# PHYTOCHEMICAL COMPOSITION, ANTINOCICEPTIVE, ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES OF *Fagaropsis angolensis* AND *Fagaropsis hildebrandtii*

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**May 2025**

## DECLARATION

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## DEDICATION

I dedicate this work to my children Rachael Maloba, Ryan Maloba and Rylan Maloba and my wife Sydney for giving me the reason to pursue with this work.

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## ABSTRACT

*Fagaropsis angolensis* and *Fagaropsis hildebrandtii* have been widely used in African traditional medicine to treat various illnesses, particularly those associated with pain, inflammation and oxidative stress. However, experimental data on the antinociceptive, anti-inflammatory and antioxidant activities of *F. angolensis* leaves, and *F. hildebrandtii stem* bark was still limited. The objectives of this study were to evaluate the antinociceptive, anti-inflammatory, antioxidant activities and phytochemical composition of *F. angolensis* leaves and *F. hildebrandtii* stem bark through an experimental controlled design. The study area was Embu and Makueni Counties. Extraction was done by maceration using water and methanol solvents. Increasing concentrations of the extracts (2-250 mg/Kg) were then evaluated for antinociceptive and anti-inflammatory activities using acetic acid-induced writhing and carrageenan-induced paw edema animal models, respectively, in comparison with aspirin 150 mg/kg and dexamethasone 10 mg/kg as standards. Antioxidant activity was assayed by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method, against standard ascorbic acid at 1mg/1ml. Standard qualitative methods were used for preliminary analysis of phytochemicals. Later, total phenolic and total flavonoid content was determined by Folin-Ciocalteu and Aluminium chloride calorimetric methods, respectively. Data was analyzed by one-way/two-way ANOVA followed by Tukey's post hoc test for multiple comparison using GraphPad Prism version 7. Statistical significance level was set at $p \leq 0.05$. Results of the percentage writhing inhibition did not show any significant difference between tested doses of both *F. angolensis* and *F. hildebrandtii* methanolic extract at 250 mg/kg and aspirin (81.95 ± 10.76% vs. 84.99 ± 3.26% and 80.24 ± 12.59 % vs. 84.99 ± 3.26%; $p>0.05$), respectively, indicative of potent *in vivo* anti-nociceptive activity. Similarly, there was no significant difference in percentage paw edema inhibition between all the *F. angolensis* and *F. hildebrandtii* extract doses and dexamethasone standard at 10 mg/kg ($p>0.05$), indicative of potent *in vivo* anti-inflammatory activity. Altogether, these results indicate that *F. angolensis* leaves, and *F. hildebrandtii* stem bark have good antinociceptive and anti-inflammatory activity at maximum concentration. *F. angolensis* and *F. hildebrandtii* water and methanol extracts exhibited potent antioxidant activity, as demonstrated by the comparable percentage radical scavenging activity to ascorbic acid ($p>0.05$) and a much lower $IC_{50}$ of 0.01 µg/ml vs. 0.90 µg/ml and 0.98 µg/ml vs. 0.987 µg/ml, respectively compared to 5.674 µg/ml for ascorbic acid. Qualitatively, phenols, steroids, flavonoids, alkaloids and coumarins were present in all the extracts of *F. angolensis* leaves and *F. hildebrandtii* stem bark. However, anthocyanins were absent in both water and methanolic extracts of both plants. *F. angolensis* methanol and water extract had significantly higher total phenolic content of 55.523±3.050 mgGAE/g vs. 48.00±0.185 mgGAE/g ($p<0.05$) while flavonoid content was 172.53±7.095 mgCE/g vs. 42.225±0.10 mgCE/g ($p<0.05$). *F. hildebrandtii* showed significantly high total phenolic levels in methanol extract compared to water extract (34.590 ± 2.490 mgGAE/g vs. 7.893 ± 1.619 mgGAE/g and total flavonoid content of 132.18 ± 0.26 mgCE/g vs. 42.68 ± 0.93 mgCE/g ($p<0.05$). These high levels of phenols and flavonoids might account for the observed antinociceptive, anti-inflammatory and antioxidant activities of the plant parts studied. The study confirmed that both leaves and stem bark of *F. angolensis* and *F. hildebrandtii* used in Embu and Makueni Counties respectively, have antinociceptive, anti-inflammatory and antioxidant activities. Study recommends plant preservation, active pharmaceutical ingredient identification, testing in non-human primates and in clinical trials.

## TABLE OF CONTENT

**TITLE PAGE** ..... **i**

**DECLARATION** ..... **ii**

**COPYRIGHT** ..... **iii**

**DEDICATION** ..... **iv**

**ACKNOWLEDGEMET** ..... **v**

**ABSTRACT** ..... **vi**

**TABLE OF CONTENT** ..... **vii**

**LIST OF TABLES** ..... **xiii**

**LIST OF FIGURES** ..... **xiv**

**LIST OF ABBREVIATIONS** ..... **xv**

**OPERATIONALIZATION OF TERMS** ..... **xvi**

**CHAPTER ONE: INTRODUCTION** ..... **1**

1.1 Introduction ..... 1

1.2 Background of study ..... 1

1.3 Problem statement ..... 4

1.4 Research objectives ..... 8

1.4.1 General objective ..... 8

1.4.2 Specific objectives ..... 8

1.5 Research questions ..... 8

1.6 Justification of the study ..... 9

1.7 Significance of the study findings ..... 11

1.8 Scope of the Study ..... 12

1.8.1 Limitation ..... 12

1.8.2 Delimitation ..... 13

1.9 Conceptional frame work ..... 13

**CHAPTER TWO: LITERATURE REVIEW ..... 15**

2.1 Introduction ..... 15

2.2 Pain ..... 15

2.2.1 Definition and types of pain ..... 15

2.2.2 Pathophysiology of pain ..... 16

2.2.3 Burden of pain ..... 17

2.2.4 Contemporary management of pain ..... 17

2.2.5 Management of pain in traditional medicine system ..... 19

2.3 Inflammation ..... 20

2.3.1 Definition and types of inflammation ..... 20

2.3.2 Pathophysiology of inflammation ..... 20

2.3.3 Burden of inflammation ..... 22

2.3.4 Contemporary management of inflammatory disorders. ..... 23

2.3.5 Management of inflammation in traditional medicine system ..... 23

2.4 Oxidative stress ..... 24

2.4.1 Definition and types of oxidative stress ..... 24

2.4.2 Pathophysiology of Oxidative stress ..... 25

2.4.3 Burden of oxidative stress ..... 26

2.4.4 Contemporary management of oxidative stress damage ..... 27

2.4.5 Management of oxidative stress in traditional medicine system ..... 28

2.5 Analysis of pharmacological efficacy of plant extracts ..... 29

2.5.1 Assay methods for antinociceptive activities ..... 29

2.5.2 Assay methods for anti-inflammatory activities ..... 30
2.5.3 Assay methods for antioxidant activities ..... 32
2.5.4 Phytochemical methods of analysis ..... 33
2.6 The Family Rutaceae ..... 34
2.6.1 Taxonomic information and distribution of Rutaceae ..... 34
2.6.2 Phytochemical composition of the plant species of the Rutaceae family ..... 34
2.6.3 Biological activities of the plant species of the Rutaceae family ..... 35
2.6.4 Traditional medicinal uses of the plant species of the Rutaceae family ..... 35
2.7 *Fagaropsis angolensis* ..... 36
2.7.1 Taxonomy and distribution of *Fagaropsis angolensis* ..... 36
2.7.2 Phytochemistry of *Fagaropsis angolensis* ..... 36
2.7.3 Biological activities of *Fagaropsis angolensis* ..... 37
2.7.4 Traditional medicinal uses of *Fagaropsis angolensis* ..... 37
2.8 *Fagaropsis hildebrandtii* ..... 37
2.8.1 Taxonomy and distribution of *Fagaropsis hildebrandtii* ..... 37
2.8.2 Phytochemistry of *Fagaropsis hildebrandtii* ..... 38
2.8.3 Biological activities of *Fagaropsis hildebrandtii* ..... 38
2.8.4 Traditional medicinal uses of *Fagaropsis hildebrandtii* ..... 38

**CHAPTER THREE: MATERIAL AND METHOD ..... 39**

3.1 Introduction ..... 39
3.2 Study area ..... 39
3.3 Collection and identification of study plant materials ..... 40
3.4 Water extraction ..... 40
3.5 Methanol extraction ..... 40

3.6 Extracts preparation ..... 41
3.7 Experimental animals ..... 41
3.8 Assay for antinociceptive activity ..... 42
3.9 Assay for anti-inflammatory activity ..... 43
3.10 Assay for antioxidant activity ..... 44
3.11 Qualitative analysis of phytochemicals ..... 45
3.11.1 Steroid test (Lieberman burchard test) ..... 45
3.11.2 Phenol test (Ferric chloride test) ..... 45
3.11.3 Saponin test (Frothing test) ..... 46
3.11.4 Tannins test (Ferric chloride test) ..... 46
3.11.5 Cardiac glycosides test (Keller killiani test) ..... 46
3.11.6 Alkaloid test ..... 46
3.11.7 Flavonoid test (alkaline reagent test) ..... 47
3.11.8 Terpenoid test (Salkowski's test) ..... 47
3.11.9 Coumarin test (Sodium hydroxide test) ..... 48
3.11.10 Anthocyanines test (Hydrochloric acid test) ..... 48
3.11.11 Anthraquinone test (Borntragger's test) ..... 48
3.12 Quantitative phytochemical assay ..... 48
3.12.1 Total phenolic content of the water and methanol extracts ..... 48
3.12.2 Total flavanoid content of the water and methanolic extracts ..... 49
3.13 Data analysis ..... 49
3.14 Ethical consideration ..... 50

**CHAPTER FOUR: RESULTS ..... 51**

4.1 Introduction ..... 51

4.2 Antinociceptive activity of *F. angolensis* leaves, water and methanol extracts ....51

4.3 Antinociceptive activity of *F. hildebrandtii* stem bark, water and methanol extracts.53

4.4 The anti-inflammatory activity of *F. angolensis* leaves, water and methanol extracts55

4.5 The anti-inflammatory activity of *F. hildebrandtii* stem bark, water and methanolic extracts .....58

4.6 Antioxidant activity of *F. angolensis* leaves, water and methanol extracts.....61

4.7 Antioxidant activity of *F. hildebrandtii* stem bark, water and methanol extracts .62

4.8 Phytochemical composition of *F. angolensis* leaves, water and methanol extracts .....64

4.8.1 Total phenolic and total flavonoid content of *F. angolensis* leaf extracts.....65

4.9 Phytochemical composition of *F. hildebrandtii* stem bark, water and methanol extracts .....67

4.9.1 Total phenolic and total flavonoid content of *F. hildebrandtii* stem bark extracts .....68

**CHAPTER FIVE: DISCUSSION OF THE RESULTS .....69**

5.1 Introduction.....69

5.2 Antinociceptive activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts.....69

5.3 Anti-inflammatory activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts.....71

5.4 Antioxidant activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts .....74

5.5 Phytochemical composition of *F. angolensis* leaves and *F. hildebrandtii* stem bark extracts .....75

**CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS ..... 78**

6.1 Conclusions ..... 78

6.2 Recommendations ..... 78

6.2.1 Recommendation for action ..... 78

6.2.2 Recommendation for further research ..... 79

**REFERENCES ..... 80**

**APPENDICES ..... 93**

**Appendix I**: Approval letter from the school ..... 93

**Appendix II**: Approval letter from NACOSTI ..... 94

**Appendix III:** Picture of *F. angolensis* ..... 95

**Appendix IV:** Picture of *F. hildebrandtii* ..... 96

**Appendix V:** Voucher specimen authentication certificate ..... 97

**Appendix VI:** Voucher specimen authentication certificate ..... 98

**Appendix VII:** Study area; Embu and Makueni Counties ..... 99

**Appendix VIII:** Dried stem bark of *F. hildebrandtii* ..... 100

**Appendix IX:** Experimental mice marked for easy identification ..... 101

## LIST OF TABLES

Table 3.1: Experimental design for determination of the antinociceptive activities for a mouse weighing 20 g. ..... 43

Table 3.2: Experimental design for determination of the anti-inflammatory activities 44

