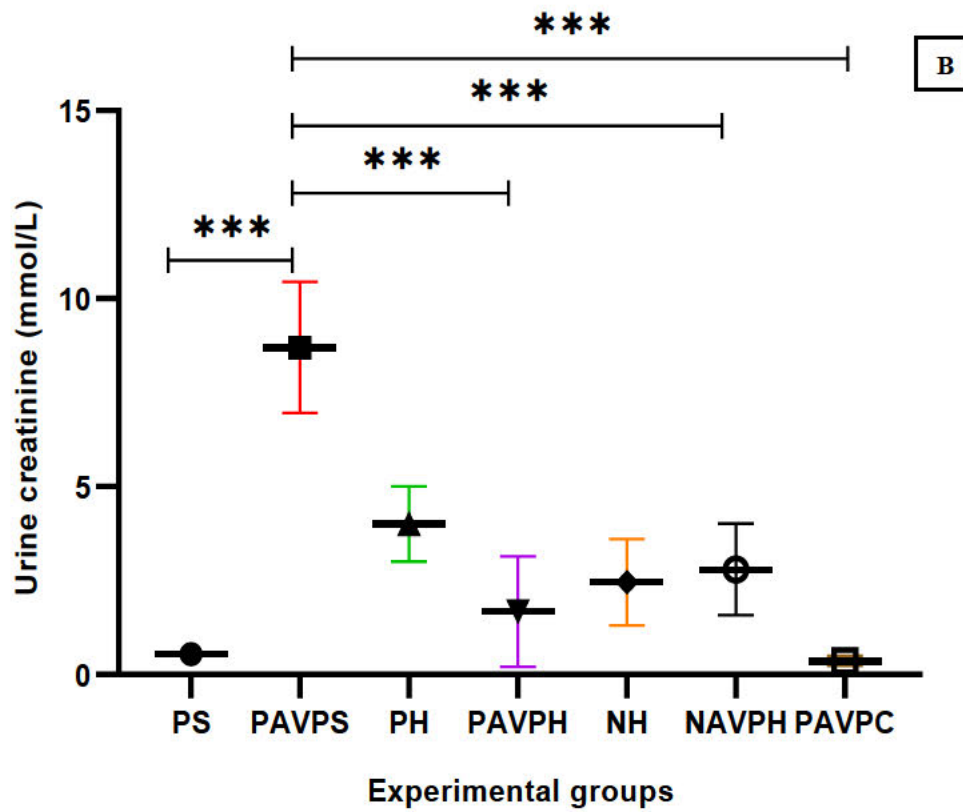
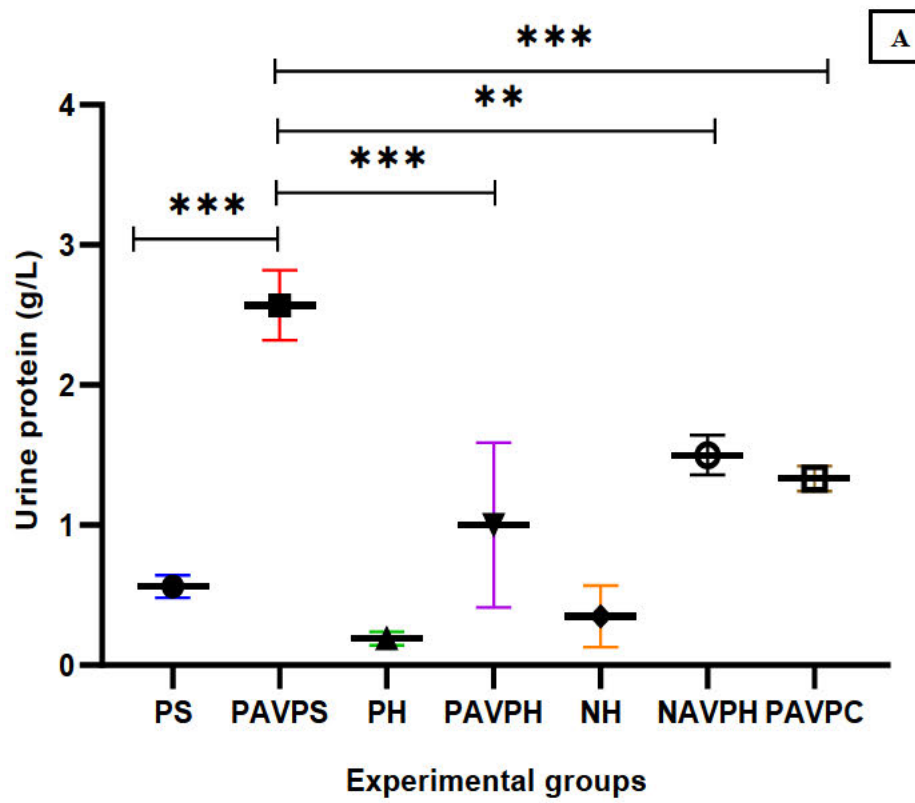


Figure 2: Urine output of all experimental groups on GD 7, 14 and 18 (mean \pm SD); ^{a-f} Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$).

KEY: Pregnant with saline delivery (PS), Pregnant with AVP delivery and saline (PAVPS), Pregnant with hesperidin delivery (PH), Pregnant with AVP delivery and hesperidin (PAVPH), Non-pregnant with hesperidin delivery (NH), Non-pregnant with AVP delivery and hesperidin (NAVPH), Pregnant with AVP delivery and captopril (PAVPC)

3.3. Effects of hesperidin on urine protein and creatinine levels

The urinary protein level of the PAVPS group (2.6 ± 0.3 g/L; $p < 0.001$) was significantly higher than the other experimental groups (Figure 3A). In contrast, a significant reduction in urinary protein levels was noted in the hesperidin and captopril-treated groups compared to the negative control group (PAVPH: 1.0 ± 0.6 g/L; $p < 0.001$; NAVPH: 1.5 ± 0.1 g/L; $p < 0.01$; PAVPC: 1.3 ± 0.2 g/L; $p < 0.001$). Urinary creatinine levels were significantly reduced in the PAVPH (1.7 ± 1.5 mmol/L), NAVPH (2.8 ± 1.2 mmol/L), and PAVPC (0.36 ± 0.1 mmol/L) groups compared to the PAVPS (8.7 ± 1.7 mmol/L) group ($p < 0.001$; Figure 3B). Furthermore, the urinary protein:creatinine ratio significantly ($p < 0.001$) increased in the PAVPS (4.3 ± 1.6 g/mmol) compared to the PS (1.0 ± 0.1 g/mmol) group. A significant ($p < 0.001$) decrease was noted in the PAVPH (0.91 ± 0.8 g/mmol), NAVPH (0.6 ± 0.2 g/mmol), and PAVPC (0.94 ± 0.1 g/mmol) groups compared to PAVPS (Figure 3C).



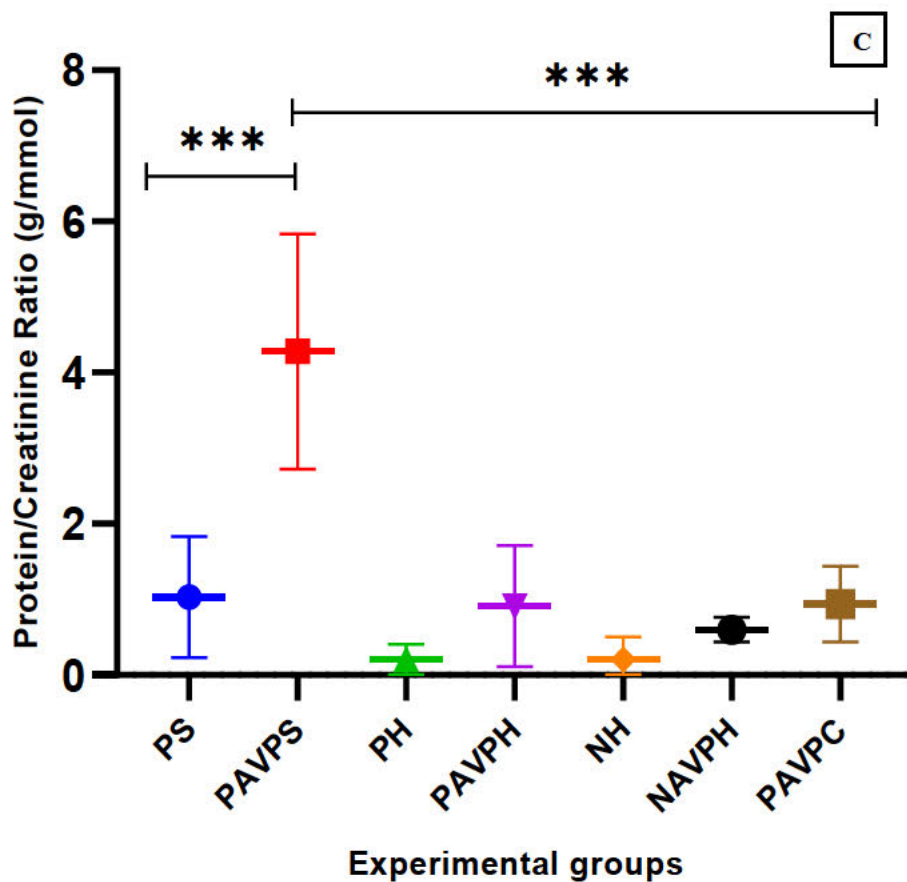


Figure 3: Urinary protein concentration (A), urine creatinine concentration (B) and urine protein: creatinine ratio levels (C) across all study groups (mean \pm SD), $**p < 0.01$, $***p < 0.001$.

KEY: Pregnant with saline delivery (PS), Pregnant with AVP delivery and saline (PAVPS), Pregnant with hesperidin delivery (PH), Pregnant with AVP delivery and hesperidin (PAVPH), Non-pregnant with hesperidin delivery (NH), Non-pregnant with AVP delivery and hesperidin (NAVPH), Pregnant with AVP delivery and captopril (PAVPC)

3.4. *Effects of hesperidin on placental and foetal outcomes*

A statistically significant difference ($p < 0.001$; Figure 4A) was noted in the placental weight of the PAVPS (0.45 ± 0.3 g) group compared to PS (0.68 ± 0.5 g), PH (0.71 ± 0.4 g), PAVPH (0.76 ± 0.4 g) and PAVPC (0.67 ± 0.3 g) groups. Similarly, the individual pup weight of the PAVPS (3.5 ± 0.4 g) group was significantly lower than the PS (5.4 ± 0.9 g), PH (5.5 ± 1.0 g), PAVPH (5.9 ± 0.2 g) and PAVPC (6.5 ± 0.1 g) groups, respectively. The number of pups in the PAVPS (9 ± 1) group was significantly lower than in PS (12 ± 1), PH (11 ± 1), PAVPH (14 ± 1), and PAVPC (12 ± 0) groups.

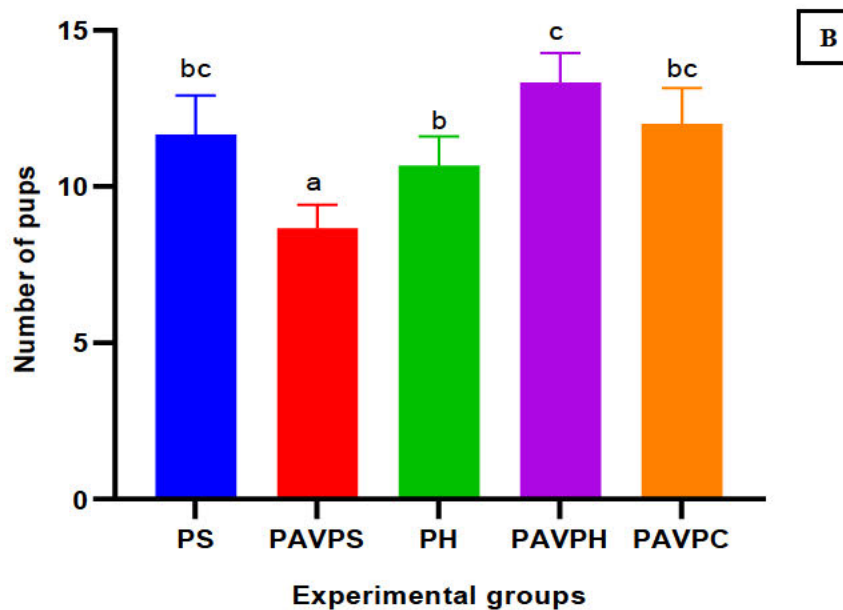
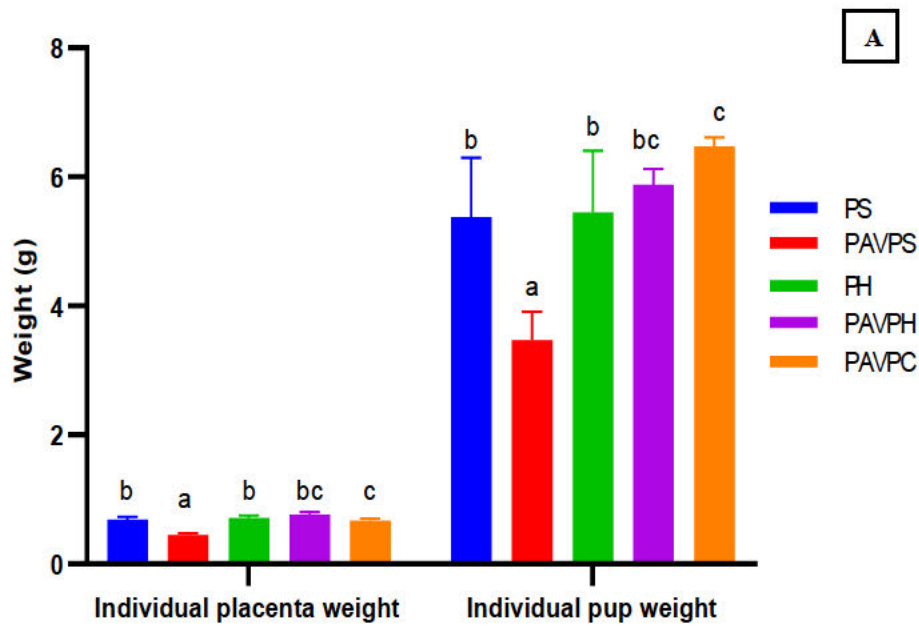