Table 4.1: Percentage radical scavenging activity (%RSA) and $IC_{50}$ of *F. angolensis* leaf extracts and ascorbic acid ..... 62

Table 4.2: Percentage radical scavenging activity (%RSA) and $IC_{50}$ of *F. hildebrandtii* extracts and ascorbic acid ..... 67

Table 4. 3: Phytochemical content of *F. angolensis* leaves, water and methanol extracts ..... 65

Table 4.4: Total phenolic and total flavonoid content of *F. angolensis* water and methanol extracts ..... 65

Table 4.5: Phytochemical content of *F. hildebrandtii* stem bark, water and methanol extracts ..... 67

## LIST OF FIGURES

Figure 1. 1: Conceptual framework, showing independent variables, intervening variables, dependent variables and the outcomes. ..... 14

Figure 4.1: Percentage inhibition of writhing by increasing doses of *F. angolensis* water and methanol leaf extracts ..... 52

Figure 4.2: Percentage inhibition of writhing by increasing doses of *F. hildebrandtii* stem bark, water and methanol extracts ..... 54

Figure 4. 3: Percentage inhibition of edema of increasing doses of *F. angolensis* water and methanol extracts ..... 57

Figure 4. 4: Percentage inhibition of edema of increasing doses of *F. angolensis* water and methanol extracts ..... 60

Figure 4.5: Antioxidant activity of *F. angolensis* leaves, water and methanol extracts ..... 62

Figure 4.6: Antioxidant activity of *F. hildebrandtii* stem bark, water and methanol extracts ..... 64

Figure 4.7: Total phenolic and Total flavonoid content of *F. angolensis* water and methanol leaf extracts ..... 66

Figure 4. 8: Total phenolic content and total flavonoid content of *F. hildebrandtii* stem bark, water and methanol exctracts ..... 68

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABS: | Absorbance |
| CDC: | Centre for disease control |
| CE/g dw | Catechin equivalent per gram of dry weight |
| COX: | Cyclooxygenase |
| CRP: | C-reactive protein |
| CVD: | Cardiovascular diseases |
| DALYs: | Disability adjusted life years |
| DMARDS: | Disease modifying antirheumatic drug |
| DNA: | Deoxyribonucleic acid |
| DPPH: | 2,2-diphenyl-2-picrylhydrazyl |
| $ED_{50}$ | Effective dose in 50% of the population |
| GAE/g dw | Garlic acid equivalent per gram of dry weight |
| HPA: | Hypothalamus pituitary gland axis |
| $IC_{50:}$ | Inhibitory concentration in 50% |
| IMID: | Immune mediated inflammatory disease |
| I.P: | Intra-peritoneal |
| Mcg/ml: | Microgram per milliliter |
| Mg/kg bw: | Milligrams per kilogram body weight |
| NCDs: | Non communicable diseases |
| NADPH: | Nicotinamide adenine dinucleotide phosphate |
| NSAIDs: | Non-steroidal anti-inflammatory drugs |
| P.O: | Per oral |
| RSA: | Radical scavenging activity |

| | |
|---|---|
| RNS: | Reactive nitrogenous species |
| ROS: | Reactive oxygen species |
| RONS: | Reactive oxygen and nitrogenous species |
| SD: | Standard deviation |
| TM: | Traditional medicine |
| UV: | Ultraviolet |
| WHO: | World health organization |

## OPERATIONALIZATION OF TERMS

**Nociception-** unpleasant sensory transmission of pain and/or emotional experience that is associated with tissue damage, through the nociceptors.

**Writhing-** it is the reflex contraction of abdominal muscle, stretching of hind legs and arching of back of mice in response to acetic acid-induced pain.

**Antinociceptive activity-** a significant reduction in number of writhing episodes of mice treated with plant extracts compared to control. Represented as percentage inhibition of writhing. Calculated by the difference in number of writhing in mice treated with normal saline, minus number of writhing in mice treated with plant extract, divided by normal saline, results multiplied by 100 %.

**Anti-inflammatory agent-** plant extracts or standard dexamethasone which on administration leads to a significant reduction in paw circumference.

**Anti-inflammatory activity-** a significant decrease in linear circumference of mice paws upon treatment with plant extract. This was calculated as the change in edema between mice treated with normal saline and plant extract, divided by normal saline, results multiplied by 100 %.

**Antioxidant agent-** plant extracts as test material and ascorbic acid as positive control.

**Percentage (%) Radical Scavenging Activity-** it is the ability of the plant extract or standard ascorbic acid to quench free radicals, measured from change in absorbance between negative control and plant extracts, divided by the negative control, results multiplied by 100 % (% RSA).

**$IC_{50}$-** inhibition of DPPH, calculated graphically from percentage radical scavenging activity and concentration of plant extracts or standard ascorbic acid.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Introduction

This chapter introduces the background of the study, the problem statement, research objectives, research questions, justification, significance of study and scope of the study.

## 1.2 Background of study

Pain is the sensory perception of noxious stimuli and is mediated by nociceptors found either peripherally or centrally (Sommer *et al.*, 2018; Farmer, 2020). Inflammation is an innate response to an invading pathogen, a toxic xenobiotic or endogenous signal such as damaged cells or traumatic injury (Seyedsadjadi and Grant, 2020). Oxidative stress refers to increased levels of oxidants such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) at the cell or tissue environment which changes normal physiological and biochemical signaling of the cell (Liguori *et al.,* 2018). These medical conditions have remained key contributors to hospitalization in most healthcare facilities due to poor management (Huang *et al.,* 2013; Avrahami *et al.,* 2022).

Pain leads to both physical and emotional suffering whereas inflammation leads to chronic diseases such as rheumatism (Dueñas *et al.,* 2016; Macdonald *et al.,* 2018). Oxidative stress drives physiological aging and neurological diseases such as Alzheimer, Parkinson's disease and dementia (Kim *et al.,* 2015; Cenini *et al*., 2019). Current conventional drugs that are used in management of pain and inflammation include, paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroid and opioids (Slater *et al.,* 2010). Although these conventional analgesics and anti-

inflammatory drugs are effective, they are associated with a number of side effects, including gastrointestinal bleeding, hepatotoxicity, nephrotoxicity and indigestion (Ghosh *et al.*, 2015). Oxidative stress is currently alleviated with conventional antioxidants including vitamins, but which have been shown to lack long term effectiveness or disease modifying properties, partly because they may not reach pharmacologically active concentration *in vivo* (Forni *et al.*, 2019). Besides these conventional drugs, several medicinal herbs are widely used in the management of diseases associated with pain, inflammation and oxidative stress, particularly in Africa, where more than 80% of the population rely on herbal medicine (Ekor, 2014). One such group of herbal plants is the genus *Fagaropsis.*

The genus *Fagaropsis* consist of at least four known species namely, *F. hildebrandtii* (Engl.) Milne-Redh*, F. angolensis* (Engl.) H.M. Gardner, *F. glabra Capuron*, and *F. velutina Capuron* (Mutinda *et al., 2*022). These plant species are found in the Eastern, Central and Southern parts of Africa, as well as Madagascar. Specifically, *F. angolensis* is widely distributed in Kenya, Tanzania, Uganda, Ethiopia, Namibia, Angola and Zimbabwe (Mutinda *et al.,* 2022). In Kenya, *F. angolensis* is distributed in the lowlands of Central Kenya and the Rift valley (Jeruto *et al.*, 2008). *F. hildebrandtii* is found in the Eastern parts of Africa especially, Somalia, Kenya and Ethiopia. In Kenya, *F. hildebrandtii* has been located in areas of Makueni and Machakos counties (WFO, 2023).

In these areas, ethnomedicinal evidence indicates that *F. angolensis* and *F. hildebrandtii* are widely used to treat various illnesses associated with pain, inflammation and oxidative stress. For instance, among communities living in Nandi County Kenya, boiled stem bark of *F. angolensis* is used in treatment of joint pain,

respiratory system infection and back ache (Kimutai *et al.,* 2016). Among the Kamba community in Makueni County Kenya, roots of *F. hildebrandtii* are used to treat chronic pain, pneumonia, arthritis, ulcers, stomach pain, malaria and women infertility (Wambugu *et al.*, 2011; Muia *et al.,* 2020). This widespread use of *Fagaropsis* species in traditional medicine practice has evoked interest in understanding pharmacological and phytochemical profiles of various plant parts. Notably, pharmacological studies of the species *F. angolensis* stem bark has confirmed antiplasmodial activity, anti-proliferative activity, antimicrobial activity and antioxidant activity (Kimutai *et al.,* 2016; Yiaile *et al.*, 2018; Alemu and Misganaw 2020; Muia *et al.,* 2020). For *F. hildebrandtii*, the roots have been investigated and found to have antimicrobial activity (Muia *et al.,* 2020). However, very little data on the antinociceptive, anti-inflammatory and antioxidant properties of the leaves of *F. angolensis* and stem bark of *F. hildebrandtii* was available.

In one previous study, the root bark of *F. angolensis* exhibited *in vitro* anti-inflammatory activity, which was ascribed to inhibition of pro-inflammatory cytokines in lipopolysaccharide stimulated peripheral blood mononuclear cells by plant norhopanes (Mukavi *et al.*, 2020). However, prior to the current study, no *in vivo* anti-inflammatory and antinociceptive studies had been done on the leaves of *F. angolensis*. The stem bark of *F. angolensis* (80% ethanol extract and fractionated 80% methanol extract) from Uganda and Ethiopia respectively, had been shown to exhibit antioxidant activities (Kuglerova *et al.,* 2011; Alemu and Misganaw, 2020). However, the leaves of *F. angolensis* growing in Embu County in Kenya had not been studied for antioxidant activities. Similarly, the antinociceptive, anti-inflammatory and antioxidant properties of *F. hildebrandtii* stem bark had not been investigated experimentally.

Previous phytochemical studies of *F. angolensis* had indicated the presence of flavonoids, alkaloids, steroids, phenols and glycosides (Yiaile *et al.*, 2018; Alemu and Misganaw, 2020). Water extract of *F. angolensis* bark from Kaptumo Sub-county in Nandi county exhibited presence of terpenoids, phenols, alkaloids, anthraquinones and flavonoids, but absence of saponins (Kimutai *et al.*, 2016). The roots of *F. hildebrandtii* have been found to be rich in alkaloids, terpenoids, glycosides, saponins, tannins, steroids, phenols and flavonoids (Muia *et al.,* 2020). However, the phytochemical content often varies depending on the plant parts, extraction methods and climatic condition of area where the plant grows (Altemimi *et al.*, 2017).

The purpose of this study was to investigate not only the phytochemical content but also the *in vivo* antinociceptive, anti-inflammatory activities as well as *in vitro* antioxidant activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark from Embu and Makueni regions of Kenya, respectively. This study findings confirmed presence of *in vivo* anti-inflammatory activity of *F. angolensis* leaves and extended established *in vitro* anti-inflammatory study findings of the root bark (Mukavi *et al.*, 2020). Therefore, it provides new evidence in support of *F. angolensis* leaves and *F. hildebrandtii* stem bark having potent antinociceptive, anti-inflammatory and antioxidant activities.

### 1.3 Problem statement

Pain interferes with various aspects of life, such as social life, mental and physical health (Duenas *et al.,* 2010). According to CDC report of 2020, the world prevalence of chronic pain among adults in the year 2019 was 20.4%. Among these adults, 7.4% had chronic pain that frequently limited life or work activities in the past 3 months.

Consequently, pain is a very common symptom in neurological diseases that requires immediate intervention (Bukhari *et al*., 2016).

Currently, acute or moderate pain is treated with various drugs including NSAIDs, paracetamol, antidepressants (amitriptyline), gabapentin and opioids analgesics. For severe pain, long term opioids are used. Neuropathic pain management involves the use of antidepressants (amitripyline), calcium channel inhibitors like gabapentin/pregabalin and topical lidocaine (Queremel and Davis, 2023). Although these drugs are relatively effective, they are sub-optimal as they are associated with several side effects including hepatotoxicity (NSAIDs) and urinary retention (amitripyline). Some like opioids are not easily accessible and can cause respiratory depression and dependency among other side effects. Moreover, some forms of cancer-related pain and migraine-related headache do not response to the currently available analgesics (Nalamachus, 2013).

Inflammatory conditions, particularly due to chronic infectious diseases and NCDs like obesity, cancer, arthritis, inflammatory bowel diseases and skin diseases present with chronic inflammation. This chronic inflammation leads to tissue damage, systemic pathological changes, increased morbidity and low quality of life and ultimately contributes to death (Furman *et al.,* 2019). Indeed, more than 50% of deaths worldwide can be attributed to inflammatory-related NCDs such as stroke, chronic kidney disease, ischemic heart disease, non-alcoholic fatty liver diseases and neurodegenerative diseases (Furman *et al.,* 2019).

Currently, the main pharmacological therapy for inflammation includes non-steroidal anti-inflammatory drugs that include aspirin, oxicams, diclofenac, ibuprofen and corticosteroids such as dexamethasone, hydrocortisone and prednisolone. However,

these drugs have several shortcomings, including side effects and high cost. Long term use of high dose aspirin and ketorolac for example, is associated with gastrointestinal erosion and interaction with other drugs including beta blockers. Dexamethasone causes bone weakening particularly in the elderly who are already predisposed to bone resorption (Punchard *et al*., 2014).

During aging and disease states, the generation of oxidants from endogenous sources is enhanced, leading to oxidative stress. Increased production of oxidants can also be induced by exogenous factors including ultraviolet (UV) light, gamma rays' radiation, electromagnetic field, alpha particle emitted by radioactive elements decay, tobacco smoke, ozone, and iron overload (Joanna *et al.,* 2019). Importantly, oxidative stress drives tissue damage, plays a role in pathogenesis of various diseases such as alzehimer and is known to accelerate the aging process (Liguori *et al*., 2018.

Currently available antioxidants including nutraceutical ascorbic acid, tocopherol, resveratrol and phenolic compounds are prescribed at various stages of disease, some as preventive while others as treatment. In the former case, antioxidants are widely used as adjuvants in the management of various health conditions including inflammation and pain. However, these antioxidants might not reach pharmacologically active concentration *in vivo.* Furthermore, these nutraceuticals neither eliminate the primary cause of disease nor limit its progression (Cynzia *et al.*, 2019). Therefore, continuous search and development of novel antinociceptive, anti-inflammatory and antioxidant agents remain an important goal if we are to improve inflammatory pain pharmacotherapy.

The use of medicinal plants in management of various diseases in human being dates centuries ago when mankind discovered therapeutic potential of plants. Among the factors contributing to high intake of these medicinal plants is acceptability, tolerance and availability. Despite over 80% of human population, especially in Africa utilizing medicinal plants, only a few have been investigated to ascertain their ascribed therapeutic effects (World Health organisation, 2019). Indeed, there is a need for experimentally controlled data on the ethno-pharmacological properties of various widely used medicinal herbs.

In Kenya, several medicinal plants are used traditionally to treat pain, and inflammatory disorders have already been documented. These include various species of the genus *Fagaropsis* which are widely used in local traditional medicine to treat malaria, back pain, joint pain, stomachache, amoebiasis, rheumatism, cough, cancer, stabbing pain, snakebite, babesiosis, gonorrhea, pneumonia, respiratory diseases, contagious bovine/caprine pleuropneumonia, ulcers and toothache (Muthaura *et al.,* 2007; Jeruto *et al.*, 2010; Wambugu *et al.,* 2011; Alemu and Misganaw, 2020; Mutinda *et al.,* 2022). However, experimentally controlled pharmacological studies to fully validate the antinociceptive and anti-inflammatory properties of these plants were limited. Therefore, this study focused on evaluating the *in vivo* bioactivity of *F. angolensis* leaves and *F. hildebrandtii* stem bark, the two plants that have been used in folk medicine for pain management in Nandi, Embu and Makueni Counties without sufficient data to fully validate their acclaimed therapeutic uses (Mutinda *et al.,* 2022).

## 1.4 Research objectives

### 1.4.1 General objective

To evaluate antinociceptive, anti-inflammatory, antioxidant activities and phytochemical composition of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts.

### 1.4.2 Specific objectives

i. To evaluate the *in vivo* antinociceptive activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts.

ii. To evaluate the *in vivo* anti-inflammatory activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts.

iii. To evaluate the *in vitro* antioxidant activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts.

iv. To determine the phytochemicals profiles of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts.

## 1.5 Research questions

i. Was there antinociceptive activity associated with *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts?

ii. Was there anti-inflammatory activity associated with *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts?

iii. Was there antioxidant activity associated with *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts?

iv Were there phytochemical compounds associated with *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts?

## 1.6 Justification of the study

Genus *Fagaropsis* has several documented medicinal values and has long been used among various ethnic groups to treat medical conditions that are associated with pain and inflammation (Jeruto *et al.,* 2008; Kimutai *et al.,* 2016; Yiaile *et al.*, 2018; Muia *et al.,* 2020; Alemu and Misganaw, 2020; Mutinda *et al.*, 2022). Notably, these studies have only determined the phytochemical composition, toxicity, distribution, pharmacological uses such as malaria treatment, cancer treatment and antimicrobial activities of the genus *Fagaropsis*. However, prior to these findings, there was no study that had sufficiently evaluated the link between ethnomedicinal use and bioactivity of *F. angolensis* leaves and *F. hildebrandtii* stem bark among the Embu, Nandi and Kamba communities in management of pain, inflammation and oxidative stress related complications (Kareru *et al.*, 2006; Kimutai *et al.,* 2016; Muia *et al.*, 2020). Mostly, leaves and stem bark of *F. angolensis* and *F. hildebrandtii* are utilized by traditional practitioners to heal complications ranging from back pain, stomachache, rheumatism, cough and infertility. The use of unvalidated plant parts for medicinal purposes can lead to toxicity, disease progression and interaction with other products such as drugs and food. This study has substantiated claimed traditional use and proposed further research.

The solvent for most plant parts by traditional medicine practitioners is water. The solvent is readily available, safe and cheap. In this study, methanol was chosen because as an organic solvent, it is easy to evaporate, partly safe and it dissolves in most of organic phytochemicals including, phenols, flavonoids and steroids as was seen from the results of this study in comparison with water.

In Kenya, *F. angolensis* is mainly found on the lower sides of Mount Kenya highlands in Meru, Embu, Taita hills, drier parts of Makueni, Kwale and the Rift valley highlands

in the area around Nandi County. Several studies, including one by Nguta *et al*. (2010) in Msambweni, Kareru *et al.* (2007) in Mbeere, Embu, and Itonga, (2011) in Meru have highlighted the use of *F. angolensis* in these Counties of Kenya. This study chose *F. angolensis* from Embu County, to address the misinformation on use of the plant among the Embu community and its surroundings. With available findings on antinociceptive, anti-inflammatory and antioxidant activities of *F. angolensis,* the study will have a greater impact on the populous Embu, Meru, Mbeere, and Kikuyu communities distributed on the lower side of Mount Kenya highlands compared to its use in the Rift valley highlands where not much research work has documented extensive use there (Jeruto *et al*., 2010).

Among these communities, pain, inflammation and complications related to oxidative stress were the main diseases managed by parts of *Fagaropsis* species. While inflammation can be controlled by the inhibition of phospholipase $A_2$, pain can be managed by inhibition of COX enzyme. The study chose both pain and inflammation, as they can both be inhibited from the arachidonic acid pathway. Relatively, oxidative stress can lead to inflammation, pain or both. In this study, despite previously reported findings on phytochemical analysis of various parts of *F. angolensis* and *F. hildebrandtii*, quantification of both phenols and flavonoids of the leaves and stem bark, respectively, was essential to determine the antioxidant activity.

There is now available experimentally controlled data that validates the widespread use of the two plants traditionally, in management of diseases associated with pain, inflammation and oxidative stress, and thus the use was well justified. The study provides a starting point for further investigation on fractionation to determine the most active fraction, constituent and binding sites.

### 1.7 Significance of the study findings

The findings of this study provide scientific evidence about the traditional uses of *F. angolensis* leaves and *F. hildebrandtii* stem bark. It provides information on pharmacological activities hence validating their traditional use in management of pain, inflammation and oxidative stress among various communities in Kenya. For the traditional practitioners, the study findings provide them with a preliminary basis of information about superiority of methanol extract against water extract and presence of activity that was associated with phytochemicals in the leaves of *F. angolensis* and stem bark of *F. hildebrandtii* extracts.

The information on pharmacological activities can be used to facilitate registration of crude herbal formulations containing extracts of *F. angolensis* leaves and *F. hildebrandtii* stem bark, which is a key requirement by the regulatory authority, Pharmacy and Poisons Board of Kenya. Besides significance to the traditional practitioners, the public interest has been addressed, as the study confirms that the use of *F. angolensis* leaves and *F. hildebrandtii* stem bark have potential beneficial effects in conditions associated with inflammation, and thus if compounded and registered after due analysis by the regulator, it will be rational to use them specifically for stipulated indications. Patients with intractable pain, inflammation or who cannot tolerate the conventional drugs can then opt for the crude extracts of the two plants following further research and under guidance of registered medical practitioner and pharmacist.

The study findings also form a solid basis for further research that can lead to discovery of novel drugs. These include clinical trials of *Fagaropsis* herbal preparations to test the effectiveness in human diseases associated with inflammation and pain, isolation and elucidation of structure, drug development and synthesis of other derivatives from

the pharmacophore unit with improved activity. This is important because strategies to preserve these endangered species have already been suggested. Moreover, the findings of the current study indicate that *F. angolensis* and *F. hildebrandtii* extracts can be used as easily accessible novel tools to elucidate novel molecular pathways involved in inflammation and pain. This can be done by genomic and proteomic analysis of cells or tissues exposed to the extracts.

### 1.8 Scope of the Study

This experimentally controlled study evaluated the antinociceptive, anti-inflammatory and antioxidant activities of *F. angolensis* leaves from Embu County and *F. hildebrandtii* stem bark from Makueni County. Water and methanol extracts were prepared for analysis, and phytochemical profile used to explain pharmacological activities.

#### 1.8.1 Limitation

Ethical consideration provides use of minimal number of mice possible that can provide clarity of data (OECD, 2002). Animal number per group were limited to 5 mice with good animal care husbandry so that they don't die especially due to choking with drug during oral administration. This small size might have limited the power of the study.

Plant extract administration was limited to oral route. The oral route of administration of plant extract and control could have the potential of affecting validity of the result as bioavailability from oral route is dependent on rate of absorption, distribution, metabolism and elimination. Pharmacokinetic factors including first pass effect and biotransformation in the gut lumen could adversely affect bioavailability for orally administered drugs.

The other limitation was the use of chemically induced acute pain and inflammation models, and therefore the data cannot be extrapolated to chronic inflammation and

neuropathic pain states. Water extraction was limited to manually operated freeze dryer as there was no automated machine for lyophilization and this may have affected the consistency of the extracts. In addition, solvents with low polarity index that is more organic were not used in the study and not all phytochemicals may have been extracted.

### 1.8.2 Delimitation

Data findings were limited to the leaves of *F. angolensis* found and used locally in Embu County and stem bark of *F. hildebrandtii* found and used locally in Makueni County. Study did not cover parts of *F. angolensis* found in Nandi County or other parts of Kenya. The following was excluded from the study; roots, seeds and flowers of *F. angolensis* and *F. hildebrandtii* as they are not commonly used.

The study only covered water and methanol extracts of both *F. angolensis* leaves and *F. hildebrandtii* stem bark. Water is a locally available solvent while methanol is an organic solvent that dissolves most phytochemicals. The study findings affirm the superiority of methanol over water as solvent. The research also focused on phytochemical screening of the crude extracts without isolation and purification of the potentially, antinociceptive, anti-inflammatory or antioxidant compounds. The secondary metabolites included phenols, alkaloids and steroids identified in this study could be characterized in subsequent studies.

## 1.9 Conceptual framework

Phytochemical contents are dependent on season of plant harvesting, species difference, plant parts and extraction solvents. The phytochemical content analysis, percentage writhing pain inhibition, percentage paw edema inhibition and percentage free radical scavenging activities are all dependent on the extracts of *F. angolensis* leaves and *F. hildebrandtii* stem bark.