Figure 4: Placental and foetal parameters in pregnant Sprague-Dawley rats. Data is shown as mean \pm SD; ^{a-c} Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$).

KEY: Pregnant with saline delivery (PS), Pregnant with AVP delivery and saline (PAVPS), Pregnant with hesperidin delivery (PH), Pregnant with AVP delivery and hesperidin (PAVPH), Non-pregnant with hesperidin delivery (NH), Non-pregnant with AVP delivery and hesperidin (NAVPH), Pregnant with AVP delivery and captopril (PAVPC).

Table 1: Indices of hepatic, renal damage, and electrolyte levels across experimental groups on gestational day (GD) 18

	Study groups							p value
	PS	PAVPS	PH	PAVPH	NH	NAVPH	PAVPC	
Copeptin (pg/mL)	96.74 ± 5.8	653 ± 25.2 ^a	129 ± 43.0	557 ± 44.0	134 ± 18.1	427 ± 88.0	494 ± 89.1	0.000
Total protein (g/L)	47.5 ± 1.1 ^a	66.3 ± 0.5 ^{cbd}	46.0 ± 2.2	52.3 ± 3.1	43.0 ± 2.9	59.0 ± 1.6	58.5 ± 0.8	0.000
Albumin (g/L)	25.58 ± 2.9	33.40 ± 1.8	33.80 ± 1.66	25.98 ± 1.8 ^b	31.45 ± 0.8	31.20 ± 0.4	33.58 ± 3.5	0.011
AST (IU/L)	59.47 ± 4.7 ^a	117.33 ± 14.0 ^e	60.07 ± 2.07	63.40 ± 9.3 ^b	76.55 ± 2.7	90.40 ± 0.6	91.93 ± 8.2	0.000
ALT (IU/L)	46.80 ± 7.8 ^a	92.23 ± 5.5 ^{bcd}	55.94 ± 3.95	61.30 ± 12.4	49.48 ± 2.7	57.95 ± 5.6	54.97 ± 4.3	0.000
Sodium (mmol/L)	136 ± 1.8	137 ± 3.5	135 ± 1	138 ± 1.0	142 ± 1.2	142 ± 0.1	139 ± 1.2	0.001
Potassium (mmol/L)	5.23 ± 0.4	4.40 ± 0.2 ^c	5.54 ± 0.26	5.20 ± 0.3	5.05 ± 0.2	5.44 ± 0.2	5.47 ± 0.6	0.032
Chloride (mmol/L)	100 ± 2.3	95 ± 2.0 ^{cd}	97 ± 1	99 ± 0.2	100 ± 2.0	101 ± 1.0	101 ± 2.0	0.009
Bicarbonate (mmol/L)	29.47 ± 2.13	28.37 ± 4.1	25.53 ± 2.94	30.35 ± 1.9	31.48 ± 2.2	30.40 ± 1.7	30.85 ± 3.6	0.271
Urea (mmol/L)	4.86 ± 0.1 ^a	8.15 ± 0.3 ^{bcd}	5.05 ± 0.61	5.81 ± 0.5	5.02 ± 0.2	6.03 ± 0.02	5.48 ± 0.2	0.000
Uric acid (mmol/L)	0.12 ± 0.3	0.24 ± 0.4	0.14 ± 0.23	0.12 ± 0.4	0.13 ± 0.3	0.17 ± 0.2	0.16 ± 0.2	0.267

Data is shown as mean ± SD; *p < 0.05 was considered statistically significant, PS vs PAVPS^a; PAVPS vs PAVPH^b; PAVPS vs NAVPH^c; PAVPS vs PAVPC^d; NAVPH vs PAVPH^e. AST - aspartate aminotransferase and ALT - serum alanine transaminase.

KEY: Pregnant with saline delivery (PS), Pregnant with AVP delivery and saline (PAVPS), Pregnant with hesperidin delivery (PH), Pregnant with AVP delivery and hesperidin (PAVPH), Non-pregnant with hesperidin delivery (NH), Non-pregnant with AVP delivery and hesperidin (NAVPH), Pregnant with AVP delivery and captopril (PAVPC)

3.5. Effects of hesperidin on hepatic, renal damage, and electrolyte levels

Table 1 represents the serum biochemical parameters and electrolyte levels across experimental groups on GD 18. Copeptin levels were significantly increased in all AVP-treated experimental groups (PAVPS, PAVPH, NAVPH, and PAVPC; $p < 0.001$; Table 1) versus the non-AVP experimental groups (PS, PH, and NH; $p < 0.001$). The total protein levels were significantly higher in PAVPS compared to PAVPH, NAVPH and PAVPC groups. A significant reduction was noted in albumin levels for PAVPH versus PAVPS groups.

Aspartate aminotransferase levels were upregulated in the PAVPS compared to PS groups. A significant decrease in aspartate aminotransferase levels was noted in the PAVPH, NAVPH, and PAVPC groups compared to the PAVPS group (Table 1). Furthermore, significantly higher serum alanine transaminase levels were obtained in the PAVPS group compared to the PS ($p < 0.001$) group. In contrast, significantly lower serum alanine transaminase levels were observed in the PAVPH, NAVPH, and PAVPC groups than in the PAVPS group (Table 1).