The outcome of the study was establishment of specific phytochemicals, magnitude of edema relieves (anti-inflammation), magnitude of pain perception (antinociceptive) and magnitude of free radical scavenging (anti-oxidation) activities. Analytical methods showed the presence of various phytochemicals including flavonoids, phenols and alkaloids which are assumed to be responsible for the recorded activities.

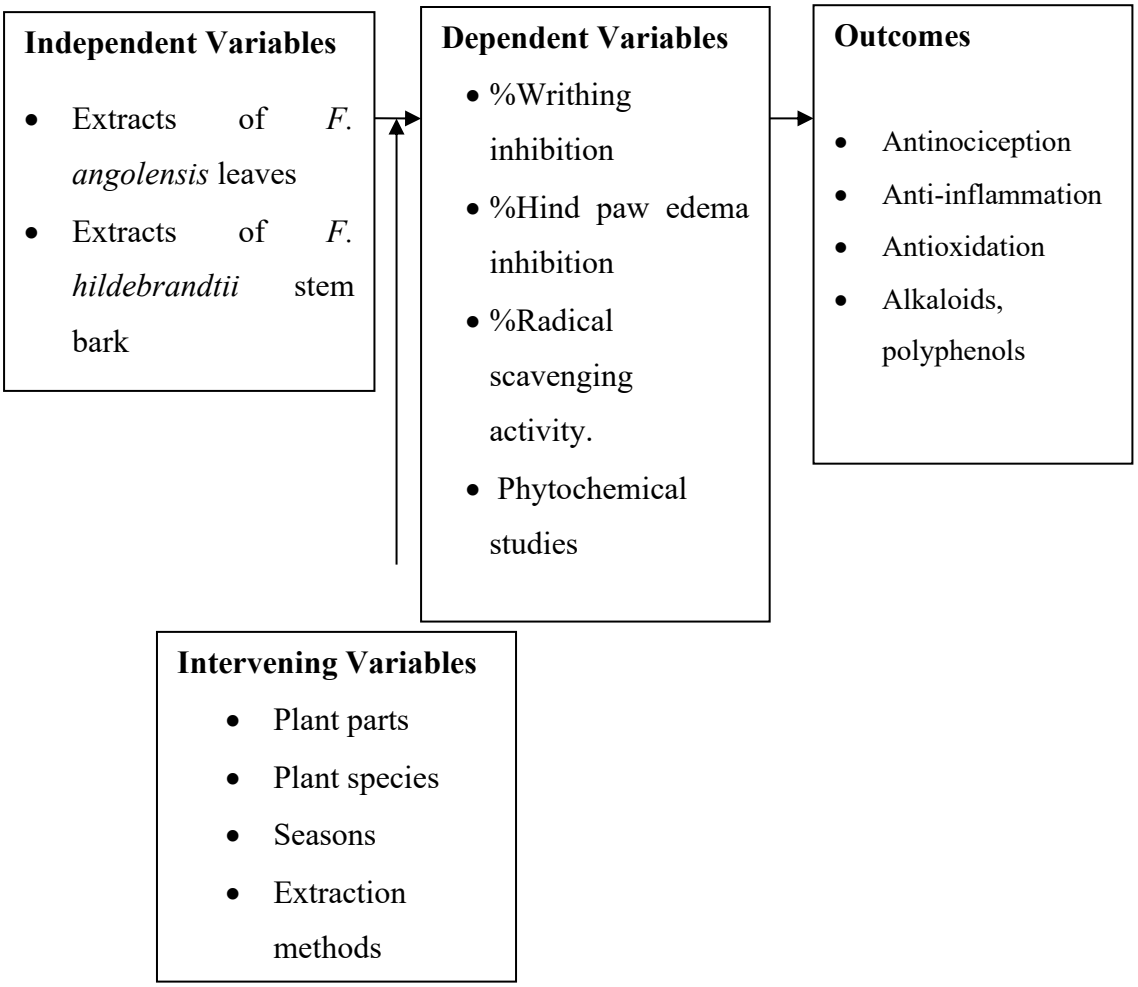



***Figure 1.1**: **Conceptual framework**, showing independent variables, intervening variables, dependent variables and the outcomes.*

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Introduction

This chapter provides a brief overview of pain, inflammation and oxidative stress. The pertinent literature was also reviewed to highlight the knowledge gap that the current study aims at filling.

## 2.2 Pain

### 2.2.1 Definition and types of pain

Pain is an unpleasant sensory and emotional experience that is associated with injury or tissue damage. During harmful stimuli, pain sensation warns us to withdraw from the injury. This is accomplished through nociception, the neural processing of harmful stimuli. It is difficult to quantify pain because pain is both an affective and a sensory component (Nalamachu, 2013). There are two categories of pain according to duration: acute pain and chronic pain. Pain that arises from a specific ailment or injury is referred to as acute pain. This is sharp in quality, lasts for less than six months and varnishes with the underlying cause (broken bones, surgery, burns, dental work, and labor). However, pain that lasts for more than six months after ailment or injury and is difficult to locate is called chronic pain, e.g. arthritis, migraine, nerve pain, back pain and fibromyalgia pain (Farmer, 2020).

There are other types of pain such as nociceptive pain, neuropathic pain and nociplastic pain. Nociceptive pain plays a role in sending warning signals to protect the body against injury. Myelinated A-delta nerve fiber is responsible for rapid transmission of pain while unmyelinated C- peripheral nerve fiber transmits slow impulse pain (Sinha *et al*., 2023). Nociceptive pain can further be divided into somatic and visceral pain. Somatic pain originates from the peripheral tissue e.g. skin, while visceral pain

originates within the abdomen or certain organs. Neuropathic pain is pain from lesion or diseases affecting the somatosensory system. Nociplastic pain is pain due to altered nociception even with no clear origin (Bonezzi *et al.,* 2020). Broadly, pain can also be categorized into physiological and pathological. Nociceptive and inflammatory pains are physiological pain as they are protective and adaptive whereas pathological pain such as neuropathic pain, is non-protective and non-adaptive (Fong and Schug, 2014).

### 2.2.2 Pathophysiology of pain

Pain is detected by sensory receptors called nociceptors. These are free nerves ending with cell bodies in the dorsal root ganglia and terminal in the superficial layer of the dorsal horn of the spinal cord. Nociceptors relay message by releasing neurotransmitters such as glutamate and substance-p (Dinakar and Stillman, 2016; Farmer, 2020). Nociception begins at the surface of the skin where nociceptors encode stimuli in the environment that can cause cell or tissue injury. Given that there are different types of environmental threats, different types of nociceptors will detect specific types of sensory information based on variation of nociceptor structure and function. Nociceptive signals are transmitted by either A delta or C fiber types that lead to either immediate or gradual perception of pain (Armstrong and Herr, 2024).

Nociceptors generate electrical signals that relay the quality, duration and intensity of noxious stimulation. The difference in nociception is achieved with specialized receptor protein and ion channel located on nerve endings. Different types of receptors with different markers identify distinct types of information. These receptors and channels mediate transduction and encoding of correspondence electric signals into attributes the brain can interpret e.g. modality, location, threshold, intensity and timing (Dinakar and Stillman, 2016; Farmer, 2020).

### 2.2.3 Burden of pain

Chronic or severe pain is considered as an ailment that is a major public health burden. Globally, 20% of the world population has shown both physical and emotional symptoms of pain. In the US, one in five adults had chronic pain in 2016 and 8% had high impact chronic pain (Kuehn, 2018; Salaffi *et al.*, 2018). There is 16.6% prevalence of pain in Spain with one in four affected in each home (Dueñas *et al*., 2016; Zelaya *et al.,* 2020). In south Africa, 21.5% of patients attending chronic care clinic had chronic pain. Among those patients, 18% of patients were adults (Kamerman *et al.,* 2020). At Kakamega teaching and referral hospital, averaged between the Numerical Rating Scale (NRS) and Faces Pain Scale-Revised (FPS-R), 80.5% of patients endorsed a non-zero level of pain and 30% of patients reported moderate to severe pain. The prevalence of pain among HIV/AIDs patients in South Africa, Uganda and Kenya was at 59% to 98% (Huang *et al*., 2013).

The physical symptoms of pain include muscles rigidity, frowning, fidgeting, teeth grinding, poor sleeping, grimacing and fearful expression. Emotional episodes on the other hand include episodes of depression, sadness, shame, rage, loneliness, grief, deep sorrow and intense distress. For instance, neuropathic pain has a high incidences of co-morbidity disorders that include depression and anxiety (Van *et al.*, 2013).

### 2.2.4 Contemporary management of pain

Currently, management of pain entails use of drugs such as NSAIDs that include, ibuprofen, naproxen, meloxicam, aspirin, opioids and other drug categories such as antidepressants, antipsychotics and anticonvulsants. Acetaminophen is used for moderate pain, moderate to severe pain when combined with opioids and reduction of fever (Slater *et al.,* 2010). Antidepressant duloxetine and amitripyline have efficacy in a variety of neuropathic pain. Antiepileptic e.g. gabapentin is used for postherpetic

neuralgia, pregabalin for postherpetic neuralgia and fibromyalgia and carbamazepine for trigeminal or glossopharyngeal neuralgia. Local anesthetics, such as lidocaine, are used for postherpetic neuralgia. NSAIDs and paracetamol are indicated for mild to moderate pain, pain associated with inflammation and temporary reduction of fever (Punchard *et al.*, 2004; Varrassi *et al.,* 2019).

Although these drugs are in great use, they have several shortcomings. They are associated with various side effects and drug-drug interactions. Some familiar side effects with commonly prescribed drug (paracetamol) are hepatotoxicity and nephrotoxicity (Punchard *et al*., 2004; Varrassi *et al.,* 2019). In a study done on pain treatment at Kakamega Teaching and Referral Hospital, paracetamol was highly prescribed at 30%, diclofenac at 14%, ibuprofen at 5% among NSAIDs (Huang *et al.,* 2013).

Certain endogenous compounds that include endorphin and dynorphin manage pain naturally. These opioids-like substances responsible for reducing pain, block μ-receptors (mu) of peripheral nerves, inhibiting release of neurotransmitter substance-p responsible for pain transmission (Dhaliwal and Gupta, 2023). Opioid drugs act like endorphin by blocking μ-receptors among other receptor types (delta and kappa) found in the periphery. This class of drugs is used for moderate to severe pain. However, they are associated with dependency, constipation and depression of respiratory system (Norn *et al.,* 2005).

While recent advances in understanding pain mechanism will help in coming up with new treatment, current management of chronic pain appears to be unsatisfactory as two thirds of chronic pain sufferers report dissatisfaction with available treatment. Consequently, there are other alternative management of pain apart from the contemporary drugs, such as the use of traditional medicine (Van *et al.,* 2013).

### 2.2.5 Management of pain in traditional medicine system

Due to emerging worldwide shortage of opioids in management of chronic pain, regulatory bodies such as Food and Drug Administration have advised practitioners on the use of alternative medicine for pain management. Medicinal plants are regarded as the alternative drugs to conventional medicine, although World Health Organization advises on use of only validated medicinal plants in formulation and dosage that cannot cause unacceptable toxicity (Lozano *et al*., 2020). Other than medicinal plants, other most common alternative pain management therapies include ayurveda medicine, yoga, acupuncture, apitherapy and traditional Chinese medicine (Hu *et al*., 2022).

Around the world, the use of medicinal plants is influenced by culture and belief that determine use of certain plants and preparation methods. Various plant families are regarded to be good in pain management and species from those particular families are generally used in pain management (Uritu *et al.*, 2018). One of the families listed as used in pain management in Makueni and Machakos Counties include Rutaceae family, where leaves, roots, stem bark and shoots of *F. hildebrandtii* are boiled in water and concoction taken orally. In most cases, medicinal plants are mixed with other plants and not used alone. In this case, *F. hildebrandtii* is mixed with *Strychinos henningsii* leaves and *Carrissa spinarum* root bark (Wambugu *et al.,* 2011). Another plant related to *F. hildebrantii* in terms of usage and found in Kenya is *F. angolensis*. The two plants were investigated in this research for antinociceptive activities as was claimed by the Kamba and Embu communities respectively, in Kenya. Previously, the antinociceptive activities of these plants had not been adequately reported.