Higher sodium levels were observed in the NH group compared to the PH group. Potassium levels were significantly increased in the NAVPH compared to the PAVPS groups. Chloride levels were significantly lower in the PAVPS compared to PS, NAVPH, and PAVPC groups. However, no significant difference was recorded for the bicarbonate levels between all experimental groups ($p > 0.05$; Table 1).

Urea levels were significantly higher in PAVPS compared to PS groups (Table 1). A decrease in urea levels was also noted in the PAVPH, NAVPH, and PAVPC groups relative to the control. An increase in uric acid levels was seen in the PAVPS compared to PS, PAVPH and NAVPH groups, albeit, not significant.

3.5. Effects of hesperidin on haematological parameters

The haematological parameters across all study groups for GD 18 are shown in Table 2. A significantly higher red blood cell count was observed in the PAVPS group compared to the PS and PAVPH groups. Higher red blood cell counts were also observed in the NAVPH group relative to the PS group. A statistically significant difference was also documented for red blood cell counts in the NAVPH compared to PAVPH groups.

Haematocrit levels were significantly higher in PAVPS compared to PS, PAVPH and PAVPC groups. A considerably higher red cell distribution width was noted in the PAVPS versus NAVPH groups. A lack of statistically significant difference was reported for the mean corpuscular haemoglobin concentration, mean corpuscular volume and platelets ($p > 0.05$; *Table 2*).

Our findings also demonstrate significantly lower haemoglobin counts in the PS, PAVPH, NAVPH and PAVPC groups compared to the PAVPS group. A substantially higher mean corpuscular haemoglobin was noted for the PAVPS group compared to PS and PAVPC groups (*Table 2*).

Moreover, the white blood cell count was significantly higher in the PAVPH and NAVPH compared to the PAVPS groups. The neutrophil levels were significantly higher in the PAVPS compared to the PAVPH group. A significant decrease in lymphocyte levels was noted in the PAVPS group compared to the PAVPH and NAVPH groups. Similarly, a significant increase was observed in monocyte levels in the PAVPS compared to the PS groups. A lack of statistically significant difference was reported for eosinophils and basophils ($p > 0.05$; *Table 2*).

Table 1: Indices of hepatic, renal damage, and electrolyte levels across experimental groups on gestational day (GD) 18

	Study groups							p value
	PS	PAVPS	PH	PAVPH	NH	NAVPH	PAVPC	
Copeptin (pg/mL)	96.74 ± 5.8	653 ± 25.2 ^a	129 ± 43.0	557 ± 44.0	134 ± 18.1	427 ± 88.0	494 ± 89.1	0.000
Total protein (g/L)	47.5 ± 1.1 ^a	66.3 ± 0.5 ^{cbd}	46.0 ± 2.2	52.3 ± 3.1	43.0 ± 2.9	59.0 ± 1.6	58.5 ± 0.8	0.000
Albumin (g/L)	25.58 ± 2.9	33.40 ± 1.8	33.80 ± 1.66	25.98 ± 1.8 ^b	31.45 ± 0.8	31.20 ± 0.4	33.58 ± 3.5	0.011
AST (IU/L)	59.47 ± 4.7 ^a	117.33 ± 14.0 ^e	60.07 ± 2.07	63.40 ± 9.3 ^b	76.55 ± 2.7	90.40 ± 0.6	91.93 ± 8.2	0.000
ALT (IU/L)	46.80 ± 7.8 ^a	92.23 ± 5.5 ^{bcd}	55.94 ± 3.95	61.30 ± 12.4	49.48 ± 2.7	57.95 ± 5.6	54.97 ± 4.3	0.000
Sodium (mmol/L)	136 ± 1.8	137 ± 3.5	135 ± 1	138 ± 1.0	142 ± 1.2	142 ± 0.1	139 ± 1.2	0.001
Potassium (mmol/L)	5.23 ± 0.4	4.40 ± 0.2 ^c	5.54 ± 0.26	5.20 ± 0.3	5.05 ± 0.2	5.44 ± 0.2	5.47 ± 0.6	0.032
Chloride (mmol/L)	100 ± 2.3	95 ± 2.0 ^{cd}	97 ± 1	99 ± 0.2	100 ± 2.0	101 ± 1.0	101 ± 2.0	0.009
Bicarbonate (mmol/L)	29.47 ± 2.13	28.37 ± 4.1	25.53 ± 2.94	30.35 ± 1.9	31.48 ± 2.2	30.40 ± 1.7	30.85 ± 3.6	0.271
Urea (mmol/L)	4.86 ± 0.1 ^a	8.15 ± 0.3 ^{bcd}	5.05 ± 0.61	5.81 ± 0.5	5.02 ± 0.2	6.03 ± 0.02	5.48 ± 0.2	0.000
Uric acid (mmol/L)	0.12 ± 0.3	0.24 ± 0.4	0.14 ± 0.23	0.12 ± 0.4	0.13 ± 0.3	0.17 ± 0.2	0.16 ± 0.2	0.267

Data is shown as mean ± SD; *p < 0.05 was considered statistically significant, PS vs PAVPS^a; PAVPS vs PAVPH^b; PAVPS vs NAVPH^c; PAVPS vs PAVPC^d; NAVPH vs PAVPH^e. AST - aspartate aminotransferase and ALT - serum alanine transaminase.

KEY: Pregnant with saline delivery (PS), Pregnant with AVP delivery and saline (PAVPS), Pregnant with hesperidin delivery (PH), Pregnant with AVP delivery and hesperidin (PAVPH), Non-pregnant with hesperidin delivery (NH), Non-pregnant with AVP delivery and hesperidin (NAVPH), Pregnant with AVP delivery and captopril (PAVPC),

Table 2: Haematological parameters across experimental groups on gestational day (GD) 18.

	Study groups							P-VALUE
	PS	PAVPS	PH	PAVPH	NH	NAVPH	PAVPC	
Red cell count (10¹²/L)	6.46 ± 0.2 ^a	7.99 ± 0.9 ^b	6.43 ± 0.2	6.44 ± 0.3 ^e	6.39 ± 0.3	8.11 ± 0.7	7.12 ± 0.5	0.000
Haematocrit (%)	41.94 ± 1.7 ^a	54.30 ± 2.6 ^{bd}	40.08 ± 1.1	41.52 ± 2.2 ^e	44.12 ± 2.2	51.30 ± 4.5	43.50 ± 2.3	0.000
RDW (%)	12.68 ± 0.4	14.76 ± 1.1	12.80 ± 0.3	12.77 ± 0.5 ^e	12.03 ± 1.1	12.07 ± 0.6 ^c	13.97 ± 1.0	0.000
MCHC (g/dL)	28.56 ± 0.1	27.97 ± 0.4	29.02 ± 0.4	28.50 ± 0.2	28.95 ± 0.9	27.54 ± 2.5	28.52 ± 0.4	0.366
MCV (fL)	63.46 ± 1.8	66.92 ± 8.1	62.36 ± 1.9	64.53 ± 1.6	62.87 ± 2.5	62.29 ± 0.5	62.42 ± 2.5	0.330
Platelets (10⁹/L)	1099 ± 94	1011 ± 279	1156 ± 89	1043 ± 123	1074 ± 102	1093 ± 153	1301 ± 244	0.644
Haemoglobin (g/dL)	11.98 ± 0.5 ^a	15.85 ± 0.3 ^{cbd}	11.64 ± 0.4	11.83 ± 0.6 ^e	13.83 ± 0.8	13.63 ± 1.0	12.40 ± 0.6	0.000
MCH (pg)	17.93 ± 0.2 ^c	19.27 ± 0.05 ^d	17.80 ± 0.3	18.31 ± 0.4	17.73 ± 0.8	17.87 ± 0.3	17.56 ± 0.8	0.009
White cell count (10⁹/L)	4.14 ± 0.5	3.59 ± 0.8 ^{cb}	3.46 ± 0.8	7.64 ± 0.3	4.13 ± 1.0	5.16 ± 0.9	3.69 ± 1.3	0.000
Neutrophils %	28.16 ± 5.3	31.00 ± 4.2 ^c	23.80 ± 1.3	29.79 ± 3.2 ^{ce}	8.40 ± 2.3	14.33 ± 4.6	25.38 ± 1.2	0.000
Lymphocytes %	63.78 ± 1.8 ^a	59.46 ± 7.8 ^{bc}	62.09 ± 4.2	87.56 ± 2.3	65.20 ± 10.5	74.98 ± 4.3	65.72 ± 4.4	0.000
Monocytes %	3.48 ± 0.9 ^a	11.90 ± 0.7 ^d	8.50 ± 3.9	6.03 ± 3.7	2.00 ± 0.5	7.81 ± 3.7	4.38 ± 3.0	0.004
Eosinophils %	0.78 ± 0.51	1.57 ± 0.82	1.22 ± 0.41	1.09 ± 0.50	1.53 ± 0.67	1.64 ± 0.65	1.43 ± 0.55	0.303
Basophils %	0.18 ± 0.11	0.18 ± 0.11	0.20 ± 0.11	0.26 ± 0.21	0.37 ± 0.28	0.29 ± 0.15	0.12 ± 0.13	0.392

Data is shown as mean ± SD. * $p < 0.05$ was considered statistically significant, PS vs PAVPS^a; PAVPS vs NAVPH^c; PAVPS vs PAVPH^b; PAVPS vs PAVPC^d; NAVPH vs PAVPH. RDW - red cell distribution width, MCHC - mean corpuscular haemoglobin concentration, MCV - mean corpuscular volume, MCH – mean corpuscular hemoglobin
KEY: Pregnant with saline delivery (PS), Pregnant with AVP delivery and saline (PAVPS), Pregnant with hesperidin delivery (PH), Pregnant with AVP delivery and hesperidin (PAVPH), Non-pregnant with hesperidin delivery (NH), Non-pregnant with AVP delivery and hesperidin (NAVPH), Pregnant with AVP delivery and captopril (PAVPC)

4. Discussion

Our findings demonstrate that hesperidin (200 mg/kg.b.w) administration for 14 days alleviated the AVP-induced increase in blood pressures associated with pre-eclampsia, improving maternal and foetal outcomes. The infusion of AVP (200 ng/h) resulted in significantly elevated blood pressure and urinary protein, characterising pre-eclampsia symptoms. Both systolic and diastolic blood pressures were elevated considerably throughout pregnancy in the PAVPS group in contrast to the PS group (Figure 1), similar to an earlier report from our lab (Ramdin et al., 2022), albeit at a lower dosage of 150 ng/h. The AVP-induced pre-eclamptic rats treated with hesperidin over 14 days significantly decreased blood pressure. Significant systolic and diastolic blood pressure reductions were reported in the treated groups on gestational days 14 and 18 following treatments with hesperidin. An earlier study reported that treatment with hesperidin for four weeks significantly suppressed age-related increase in blood pressure in spontaneously hypertensive rats (Ikemura et al., 2012). Similarly, spontaneously hypertensive rats administered with hesperidin-rich diets for more than 15 weeks also significantly decreased blood pressure. A more recent study reported that hesperidin reduced blood pressure in a dose-dependent manner and increased plasma ACE activity and angiotensin II levels in two-kidney, one-clipped (2K-1C) hypertensive rats (Wunpathe et al., 2018).

We report significantly reduced urinary outputs in PAVPS rats compared to the PS rats (Figure 2). The reduced urinary output may be attributed to AVP's antidiuretic effect *via* V2 receptor activation and increased expression of aquaporin-2 channels, which results in water retention and consequent decreased urinary output (Guelinckx et al., 2016). We further demonstrate a significant increase in urinary production following treatment with hesperidin. Interestingly, our results are corroborated by an earlier study conducted by Galati et al. (1996), who demonstrated significant anti-hypertensive and diuretic effects of hesperidin in rats following oral administration of the drug at a dose of 200 mg/kg body weight and ascribed this hypotensive effect to increased diuresis (Galati et al., 1996).

Although proteinuria is no longer considered a diagnostic indicator of pre-eclampsia (Gynecologists, 2013), it remains an integral diagnostic predictor of the development of pre-eclampsia (Guida et al., 2018; Özkara et al., 2018; Tanacan et al., 2019). We demonstrate significantly increased urinary protein and creatinine levels in the PAVPS group, followed by decreased urinary protein and urine creatinine levels after treatment with hesperidin (Figure 3A and 3B). Furthermore, a significantly increased urine protein: creatinine ratio was shown in the PAVPS compared to the PS groups. Interestingly, a significant decrease was noted in the

hesperidin AVP-treated groups compared to the PAVPS group (Figure 3C). These findings suggest that hesperidin can ameliorate the progression of pre-eclampsia symptoms and its related kidney dysfunction by reducing proteinuria.