## 2.3 Inflammation

### 2.3.1 Definition and types of inflammation

Inflammation is an adaptive response to either infection or tissue damage. The role of inflammation is restorative and healing. In certain cases, like autoimmune disease, the immune system acts as if normal tissue is infected or somehow unusual, causing damage. There are two types of inflammation, acute and chronic inflammation. Acute inflammation is a self-limiting process unlike chronic inflammation which could be destructive. The five cardinal signs of inflammation are; pain, heat, redness, loss of function and swelling (Mendes *et al.,* 2018).

### 2.3.2 Pathophysiology of inflammation

Inflammation results from activation of the immune system in response to broad range of stimuli. Immune system has both cellular and hormonal components. The cause of inflammation is influenced by immune condition of the host, the virulence of an infection and the fine-tuning of local tissue reaction. Immunity is a compromise between insufficient and exaggerated immune reactions. The balance between the two is achieved by T-cell and B-cell selection in the bone marrow and thymus on one hand and check point control in peripheral lymphatic tissue on the other hand (Medzhitov, 2008; Mendes *et al.,* 2018).

Acute inflammation is triggered by infection or tissue injury and involves delivery of blood compounds such as the fibrinogen, albumin, globulins and neutrophils to the site of infection or injury. Neutrophils move to the site of infection through selective extravasations. At that site, they become activated either by direct contact with pathogen or through the action of cytokine secreted by tissue residence cells. Thereafter, neutrophils attempt to kill the invading agent by releasing toxic content of the granules which include reactive oxygen species, cathepsin G, reactive nitrogen

species and elastase. Finally, lipoxins are involved in resolution of inflammation. They stimulate monocytes at the site which remove dead cells and initiate remodeling (Medzhitov, 2008; Mendes *et al*., 2018).

Lesion formation at the site of injury or infection varies with type of leukocytes produced. That is, lesions are characterized based on specific leukocytes at the injury site. The events are controlled by certain mediators and modulators that include growth factors, cytokines, complement system, eicosanoids and peptides. These extracellular and intracellular events are co**o**rdinated with ability to assemble or dissemble cells as they move from inactive to active role depending on; stage of inflammation, initiating agent, tissue or organ that is involved, if inflammation is acute, chronic or long lasting and whether it involves formation of scar or granuloma (Medzhitov, 2008; Mendes *et al.,* 2018).

Chronic inflammation could either be continuation of acute inflammation or it can occur on itself. The composition of white blood cell changes soon after episodes of acute inflammation. Macrophages, lymphocytes and plasma cells in the tissue sites producing inflammatory cytokines, growth factors and enzymes, begin to replace neutrophils contributing to tissue damage or secondary repair including fibrosis and granuloma formation. In response to foreign organism invasion, antigens, macrophages and dendritic cell release cytokines such as IL-1 and TNF alpha (Chen *et al*., 2017). These cytokines induce injury sites to release selectin and integrins which stimulate chemotaxis of circulating leukocytes and leads to phagocytosis and presenting cells to lymphocytes. Circulating leukocytes are activated to release further cytokines (Pahwa *et al.,* 2023). Controlled inflammatory response is beneficial but can be detrimental if not controlled causing septic shock. Like in case of inflammation due to infection, the target is to get rid of the infection by immune cell mobilization (Medzhitov, 2008).

Therefore, it is good that acute inflammation is controlled and that it does not lead to chronic inflammation. This is because persistent inflammation will destroy various tissue organs resulting in non-communicable diseases (NCDs). Damage to the vascular endothelium is a risk factor for cardiovascular diseases, type II diabetics and central obesity (Phillips *et al.*, 2019). Cardiovascular diseases are also associated with increased plasma C-reactive protein (CRP) concentration and increased low density lipoprotein cholesterol. Obesity increases inflammatory cells, ROS circulation and DNA denaturation resulting in cancer. Inflammation also directly plays a role in cancer pathology (Singh *et al.,* 2019).

### 2.3.3 Burden of inflammation

The world health organization asserts that immune mediated inflammatory diseases (IMID) is the greatest threat to human health as it is involved in various diseases including diabetic, cardiovascular diseases, rheumatoid arthritis, cancer and respiratory diseases (Furman *et al.*, 2019). IMID is characterized by acute or chronic inflammation that can affect any organ. The prevalence of IMIDs in western society is about 5–7%, with psoriasis having similar prevalence among men and women (Furman et al. 2019). People with IMIDs have shorter lifespans than the general population. The expected survival of rheumatoid arthritis patients is likely to decrease by 3-10 years according to severity of the disease and the age of disease onset. Covid-19 has claimed almost 4,000 Kenyan among 180,000 reported cases of severe acute respiratory syndrome. Those death cases were related to respiratory inflammation, with difficulty in breathing (Brand *et al*., 2021).

Inflammation can lead to formation of granuloma, atherosclerosis and tissue necrosis. Mediators of inflammation can lead to production of reactive oxygen species and reactive nitrogenous oxygen species which in a non-homeostasis state can lead to

damage of DNA (Pereira *et al.,* 2015; Nediani and Giovannelli, 2020). Chronic inflammatory conditions such as auto-immune disease are characterized by presence of pain that leads to physical suffering, impaired function and reduced quality of life. These are relapsing conditions which have a negative effect on productivity and employment status of most patients. They can reduce the financial capacity of the patient and the family at large. Non-health care or indirect cost includes earlier retirement and decreased productivity (Philip *et al.,* 2011).

### 2.3.4 Contemporary management of inflammatory disorders.

Acute inflammation is usually part of the healing process and does not need treatment. NSAIDs such as meloxicam, antihistamines such as chlorpheniramine and steroidal drug prednisolone could be used to manage acute inflammation, if necessary. Currently available drugs for chronic inflammation include NSAIDs (such as ibuprofen, naproxen, aspirin), corticosteroids such as dexamethasone, statins such as atorvastatin, metformin, disease modifying antirheumatic drugs (DMARDs) for instance. Janus kinase inhibitors (such as olumiant) and herbal supplements such as ginger. Dexamethasone produces therapeutic effects at physiological concentration, resulting in adverse effects such as Cushing syndrome (Punchard *e al.,* 2004; David *et al.,* 2014). Drugs such as NSAIDs have also become ineffective in long term protection as they interfere with essential cellular pathways, cause toxicity and reduced sensitivity in long term use (Ghosh *et al.,* 2015). Other than ginger, various herbal plants have also been used in management of inflammation with scanty data to support their use.

### 2.3.5 Management of inflammation in traditional medicine system

Over 80 % of human population largely relies on traditional medicine (TM) or non-conventional medicine for their primary health needs especially in low-medium income

countries of Africa. These traditional medicines include complementary (alternative) medicines and practices that are not considered part of conventional medicine. The World Health Organization(WHO) defines TM as, total sum of the skills, practices and knowledge that is based on earlier beliefs, theories indigenous to a particular or different heritage or cultures, whether explicable or not, that is used to maintain a good health as well as to keep off ailments, diagnose, improve or treat physical and psychological illness(World Health Organisation, 2019). This is a holistic approach that is recognized as a heterogeneous set of non-conventional products and practices which have potent healing effects. Though the practices are mostly ancient, some remain as useful in treating various ailments to date. In some remote area of Africa, Traditional Medicine is still considered to be the first contact during primary healthcare before seeking other treatment alternatives (James *et al.*, 2018).

Species of *Ipomoea* and *alstonia* found in Egypt have been found to contain lipoidal and phenolic compounds responsible for anti-inflammatory effects. Some of the plant species found in Kenya with anti-inflammatory effect include organic extract of *O. sinuatum, Mondia whitei, Galinsoga parviflora* and *Xzanthoxylum chalybem* (Oguntibeju, 2018). Other forms of traditional complementary medicine that are used in treating inflammation are biological based therapies, mind and body medicine, energy giving medicine and manipulative body-based therapies (Seifert *et al.,* 2020). This study was undertaken to substantiate ethnomedicinal claim of *Fagaropsis* species in management of inflammatory related complication.

## 2.4 Oxidative stress

### 2.4.1 Definition and types of oxidative stress

Free radicals are highly reactive atoms or molecules with one or more unpaired electrons in the outer shell and are formed when oxygen interacts with molecules like

nitrogen, chloride, bromide and hydrogen. Oxidative stress can be classified into four types: high, intermediate, low and basal. High oxidative stress is cytotoxic, intermediate/low oxidative stress is a stress signal, while basal oxidative stress is considered physiological (Magnani *et al.,* 2020).

### 2.4.2 Pathophysiology of Oxidative Stress

During aerobic respiration in the body, unstable biomolecules or chemical products such as nitrogenous compounds, hydrogen and oxygen species are generated. These free radicals are produced in cells through loss or gain of single electron hence behaving like oxidant. When excessive release of free radical goes beyond physiological capacity of the body to get rid through cell quenching, oxidative damage occurs (Liguori *et al.,* 2018; Kruk *et al*., 2019).

Endogenous sources of reactive oxygen and nitrogenous species (RONS) are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase, lipoxygenase and angiotensin II. NADPH oxidase is a source of free radical; superoxide anion which is formed by an electron reduction of molecular oxygen with electrons supplied by NADPH during cellular respiration. Most of these free radicals are dysmutated into the hydrogen peroxide by superoxide dismutase (Kruk *et al*., 2019). Hydrogen peroxide is not a free radical because it lacks unpaired elements, but it is able to form highly reactive oxygen species (ROS) and hydroxyl ions through fenton reaction. Endogenous antioxidant enzymes are superoxide dismutase, catalase and glutathione peroxidase. Superoxide dismutase converts singlet oxygen to hydrogen peroxide which is decomposed to water and oxygen by catalase preventing hydroxyl radical formation (Forni *et al*., 2019).

Non-enzymatic reactions can also be responsible for free radical production for instance, when oxygen reacts with organic compounds or when cells are exposed to ionization radiation or even during mitochondrial respiration. Free radicals can be produced from either endogenous or exogenous sources. Cancer, ischemic, infection, inflammation, excessive exercise, immune cell activation, mental stress and aging are all endogenous sources of oxidative stress (Piedrafita *et al.*, 2015). Exogenous sources of oxidative stress can occur due to exposure to environmental pollutants, drugs (bleomycin, cyclosporine, tacrolimus and gentamycin), alcohol, cigarette smoking and radiation. When these exogenous compounds penetrate the body, they are metabolized to release free radicals (Magnani *et al*., 2020).

During exposure to stressful environment, antioxidants may cause an imbalance between reactive oxygen species (ROS) production and free radical scavenging that leads to activation of enzymatic and non-enzymatic responses in organelles. These include changes in activity of superoxide dismutase, peroxidase and catalases and non-enzymatic synthesis of low molecular compounds (ascorbic acid, glutathione, phenolic compound, flavonoid and carotenoid) and high molecular compound such as tannin (Nediani and Giovannelli, 2020). Failure to produce free radicals' scavengers can lead to oxidative stress.

### 2.4.3 Burden of oxidative stress

Several diseases are associated with the presence of oxidative stress. They include both communicable diseases and non-communicable diseases. Cancer, covid-19, chronic obstructive pulmonary disease, chronic kidney disease, neurodegenerative disease, cardiovascular diseases and inflammatory bowel diseases are all linked to oxidative stress (Liguori *et al.,* 2018).

The prevalence of ulcerative colitis and Crohn's disease in Europe was as high as 505 and 322 per 100,000 person-years, respectively. The highest incidence of inflammatory bowel disease was reported in Faroe Island with an incidence of 80 per 100,000 person-years. In Europe, there was an incidence of 24.3 for ulcerative colitis and 12.7 per 100,000 person-years for Crohn's disease (Uritu *et al.*, 2018).

Globally, within the age of 0-74 years, the risk of developing cancer is 20.2%. A total number of 18 million new cases were diagnosed in 2018, the most frequent of which was lung cancer at 2.09, breast cancer at 2.09 and prostate cancer at 1.28 million cases. The World Health Organization (WHO) updated corresponding to the year 2016 on the top 20 causes of disease burden, that is estimated as cause specific disability adjusted life years (DALYs), cancer imposed the largest worldwide burden (244.6 million DALYs). In men, 137.4 and in women, 107.1 million DALYs were reported (Mattiuzzi and Lippi, 2019).