Abnormal placentation in pre-eclampsia results in decreased placental perfusion, consequent hypoxia, and foetal growth restriction (Cheng and Wang, 2009; Cotechini et al., 2014). We demonstrate significantly lower individual placental and pup weights in the PAVPS versus the PS groups (Figure 4A). Furthermore, the number of pups were markedly lower in the PAVPS compared to the PS groups (Figure 4B). The smaller placentae may be associated with reduced uteroplacental blood flow due to vasoconstriction of spiral arterioles, which directly influences foetal growth reflecting human pre-eclampsia (Ferrazzani et al., 2011). We report a significant increase in the weight of the placenta in the hesperidin-treated groups, as well as a significant increase in the pup weight and the number of pups in the hesperidin-treated groups. Our results suggest that hesperidin can be used as a treatment in pre-eclampsia-induced foetal growth restriction, as we reveal significant beneficial effects of foetal weight gain.

Copeptin, a stable protein by-product of AVP synthesis released in a 1:1 ratio with AVP, is a biomarker for measuring AVP secretion (Dobša and Cullen Edozien, 2013). Our study shows a significant increase in copeptin levels in all AVP-treated groups, and non-significantly lower copeptin levels were noted in the hesperidin-treated groups. AVP stimulates the renin-angiotensin-aldosterone system (RAAS), which causes vasoconstriction, which is mediated by the V1a receptor and, as a result, increases peripheral resistance and systemic blood pressure, as observed in our study (Qian, 2018). The significant increase in copeptin suggests that hesperidin could act as a vasopressin V1a receptor antagonist, resulting in vasodilation and thereby decreasing blood pressure.

Approximately 3% of pregnancies are affected by liver diseases which result in maternal and foetal mortality (Mikolasevic et al., 2018). As a result, liver function tests are critical indicators of liver dysfunction, such as intrahepatic cholestasis of pregnancy or acute fatty liver of pregnancy, which manifests in late pregnancy (Mikolasevic et al., 2018). These liver injuries are typical in severe cases of pre-eclampsia patients who present with HELLP syndrome (Brown et al., 2018). We observed significantly higher serum alanine transaminase and aspartate aminotransferase levels in the PAVPS compared to the PS-treated groups. The depleted serum level of both liver function enzymes in normal and hesperidin-treated groups suggests the protection of hepatocellular injury and the safety of the compound in healthy rats.

In our study, we notice a decrease in the serum concentrations of potassium and chloride in the PAVPS group, which propose a dysregulation in their transport across the vascular smooth-muscle cell membrane (Indumati et al., 2011). Elevated serum urea and uric acid have been linked with the development of nephropathy (Hovind et al., 2011). We demonstrate an increase in urea levels in the PAVPS group compared to the treated groups. The increased serum level of urea and acid were ameliorated with the treatment of hesperidin which further supports the ability of hesperidin to abate the progression of pre-eclampsia complications.

We note significantly elevated red blood cell counts and haematocrit levels in the PAVPS group. AVP promotes the proliferation and differentiation of red blood cell precursors, which justifies increased red blood cell counts and haematocrit levels (Mayer et al. 2017). Elevated haematocrit and red blood cell levels result in an upsurge of blood viscosity and peripheral resistance, leading to increased blood pressure (Emamian et al. 2017). Recently red cell distribution width has been associated with hypertension and many other cardiovascular risk factors (Kurt et al., 2015). Furthermore, high red cell distribution width levels are believed to reflect increased inflammation (Kurt et al., 2015). We report a higher red cell distribution width percentage in the untreated PAVPS group, which correlates with an earlier study that revealed that red cell distribution width was associated with the presence and the severity of pre-eclampsia (Kurt et al., 2015). Despite the non-significant differences in platelet counts, we note a decreased level in the PAVPS group compared to the other groups. Reduced platelet count is associated with developing HELLP syndrome in severe pre-eclampsia, characterised by haemolysis, elevated liver enzyme, and low platelet count (Weiner et al. 2016). We note increased haemoglobin and mean corpuscular haemoglobin levels in the untreated control group. An earlier study suggested that an increased free haemoglobin concentration was the cause of vasoconstriction in pre-eclampsia (Sarrel et al., 1990). Furthermore, our findings are supported by a study that showed that women with high haemoglobin concentration carried an increased risk of pregnancy-induced hypertension (Aghamohammadi et al., 2011).

We report higher levels of neutrophils in the untreated PAVPS group. Earlier evidence suggests that neutrophils are activated in the placental bed and maternal circulation of women with pre-eclampsia (Butterworth et al., 1991; Greer et al., 1989; Sacks et al., 1998). Furthermore, neutrophil activation is associated with a free radical release that either can affect endothelial function directly or contribute indirectly through the production of lipid peroxides (Sacks et al., 1998). We report elevated white blood cell and lymphocyte levels in the hesperidin-treated groups; however, a significantly lower level in the untreated group. The significant increase in

the white blood cell and lymphocytic count caused by hesperidin reflects the compound's leukopoietic and possible immunomodulatory effects (Berinyuy et al., 2015).

5. Conclusion

These results indicate the ability of hesperidin to improve blood pressure and reduce proteinuria, thus improving maternal and foetal outcomes. The pharmacological action of hesperidin in ameliorating liver dysfunction and improving haematological parameters suggests its promising use in treating pre-eclampsia. However, this plant-derived compound must be effectively studied at the clinical level to establish its usefulness in treating and preventing pre-eclampsia and other hypertensive disorders of pregnancy.

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CRedit authorship contribution statement

Conceptualization: SB and NG; methodology: SS; Experimental work, analysis, and original draft preparation: RR; Review and editing: SB, RM, TN, and NG. All authors have read and agreed to the final version of the manuscript.

Declaration of Competing interest

The authors declare no conflict of interest

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CHAPTER 6

SYNTHESIS

Globally, PE affects up to 8% of pregnancies (1), with a higher prevalence in low and middle-income countries (2). The exact aetiology of PE remains unknown; however, its pathogenesis is associated with reduced trophoblast invasion, non-physiological transformation of spiral arteries with subsequent placental perfusion, and fetal hypoxia (3). These physiological anomalies result in the onset of clinical manifestations of hypertension and maternal organ injury. There is still no cure for PE, nonetheless, current management strategies aim to lower blood pressure and alleviate maternal and neonatal complications (4).

Synthetic antihypertensive agents are widely used for the management of hypertension in pregnancy; however, their use is accompanied by various adverse side effects (5-7). Considering this, alternative treatments effective in reducing the development and progression of hypertension and its symptoms in pregnancy are necessary.

Indigenous plants are widely recognised as valuable sources of medicinal compounds, whose healing properties are rooted in ancient times (8). Traditional medicine remains the central core of healthcare service in Africa due to the low socioeconomic status of almost 80% of its vulnerable population (9, 10). Furthermore, the bioactive compounds found in medicinal plants may be therapeutically effective and perhaps coexist as precursors and/or ground-breakers for drug synthesis in allopathic medicine (12).

Noteworthy, bioactive compounds isolated from medicinal plants also exhibit antioxidant, anti-inflammatory, and anti-hypertensive properties, which may be beneficial for the development of new and more accessible pharmaceutical drugs in treating HDPs such as PE (11). In view of this, *in vivo* studies are indispensable in establishing the clinical effectiveness of plant extracts and phytochemicals to validate their use in traditional medicine. Thus, understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of the bioactive extracts or molecules from medicinal plants that have biological activity is warranted (2). The overall findings of this study are summarised below and schematically illustrated in Fig. 6.1.

Paper 1 (Associated with Chapter 2: Section B) - South African medicinal plants displaying angiotensin-converting enzyme inhibition: potential application in the management of pre-eclampsia

Published in *Journal of Ayurveda and Integrative Medicine* (Impact factor: 1.28)

This chapter is divided into sections A and B. Section A provides a detailed literature review relating to the pathogenesis of PE development, its association with the Renin-Angiotensin-Aldosterone system, the use of animal models for the study of PE, treatment/management of PE as well as the use of medicinal plants and phytochemicals as alternatives to synthetic drugs. Section B consists of a peer-reviewed manuscript (Chapter 2) which provides an extensive review of indigenous medicinal plants with ACE inhibitory activity in South Africa and outlines their potential use in the treatment of hypertension in pregnancy. We also provide evidence on the side effects of current synthetic anti-hypertensive drugs and their side effects.

To-date, ACEIs have proven to be valuable in the management of hypertensive disorders, however, these drugs present several limitations such as reduced efficacy with prolonged use, various adverse side effects, and teratogenic effects if used during the last two trimesters of pregnancy (12). Based on the side effects associated with the use of synthetic anti-hypertensive drugs, we propose the need for safer alternatives. Over 3000 plants are currently utilized in the treatment and management of numerous illnesses by several traditional healers and more than 100 plant species have been documented to treat hypertension traditionally in South Africa (13). We advocate the use of phytotherapy as an alternative source of treatment and for the development of new therapeutic compounds for the management of HDPs.

Manuscript 2 (Chapter 3: Aligned with objectives 1 and 2) - Antioxidant and angiotensin-converting enzyme inhibitory potential of South African traditional medicinal plants and plant-derived compounds.

Published in *South African Journal of Botany* (Impact factor: 3.1)

Chapter 3 provides a comprehensive evaluation of the antioxidant and ACE inhibitory activities of selected medicinal plants and their major secondary metabolites indigenous to South Africa. Given the crucial role that oxidative stress plays in the etiology of human hypertension development, it is more beneficial for an antihypertensive drug to have an antioxidant effect. We, therefore, evaluated the antioxidant and ACE inhibition activity of six plant extracts and six phytochemicals. Our findings demonstrate free radical scavenging activity for all plant

extracts, with *D. rotundifolia* showing the most vigorous activity. Furthermore, oleanolic acid, 3 β -taraxerol and β -amyryn were shown to possess intense free radical scavenging activity in this study. All plants and compounds tested, exerted counteractive effects on NO formation which may help reduce the adverse physiological effects introduced by excess NO production. These findings are important as therapies targeting free radicals by lowering reactive nitrogen species or ROS production may help reduce vascular injury therefore preventing hypertensive end-organ damage. Our data demonstrated a >50% ACE inhibition activity in five methanol plant extracts (*A. afra*, *C. anisata*, *D. rotundifolia*, *R. chirindensis* and *S. birrea*), hesperidin, and nicotinamide. Our findings may pave the way for new natural ACE inhibitory alternatives.

Manuscript 3 (Chapter 4: Aligned with objective 3) - Mating Success of Timed Pregnancies in Sprague Dawley Rats: Considerations for Execution.

Published in *Reproductive Biology*: (Impact factor: 2.1)

Rodent models are an essential tool in our understanding of human pregnancy and pregnancy-related disorders. However, establishing a precise gestational day 0 in experimental animals, with an adequate sample size for human pregnancy correlation, is a challenge for researchers (14). This chapter provides recommendations for timed-pregnancy experiments to achieve an appropriate sample size for the study of human pregnancy disorders.

Our findings show that the implementation of LB and Whitten effect mating phenomena, combined with a 1 female:1 male ratio, and a 48-hour mating period are effective indicators for a high pregnancy success rate in Sprague-Dawley rats. Based on the outcomes, we offer practical considerations to support the planning and execution of timed-pregnancy studies with a high pregnancy success rate, making such experiments less laborious and reducing animal wastage.

Manuscript 4 (Chapter 5: Aligned with objectives 4 and 5) - Hesperidin improves physiological outcomes in an arginine vasopressin rodent model of pre-eclampsia

Accepted in the *Journal of Fundamental and Clinical Pharmacology*: (Impact factor: 2.9)

This chapter provides a complete physiological report of hesperidin for the treatment of PE. Our findings demonstrate that hesperidin administration alleviated the AVP-induced high blood pressures associated with PE and improved maternal and foetal outcomes. We demonstrated an increase in urinary production following treatment with hesperidin which suggest its hypotensive effects could be attributed to its diuretic effects. We also demonstrate

significantly decreased urinary protein and urine creatine levels after treatment with the phytocompound which suggests that hesperidin may ameliorate the progression of PE symptoms and its related kidney dysfunction by reducing proteinuria.

Abnormal placentation in PE results in decreased placental perfusion, consequent hypoxia, and foetal growth restriction (15, 16). We report a significant increase in the weight of the placenta in the hesperidin-treated groups, as well as a significant increase in the pup weight and the number of pups in the hesperidin-treated groups suggesting that hesperidin can ameliorate PE-induced foetal growth restriction.

The decrease in copeptin levels in treatment groups suggests that hesperidin could act as a vasopressin V1a receptor antagonist resulting in vasodilation and thereby decreasing blood pressure. The depleted serum level of both liver function enzymes in hesperidin-treated groups suggests the protection against hepatocellular injury. The increased serum level of urea and acid were ameliorated with the treatment of hesperidin which further supports the ability of hesperidin to abate the progression of PE complications. Furthermore, the significant increase in the white blood cell and lymphocytic count caused by hesperidin reflects the compound's leukopoietic and possible immunomodulatory effects (17).