### 2.4.4 Contemporary management of oxidative stress damage

Currently used antioxidants include ascorbic acid which acts through scavenging hydroxyl and superoxide anions. Others include, tocopherol involved in lipid peroxidation of cell membrane, phenolic antioxidants like stilbene derivative (phenolic acid, flavonoid and resveratrol), mineral compounds (selenium, zinc), vitamins (A, C, E and K), carotenoid and cysteine derivatives (carbocisteine). Mineral compounds are involved in upregulation of enzymatic processes involved in antioxidation processes (Liguori *et al.*, 2018; Tian and Liu, 2020). Vitamin E provides protection through acting against lipid peroxidation. Carotenoid activity is based on the scavenging of peroxyl radical. In the body, it reacts very fast with oxygen radical, hydrogen peroxide and ozone enzymatically through ascorbate peroxidase (Forni *et al.*, 2019). These exogenous compounds are taken as supplements either orally or as injectables.

Antioxidants are used for various indications such as management of neurological pain, inflammation, treatment of cough, cognitive problems and as supplements to boost the immune system. Some flavonoids are used to manage alzheimer. They include resveratrol, catechin and myricetin (Simunkova *et al*., 2019). Specifically, derivatives of cysteine such as carbocisteine found in cough syrup as mucolytic, have both antioxidant and anti-inflammatory activities.

Currently used antioxidants such as vitamin C and vitamin E are less effective in alleviating oxidative stress damage. Partly because, these antioxidants may not reach pharmacologically active concentration *in vivo.* Furthermore, these nutraceuticals neither eliminate the primary cause of disease nor limit its progression (Furman *et al.,* 2019).

Several plants with medicinal values are also used in management of metabolic and cellular diseases due to the presence of antioxidant activities. Odongo *et al*. (2017), established that methanolic leaf extract of *Rhus vulgarias* collected in Vihiga County, Kenya had good antioxidant and anti-inflammatory activities. Thus, there exist medicinal plants that can be used to manage oxidative stress when validated experimentally.

### 2.4.5 Management of oxidative stress in traditional medicine system

Among the Asian countries, spices have been added to food or eaten raw as antioxidants but there is other implicated importance in use of spices. In one study, clove was considered to have the best antioxidant activity followed by peppermint, allspice, cinnamon and rosemary among other spices (Paur *et al.*, 2011). Similarly, among medicinal plants found in Congo Basin, 70 species belonging to 47 families are used in treatment of complications related to oxidative stress such as hypertension, diabetics, anemia, pain, asthma and cough. Three plant species namely; *Syzygium brazzavillense,*

*Parinari congensis* and *Croton dybowskii* were noted to be widely used among traditional healers as antioxidant (Tchicaillat-Landou *et al.,* 2018).

In South Africa, plants with anti-antioxidant and anti-inflammatory activities have been used to manage pain. In an *in vitro* assay of medicinal plants used in pain and inflammation management, *Peltophorum africanum* extract had better antioxidant activity compared to standard ascorbic acid (Adebayo *et al.*, 2015).

Notably, ethnomedicinal studies indicate that plants with antioxidant activities have both antinociceptive and anti-inflammatory uses. Similarly, *Fagaropsis* species have been used in management of pain and inflammatory related complication with inadequate data to support the use. The findings of this research approved the presence of antinociceptive, anti-inflammatory and antioxidant activities in the leaves of *F. angolensis* and stem bark of *F. hildebrandtii* as claimed by traditional medicine healers with reservation for further primate and clinical studies.

### 2.5 Analysis of pharmacological efficacy of plant extracts

#### 2.5.1 Assay methods for antinociceptive activities

To evaluate either peripheral or central antinociceptive activity of plant extracts, different models of assay are used. Acetic acid-induced writhing model is used to test peripheral antinociceptive activity of plant extracts (Bukhari *et al.*, 2016). In this model, pain induction is through injection of acetic acid peritoneally. The release of endogenous prostaglandins as well as other pain mediators such as histamine, bradykinin and serotonin are responsible for pain. Peritoneal receptors are mediators of abdominal writhing (Yasmen *et al*., 2018).

Hot plate method is used to assay central antinociceptive activity. In this method, mice are placed on a cylindrical open-ended container with metallic floor. Metal plate is heated and the respond to pain is noted when the mice licks the tip of the tail or jumps

when the footpad gets heated ( Muhammad *et al.,* 2012; Cheng *et al.,* 2016; Yimer *et al.,* 2020).

Tail immersion (tail flick test) involves immersion of a small portion of mice tail into hot water or exposure to radiant heat at $55^0$C. Jerk and flick tail movement away from the heat source is recorded as a measure of pain experience (Islam *et al.,* 2014).

Formalin-induced paw licking tests or formalin pain induction method are both used for central antinociceptive activity assay although because of ethical reasons, formalin induced paw licking model has received much criticism and reservations. High concentration of formalin more than 5 % is not recommended (Islam *et al.*, 2014).

In this study, acetic acid-induced writhing model was used because of its' high sensitivity and response to low dose in peripheral antinociceptive activity assay (Musa *et al.*, 2007; Yimer *et al.,* 2020).

**2.5.2 Assay methods for anti-inflammatory activities**

Anti-inflammatory activities of plant extracts can be evaluated by various models such as carrageenan-induced paw edema. This is a biphasic model where the initial phase that occurs at 1 hour to 1.5 hour is characterized by non-phagocytic edema followed by the second phase lasting for 2 to 5 hours, which is predominantly characterized by increased edema formation. The first phase is due to production of histamine, bradykinin and serotonin. The second phase is mainly due to production of prostaglandins. This model is closely related to histamine induced paw edema (Muhammad *et al*., 2012).

Croton-oil ear edema model is used for topical induction of inflammation. Five (5) % croton oil is applied topically to the inner left ear of the mouse. Experimental drug is applied 30 minutes later, on the left inner ear, after 6 hours, the mice is euthanized. The

response is measured by taking the weight of two plugs measuring 6mm in diameter collected from both left and right ear (Oliveira *et al.*, 2013).

Xylene induced ear edema, which is mostly used for steroidal drug assay, act by inhibiting phospholipase $A_2$ enzyme. After I hour of treatment group administration, 50 µl of xylene is smeared on the right ear, the left ear remaining as control. In 1 hour, the mice are euthanized and then the two ears are cut off along the auricle. The auricle with a diameter of 8 mm is weighed to calculate ear edema (Ishola *et al*., 2014; Cheng *et al.*, 2016).

Egg albumin inflammation model is also used for acute inflammatory response investigation. Egg albumin is injected into the paw of mouse and vernier calipers are used to measure paw circumference. This is also a biphasic model with the first phase occurring in the first and second hour, while the second phase occurring from the third up to the fifth hour (Tolu and Dapper, 2016).

Another model that is used to evaluate acute inflammation is the acetic acid-induced capillary permeability model. Upon administration of experimental groups, Evans blue (0.5%) is injected into the tail after 1 hour. The mouse is then injected with 0.7 % acetic acid peritoneally. After 20 minutes, the pleural cavity is washed with normal saline and the liquid collected and centrifuged at 3000 rpm for 10 minutes and absorbent measured at 590nm (Sun *et al.,* 2018).

In this study, the carrageenan-induced paw edema model was chosen because it is the standard inflammation induction model for both non-immune and acute inflammatory response for investigating both plant extract and synthetic drugs (Patil *et al.*, 2019). Since inflammation occurs in polyphase, that could either be short lived or long lasting, this sequential model was precise in evaluating activity as seen from the results.

### 2.5.3 Assay methods for antioxidant activities

Antioxidant in plant extracts is evaluated to ascertain the ability of the extract to scavenge for free radical, hence stabilizing corresponding compounds. The level of antioxidant can be assayed by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method where percentage radical scavenging activity of plant extract is compared to the standard ascorbic acid. Absorbance of the extracts and the standard ascorbic acid is compared graphically in finding the $IC_{50.}$ Other model for antioxidant evaluation include ferric reducing antioxidant power (FRAP) method, Reducing power method and 2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging method (Chaves *et al.*, 2020).

In reducing ferric antioxidant power, 0.2 ml of extract is mixed with FRAP reagent, the mixture is incubated for 30 minutes and absorbance read at 593nm on a spectrophotometer. Blank is prepared by substituting the same amount of extract with methanol (Chaves *et al.,* 2020). Reducing power method involves preparation of mixture of extract and 0.2 mM phosphate buffer solution at pH of 6.6 and 2.5 ml of 1 % potassium ferrocyanide. The mixture is incubated for 20 minutes in a water bath. Then 2.5 ml of 10 % trichloroacetic acid is added and the mixture centrifugated at 3000 rpm for 10 minutes. 2.5 ml of supernatant is then mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % $FeCl_3$. Absorbance is read at 700 nm (Chaves *et al*., 2020).

The assay method, 2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method involves mixture of ABTS, and potassium persulfate kept in the dark for 16 hours at room temperature. Mixture is then diluted with methanol until absorbance value of 1-1.5 at 734 nm. Scavenging properties of plant extract on ABTS radicals is measured against standard drug (Chaves *et al*., 2020). The DPPH model was adopted

in this study because of ease, high sensitivity and usage of stabile radicals (Baliyan *et al.,* 2022).

### 2.5.4 Phytochemical methods of analysis

Phytochemical analysis is done to identify the main constituent classes of secondary metabolites in plant extracts. This is done by either qualitative or quantitative methods. Preliminary analyses are confirmed by qualitative color change of test reagents. Standard analytical methods such as Liebermann Burchard test and dragendoff's test are used to test for the presence of steroids and alkaloids, respectively (Evans, 2009). Other analytical methods such as Gas chromatography, Mass spectrometry, High Performance Liquid Chromatography, High Performance Thin Layer Chromatography, Optimum Performance Laminar Chromatography, X-ray crystallography, Nuclear Magnetic Resonance spectroscopy, IR spectroscopy and UV spectroscopy can be used to quantify or identify specific constituent of phytochemicals ( Nortjie *et al.,* 2022).

In this study, total phenolic content and total flavonoid content was done according to Folin-Ciocalteu and Aluminium chloride methods, respectively. Values of total phenolic and total flavonoids were extrapolated on garlic acid and catechin standard graphs, respectively (Harborne, 1998; Abubakar and Haque, 2020). Follin-Ciocalteu method allows the estimation of all the flavonoids, anthocyanin and non-flavonoid phenolic compounds of all the phenolic compounds available in the sample. In determining the total flavonoid content, aluminum chloride method based upon the formation of stable complex between aluminum chloride, keto and hydroxy group of both flavones and flavonoids was adopted because it allows determination of all flavonoids in plant extracts (Fattahi *et al.,* 2014).

## 2.6 The Family Rutaceae

### 2.6.1 Taxonomic information and distribution of Rutaceae

Rutaceae is commonly known as citrus flowering family plants. It is one of the largest family of the plant order Sapindales with about 2100 species in about 154 genera. The genus comprises of trees, shrubs, lianas and infrequently herbs that occur in tropical and other sub-tropical areas of the world (Simpson, 2010; Tamokou *et al.*, 2017).

In Kenya, there are twenty-seven indigenous plant species reported within ten genera of Rutaceae family. The most common genera of Rutaceae plant family in Kenya are *Calodendrum, Clausena, Diphasia, Diphasiopsis, Teclea, Toddalia, Toddaliopsis, Vepris, Zanthoxylum* and *Fagaropsis* (Mailu *et al.*, 2020).

### 2.6.2 Phytochemical composition of the plant species of the Rutaceae family

Plants species of the family Rutaceae contain various secondary metabolites. Citrus plants from this family contain acetophenones, stilbenes, condensed tannins, terpenoids, flavonoids and low molecular phenolics (Rajkumar *et al.*, 2012). Extracts of *Citrus maxima* showed the presence of the following Phyto-constituents; alkaloids, saponins and carbohydrates. Limonoids also found in this family, have anti-cancer activity. Other phytochemicals in this family include glycosides, carbohydrates, tannin, alkaloids, quinone, phenol and saponins (Wekesa *et al.,* 2023).