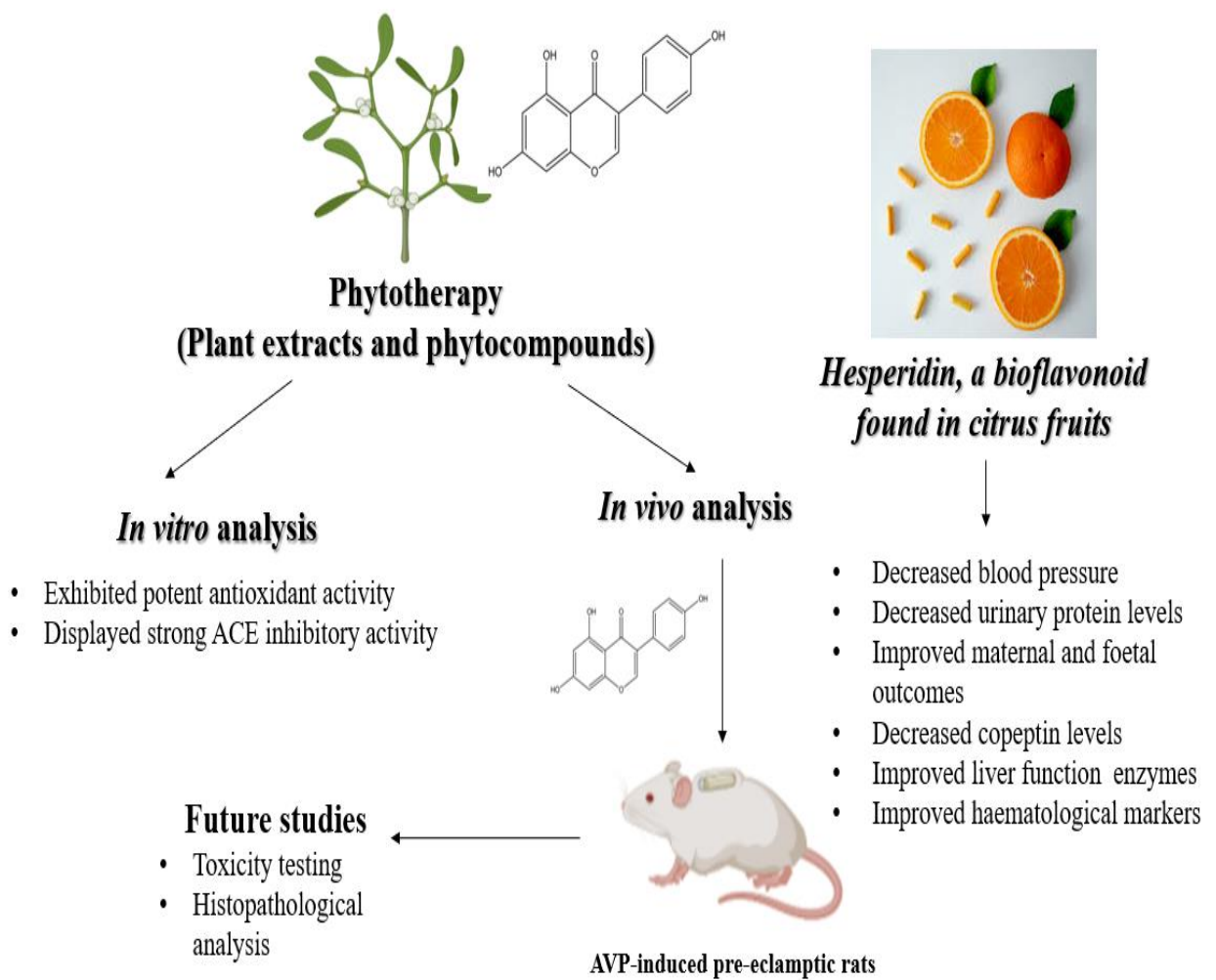


Figure 7.1: Schematic diagram outlining the main overall findings of this study.

Conclusion

Phytotherapy in the treatment of various disorders has been rediscovered due to the adverse side effects of current synthetic drugs. Our findings revealed that the medicinal plant, *D. rotundifolia*, and phytochemicals, hesperidin, and nicotinamide demonstrated significant ACE inhibitory and antioxidant potential. The ACE inhibition activity of Hesperidin was comparable to Captopril highlighting its antihypertensive effect. Furthermore, hesperidin effectively improved PE phenotype *in vivo* (Figure 7.1). Our findings, therefore, support the ethnopharmacological use of hesperidin in the management of PE. This finding is especially significant for LMICs where conventional ACEI drugs are inaccessible due to financial constraints and has great potential to reduce the maternal and fetal mortality rates thus ensuring that Africa can meet the global sustainable development goal targets by 2030. Furthermore, we provide a workflow for the evaluation of other phytochemicals that can be used in the treatment of HDPs.

Recommendations and future studies

Nevertheless, additional investigations are required to elucidate its effect on body organs, biochemical profiles, and relevant gene expressions *in vivo* to determine its comprehensive effect on PE management. The following recommendations must be considered to further understand the use of hesperidin in the treatment and management of hypertension, especially in pregnancy.

1. Conduct a complete histopathological analysis on kidney, cardiac and placental tissues to confirm our findings of the protective effect of hesperidin.
2. Evaluate the safety of hesperidin on pregnancy-induced hypertension by conducting the relevant toxicity studies
3. Conduct *in vivo* studies investigating the antihypertensive effect of hesperidin in other *in vitro* and *in vivo* models of PE to fully understand the integrative pathways in the pathogenesis and treatment of PE.

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APPENDICES

APPENDIX 1- ETHICS APPROVAL



07 September 2020

Ms Rebecca Reddy
Department of Basic Medical Sciences
Ritson Campus
Durban University of Technology

Dear Ms Reddy,

Protocol reference number: AREC/015/020D

Project title: Evaluation of selected South African Medicinal Plants in the treatment of pre-eclampsia.

Full Approval – Research Application

With regard to your revised application received on 25 August 2020, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 06 September 2021.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....
Dr Sanil D Singh, PhD
Chair: Animal Research Ethics Committee

/kr
cc Supervisor: Dr Roshila Moodley (UKZN)
cc Supervisor: Dr Nalini Govender (DUT)
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