Most of the compounds isolated from *Z. gilletii* had a benzophenanthridine alkaloid skeleton that is typical alkaloids from the genus *Zanthoxylum* (Rutaceae). Alkaloids, coumarins, flavonoids, lignans, amides, fatty acids and alcohol have all been isolated from the extracts of the genus *Zanthoxylum* (Wekesa *et al.*, 2023). Phytochemicals identified in the ethanol root bark extract of *V. nobilis* from Rutaceae family were tannin, reducing compounds, alkaloids, steroids, glycosides, polyuronides, starch, coumarins derivatives and flavonoids (Omujal *et al.*, 2020).

### 2.6.3 Biological activities of the plant species of the Rutaceae family

In the past 12 years, 655 coumarin have been isolated. Coumarin isolated from Rutaceae family have anticancer, anti-inflammation, antimicrobial and endocrinal condition management (Santos *et al.*, 1995). *Zanthoxylum limonella* extract have antimalarial activity, mosquito repellant larvicidal activity, antitumor, antimicrobial and ant-inflammatory activity (Supabphol and Tangjitjaroenkun, 2015). Antipyretic, analgesic and anti-inflammatory activities have been reported in the leaves, anti-inflammatory activity in the root bark and leaves, antimicrobial activities in the leaves, stem bark and roots of *V. nobilis* (Omujal *et al*., 2020).

Leaves, stem bark and roots of *C. macroptera* extracts have been shown to exhibit, antioxidant, cytotoxic, antimicrobial, thrombolytic, hypoglycemic, anxiolytic, antidepressant, cardioprotective and hepatoprotective activities (Aktar and Foyzun, 2017). Parts of *A. monophylla* have the following pharmacological activities; leaves and fruits; rheumatic pain and skin infection, leaves; swelling, joint pain, fever and rheumatic pain; roots; cough phlegm (Shelar and Singh, 2023). Ethnopharmacological studies of the genus *Fagaropsis* had shown that plants parts can be used in treatment of malaria, cancer and respiratory diseases (Mutinda *et al.,* 2022)..

### 2.6.4 Traditional medicinal uses of the plant species of the Rutaceae family

The plants of Rutaceae family are known in traditional medicine for mitigation of pain and inflammation in south Africa. These plants include *Ricinus communis, Aloe ferox, Pentanisia prunelloid, Dodonaea viscosa, Ruta graveolas* and *Solanum aculeastrum* (Aremu and Pendota, 2021). In addition, the extracts from various plant species of Rutaceae are used for the management of cancer, upper respiratory tract infections, gastrointestinal disorders, malaria and urogenital disorders in Meru County. These plants include, *Zanthoxylum usambarense, Fagaropsis angolensis, Clausena anista,*

*Toddalia asiatica* and *Zanthoxylum chalybeum* (Muthaura *et al.,* 2007). *Fagaropsis species* are used in snakebite, contagious bovine, improving milk production in cows, gonorrhea, diarrhea, rheumatism, pneumonia, breast cancer, toothache, babesiosis, joint aches and cough (Mutinda *et al*., 2022).

## 2.7 *Fagaropsis angolensis*

### 2.7.1 Taxonomy and distribution of *Fagaropsis angolensis*

*F. angolensis* is a deciduous tree with spreading crown. It grows 7 - 24 m tall but can be as tall as 40 m. It belongs to the phylum; Spermatophyta, order; Sapindales, familiy; Rutaceae and Genus; *Fagaropsis* from the larger Plantae kingdom. *F. angolensis* is a habitat of lowlands in East, Central, Southern Africa and the Central parts of Africa. It is found in Kenya, Tanzania, Rwanda, Eastern Zambia, Mozambique, Zimbabwe, Malawi, Eastern DRC, Ethiopia and Uganda. In Kenya, the plant has been located in Nandi, Embu, Kwale, Taita Taveta and Meru Counties (WFO, 2023).

### 2.7.2 Phytochemistry of *Fagaropsis angolensis*

Phytochemicals are powerful groups of compounds that belong to the secondary metabolites of plants. They include diverse chemical entities such as flavonoids, steroids, saponins, vitamins and polyphenols. These phytochemicals play a role in reproduction, symbiotic association and interaction with other plants. The stem bark of *F. angolensis* contains alkaloids, flavonoids, phenols, tannins, steroids, glycosides, terpenoids and steroids (Alemu and Misganaw, 2020). Water and methanolic extract of leaves, leaf stalk, root bark, root stem and whole roots contains alkaloids, flavonoids, glycosides, phenols, steroids, tannin and terpenoids (Yiaile *et al.,* 2018). Kimutai *et al.,* (2016) established the presence of terpenoids, flavonoids and phenols in acetone and water extracts using thin layer chromatography. Methanol-dichloromethane extraction

of root bark of *F. angolensis* led to discovery of two new norhopane and a nornehopane (Mukavi *et al.*, 2020).

### 2.7.3 Biological activities of *Fagaropsis angolensis*

The root bark extracts of *F. angolensis* have been shown to be effective in the treatment of inflammatory complications including pain. In one study, the extract reduced the levels of tumor necrotic factor alpha, IL-1β, IL-2 and granulocyte macrophage colony stimulating factor in lipopolysaccharide stimulated peripheral blood mononuclear cells (Mukavi *et al.*, 2020). Stem bark extracts have antimicrobial, antispasmodic and antineoplastic activity (Kuglerova *et al.,* 2011; Kimutai *et al.*, 2016; Yiaile *et al.,* 2018; Alemu and Misganaw, 2020). Anticancer activity was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method while antimicrobial activity was assayed by agar plate disc diffusion method and broth micro dilution methods (Kuglerova *et al.*, 2011; Yiaile *et al.*, 2018).

### 2.7.4 Traditional medicinal uses of *Fagaropsis angolensis*

In Embu, Meru and Mbeere communities, *F. angolensis* is used to treat malaria and backache. Kikuyus mainly use it for malaria treatment. Among the Nandi community in former Kaptumo Division, *F. angolensis* bark is boiled in water for treatment of pneumonia, rheumatism and chest problems (Kareru et al. 2006; Kimutai et al. 2016). In addition, *F. angolensis* is used to treat cancer, cough and livestock conditions including bovine or caprine pleuropneumonia, babesiosis and diarrhea (Kewessa *et al.,* 2015; Mihertu and Teshome, 2017).

## 2.8 *Fagaropsis hildebrandtii*

### 2.8.1 Taxonomy and distribution of *Fagaropsis hildebrandtii*

*Fagaropsis hildebrandtii* is a wild deciduous shrub or tree species that grows up to 2-7 meters tall. The plant originates from the larger plantae kingdom, Sapindale order,

Rutaceae family, *Zanthoxyloideae* sub-family and Genus *Fagaropsis*. The plant is found in Tropical areas of the East Africa, including Somali, Kenya and Ethiopia. The plant has a nice smelly fruit when unripe which turns red when ripe. In Kenya, *F. hildebrandtii* has been located in tropical wet and dry parts of Makueni, Kiambu and Nairobi Counties (WFO, 2023).

### 2.8.2 Phytochemistry of *Fagaropsis hildebrandtii*

The water and hexane root bark extracts has been established to contain alkaloids, glycosides, flavonoids, terpenoid, tannin, steroids, saponin, phenols (Muia *et al.,* 2020). In this study, apart from the preliminary study on phytochemical, quantitative study was done on phenols and flavonoids.

### 2.8.3 Biological activities of *Fagaropsis hildebrandtii*

The root extract of *F. hildebrandtii* has antimicrobial activity against *S. aureus* and *S. typhimurium* but not sensitive to *C. albicans*. The extract was prepared from water and hexane solvents and studied by cup-plate agar diffusion test. In this study, the extracts of *F. hildebrandtii* harvested in Makueni county at doses of 300-2000 mg/kg was associated with changes in kidney and liver biochemistry on prolonged administration (Muia *et al*., 2020).

### 2.8.4 Traditional medicinal uses of *Fagaropsis hildebrandtii*

Parts of the plant used are the leaves, stem bark, roots, sap and shoot. Plant concoction is made by either boiling the plant part in water or just soaking it in water. Uses of the plant include treatment of asthma, dysentery, treatment of postpartum hemorrhage, prevention of milk let down and first trimester abortion in pregnant women (Mutinda *et al*., 2022). Other uses include treatment of internal abscess, chronic joint pains, chest pains, stomach pains, ulcers, epilepsy, malaria, pneumonia, respiratory infections and infertility (Wambugu *et al.,* 2011; Muia *et al.,* 2020).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Introduction

This chapter elaborates on where and how plant parts were collected, the extraction procedure and assay methods for antinociceptive, anti-inflammatory, antioxidant and phytochemical constituents found in the leaves of *F. angolensis* and stem bark of *F. hildebrandtii*.

## 3.2 Study area

Leaves of *F. angolensis* were collected from Michegethiu village near Kiang'ombe hills in North Mbeere sub county, Embu County in Kenya (031.545S;03727.112E). Embu County is found in the former Eastern province. It borders Kirinyaga County to the West, Kitui County to the East, Tharaka Nithi County to the North and Machakos County to the South. The County lies at 1750m above sea level. The area has an annual rainfall of 1206 mm and humidity of 70% (Appendindx VII). In Meru community, *F. angolensis* is called, "Mukuriampungu" while in Embu community it's called "Mukuria Hungu". Stem bark of *F. hildebrandtii* was collected in Makueni County, Kilome sub-County, Kasikeu ward, Uvaleni location in Kythani village in Kenya (15250.2S;372225.3E). The County is also found in the former Eastern province and borders Kajiado County to the West, Taita Taveta County to the South, Kitui County to the East and Machakos County to the North. The County lies at 1787 m above sea level. The area has an annual rainfall of 800 mm – 900 mm and humidity of 68% (Appendindx VII). Among the Kamba, *F. hildebrandtii* is locally called "Muvindavindi".

### 3.3 Collection and identification of study plant materials

The leaves of *F. angolensis* (Appendix III) and stem bark of *F. hildebrandtii* (Appendix IV) were collected from Embu and Makueni Counties respectively with help of local herbalist. The leaves were randomly collected by plucking and stem barks by debarking. Voucher specimen was prepared by packaging in airtight carrier bags and transported to the department of botany East Africa Herbarium in Nairobi for identification and authentication (Appendix V, *F. angolensis* and Appendix Vi, *F. hildebrandtii*). The collected plant parts were air-dried at room temperature of 20 ºC and humidity of 45 % at the Department of Pharmacognosy, Mount Kenya University and later ground into dry and fine powder using an electrical motor-driven laboratory plant mill (Buchi, Switzerland AG). The powdered plant material was then kept in airtight bags awaiting water and methanol extraction.

### 3.4 Water extraction

Water extraction was done according to methods described by Evans (2009). Briefly, 250 g of the finely powdered *F. angolensis* leaves, and *F. hildebrandtii* stem bark (Appendix VIII) was soaked in 500 ml distilled water for 2 days and on third day, it was heated at 60 °C for 5 minutes. The mixture was then filtered through the Whatman filter paper No.1 and thereafter lyophilized using a freeze dryer (Thermo Fisher Scientific).

### 3.5 Methanol extraction

Methanol extraction was done according to the procedure described by Harborne, (1998). Briefly, 250 g of the powdered leaves and stem bark of *F. angolensis* and *F. hildebrandtii* respectively were soaked in 1000ml of methanol in a 2-litre conical flask. The flask was then covered with an aluminum foil paper and gently agitated once daily for two days. Thereafter, the mixture was decanted then filtered through a Whatman

filter paper No.1 and concentrated *in vacuo* with use of a rotary evaporator (Stuart® RE300) set at 50 °C. The extract was then dried by evaporation in a closed hot-air oven (i-therm AI-7941) set at 35 °C.

### 3.6 Extracts preparation

Four dose levels of 2 mg per kg body weight ,10 mg per kg body weight, 50 mg per kg body weight and 250 mg per kg body weight of the study plant extract were prepared as per the method described by Earnest and Ajaghaku (2014).

Dose for the mice = (Weight of mice in g /1000 g) × specific plant extract dose per kg body weight).

For example, for a dose level of 250 mg per kg body weight that was given to a mouse that weighed 20 g, 5 mg plant extract was weighed and dissolved in 0.20 ml of physiological saline as per the (OECD, 2002) guidelines to avoid fluid overload. The reference drugs: aspirin and dexamethasone were prepared following the same procedure. The two plant extracts were kept and analyzed separately: avoiding mixing of results through contamination.

### 3.7 Experimental animals

In this research, to ensure uniformity of the results, 8–12-week-old male Swiss-albino mice weighing 20 ± 2 g were purchased from laboratory animal section for breeding at the Public Health department, Pharmacology & Toxicology laboratory at Kabete Campus, University of Nairobi. The Swiss-albino mice were then kept in rectangular laboratory cages (Appendix IX) that measured 35 cm × 25 cm × 18 cm, for 1 week for acclimatization to the laboratory conditions before the study. Soft wood shavings were spread on the floor of the cages to provide warmth. There were enough rodent pellets that were dry and clean water *ad-libitum*. The study mice were not fed on pellets the night before study. Experimental protocol approval was obtained from the biosafety,

animal ethics committee of Mount Kenya University before commencement of the study.

### 3.8 Assay for antinociceptive activity

In this study, acetic acid-induced writhing model as described by Rashid *et al.* (2015) was used to evaluate the analgesic activity of the plant extracts. Briefly, according to OECD 423 guideline, male Swiss albino mice was randomly assigned to 6 treatment groups (5 mice per group) as follows: Negative control group [A] received normal saline (*p.o*); positive control group [B] was given aspirin (150 mg/Kg body weight *p.o*) experimental groups (C-F) received the plant extract at 2 mg/Kg body weight, 10 mg/Kg body weight, 50 mg/Kg body weight and 250 mg/Kg body weight respectively. Dose levels were selected based on acute toxicity study previously done by Muia *et al*., (2020) and Alemu and Misganaw, (2020). All the mice groups weighing 20 g were injected (*ip*) with 60 mg/kg of 200 µl acetic acid, 30 minutes after administration of plant extract/aspirin standard/normal saline (table 3.1). The writhing frequency in each of the mice was then determined five minutes post acetic acid injection and thereafter 15 minutes. A reduction in the number of writhing in mice given plant extract when compared to the control group was considered as evidence of antinociceptive potential. The antinociceptive activity of plant extracts and standard aspirin was expressed as percentage change in writhing in study groups compared to the negative control (Cheng *et al.,* 2016).

**Table 3.1: Experimental design for determination of the antinociceptive activities for a mouse weighing 20 g.**

| Group | Treatment administered |
|---|---|
| A;Negative control | Normal Saline (*p.o.*) + 60mg/kg of 200 µl acetic acid |
| B;Positive control | Aspirin (150 mg/Kg bw; *p.o.*) + 60mg/kg of 200 µl acetic acid |
| C;Experimental group | Plant extract (2 mg/Kg bw; *p.o.*) + 60mg/kg of 200 µl acetic acid |
| D;Experimental group | Plant extract (10 mg/Kg bw; *p.o.*) + 60mg/kg of 200 µl acetic acid |
| E;Experimental group | Plant extract (50 mg/Kg bw; *p.o.*) + 60mg/kg of 200 µl acetic acid |
| F;Experimental group | Plant extract (250 mg/Kg bw; *p.o.*) + 60mg/kg of 200 µl acetic acid |

***NB: All mice received acetic acid ip***.

*Each group is comprised of 5 mice; p.o=oral route; i.p = intra peritoneal route. The units of administration were 200 µl. Extract: Water or methanol extract of F. angolensis and F. hildebrandtii prepared in normal saline.*

### 3.9 Assay for anti-inflammatory activity

The anti-inflammatory activity of water and methanol extract of *F. angolensis and F. hildebrandtii was* evaluated using carrageenan-induced right hind paw edema mouse model as previously described by Yimer *et al.*, (2020) with slight modifications where necessary. Briefly, 30 Swiss albino male mice weighing 20 ± 2 g were randomly allocated into 6 groups (Group A to F) containing 5 mice per group. Edema was induced through sub plantar injection of freshly prepared 100 µl 1 % of carrageenan in distilled water into the right hind paw of each mouse one hour after administration of the plant extract/dexamethasone standard/normal saline. The negative control [group A] was administered with physiological saline *p.o,* positive control [group B] received 10

mg/Kg body weight of dexamethasone *p.o.* and experimental groups C-F received the plant extracts at increasing single *p.o* doses of 2 mg/Kg body weight, 10 mg/Kg body weight, 50 mg/Kg body weight and 250 mg/Kg body weight, respectively (table 3.2). The linear paw circumference was measured at hourly intervals for 5 hours. The percentage edema inhibition was then calculated as previously described by Rahman *et al.* (2011).

**Table 3.2: Experimental design for determination of the anti-inflammatory activities**

| Group | Treatment Administered |
|---|---|
| A;Negative Control | Normal Saline (*p.o.*) + 100 µl of carrageenan (sub plantar injection) |
| B;Positive Control | Dexamethasone (10 mg/Kg bw *p.o.*) + 100 µl of carrageenan (sub plantar injection) |
| C;Experimental group | Extract (2 mg/Kg bw *p.o.*) + 100 µl of carrageenan (sub plantar injection) |
| D;Experimental group | Extract (10 mg/Kg bw *p.o.*) + 100 µl of carrageenan (sub plantar injection) |
| E;Experimental group | Extract (50 mg/Kg bw *p.o.*) + 100 µl of carrageenan (sub plantar injection) |
| F;Experimental group | Extract (250 mg/Kg bw *p.o.*) + 100 µl of carrageenan (sub plantar injection) |

***NB: All mice received carrageenan sub plantar.***

*Each group consisted of 5 mice; p.o =oral route. Extract: Water or methanol extract of F. angolensis and F. hildebrandtii prepared in normal saline.*

### 3.10 Assay for antioxidant activity

The antioxidant activity of *F. angolensis* and *F. hildebrandtii* extracts was evaluated using 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay as described by Moriasi *et al.* (2020) with few modifications. *F. angolensis* and *F. hildebrandtii* leaves and stem bark extracts, respectively and ascorbic acid as standard (10 mg/10ml)

were prepared in concentration of 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml and 1000 µg/ml in methanol. 1.4 ml extract/ascorbic acid standard was transferred into cuvette containing 1.6 ml of DPPH (0.1 mM) prepared in methanol. A mixture of 1.6 ml 0.1 mM DDPH solution in methanol (Scharlau) and 1.4 ml of methanol served as the negative control. The test samples and the standard were prepared in triplicate which was then kept in a dark closet at room temperature for approximately 15 minutes allowing reaction to take place. Absorbance was measured at wavelength of 517 nm against methanol as blank using an ultraviolet-visible spectrophotometer (Shimadzu 1601) in triplicates. The percentage radical scavenging activity, (% RSA) was calculated and $IC_{50}$ computed from the graph of concentration against % RSA (Rajurkar and Hande, 2011).

### 3.11 Qualitative analysis of phytochemicals

To determine phytochemical composition of each extract, qualitative method as was done by Evans, (2009) and Sathisha *et al*., (2011), was used with slight variation. Presence or absence was denoted by (+) and (-), respectively.

#### 3.11.1 Steroid test (Lieberman burchard test)

Two (2) mg of plant extract was added to 1ml of acetic anhydride and the mixture boiled in a water bath using boiling tubes for 5 minutes. The extracts were then filtered while hot, cooled and to 2 ml of each filtrate and equal volume of concentrated sulphuric acid was added. A yellow ring at the separating level of the two liquid which turns red after 2 minutes indicated the presence of steroids (Evans, 2009; Sathisha *et al.*, 2011).

#### 3.11.2 Phenol test (Ferric chloride test)

One (1) mg of plant extract was added to 2 ml of distilled water. In 2 ml of each extract solution, 5 drops of 10 % Ferric chloride solution was added and an immediate

formation of a green precipitate indicated presence of phenols (Evans, 2009; Sathisha *et al*., 2011).

### 3.11.3 Saponin test (Frothing test)

Five (5) mg of plant extracts were added to a test tube with 4 ml of distilled boiling water. Water mixture was then allowed to cool. It was agitated vigorously and left to stand. Persistent frothing suggested presence of saponins (Evans,, 2009; Sathisha *et al.,* 2011).

### 3.11.4 Tannins test (Ferric chloride test)

Two (2) mg of plant extract was placed in a test tube. Thereafter, three drops of 5 % ferric chloride solution were added in each test tube. Development of a brown–green precipitate with ferric chloride suggested presence of tannins (Evans, 2009; Sathisha *et al.,* 2011).

### 3.11.5 Cardiac glycosides test (Keller killiani test)

Three (3) mg of extract was added into 5 ml of distilled water. Two (2) ml of glacial acetic acid was then added followed by 2 drops of ferric chloride. One (1) ml concentrated sulphuric acid was added to the test tube along the inner wall. The presence of purple color indicated presence of glycoside whose aglycone moiety has unsaturated lactone ring (Evans, 2009; Sathisha *et al.,* 2011).

### 3.11.6 Alkaloid test

**(a) Mayer's test**

One (1) mg of plant extract was mixed with about 2 ml of 1 % hydrochloric acid, warmed and filtered. Two (2) ml of the filtrate was mixed with 1 ml Mayers reagent consisting of mercuric chloride (1.36 g) and of potassium iodide (5.00g). The

appearance of cream-colored precipitate was a positive indication for the presence of alkaloid (Evans, 2009; Sathisha *et al.,* 2011; Abubakar and Haque, 2020).

**(b) Dragendoff's test**

Two (2) mg of each extract was dissolved into 5 ml of distilled water. Two (2) ml of Concentrated hydrochloric acid was then added to each of the solutions and the mixture filtered. Three (3) ml of the filtrate was collected in a test tube and 1ml of dragendoff reagent consisting of potassium bismuth iodide, bismuth nitrate (Bi $(NO_3)_3$), tartaric acid and potassium iodide (KI) was added. Orange precipitate indicated presence of alkaloids (Evans, 2009; Sathisha *et al.*, 2011; Abubakar and Haque, 2020).

**(c) Wagner's test**

Two (2) drops of Wagners reagent (2 gm of iodine and 6 gm of potassium iodide in 100 ml of distilled water) was added to 1 mg of the extract and mixed well. Appearance of reddish precipitate indicated the presence of alkaloids (Evans, 2009; Sathisha *et al.,* 2011; Abubakar and Haque, 2020)..

**3.11.7 Flavonoid test (alkaline reagent test)**

Five (5) drops of 5 % sodium hydroxide solution were added to 3 mg of each plant extract. Thereafter, 0.5 ml of 2 M hydrochloric acid was then added. Immediate development of a red color indicated the presence of flavonoid (Evans, 2009; Sathisha *et al.*, 2011; Abubakar and Haque, 2020).

**3.11.8 Terpenoid test (Salkowski's test)**

Three (3) mg of the plant extract in a test tube was mixed with 1 ml of chloroform followed by careful addition of 5 drops of concentrated sulphuric acid. The formation

of blue-green ring indicated the presence of terpenoids (Evans, 2009; Sathisha *et al.,* 2011; Abubakar and Haque, 2020).

### 3.11.9 Coumarin test (Sodium hydroxide test)

In 5 gm of plant extract, 3 ml of 10 % sodium hydroxide was added. Formation of yellow color confirmed presence of coumarin (Evans, 2009; Sathisha *et al.*, 2011; Abubakar and Haque, 2020).

### 3.11.10 Anthocyanines test (Hydrochloric acid test)

Two (2) ml of 2N HCL was treated with approximately 2 mg of plant extract. Thereafter, the mixture was added to 2 ml of 10 % ammonium. Blue violet color formation indicated presence of anthocyanines (Evans 2009; Sathisha *et al.*, 2011; Abubakar and Haque, 2020).

### 3.11.11 Anthraquinone test (Borntragger's test)

In 5 mg of plant extract, 10 ml of benzene was added and lastly, 5 ml of 10 % ammonium was added and agitated. Pink color indicated presence of Anthraquinones (Evans, 2009; Sathisha *et al.,* 2011; Abubakar and Haque, 2020).

## 3.12 Quantitative phytochemical assay

### 3.12.1 Total phenolic content of the water and methanol extracts

Total phenolic content of the water and methanol extracts of the leaves of *F. angolensis* and stem bark of *F. hildebrandtii* were determined by Folin-Ciocalteu assay method. The reaction mixture was reconstituted by mixing 500 µl of plant extracts (1000 mg/ml) or Garlic acid (Loba chemie) as standard at concentrations levels of 150 µg/ml, 75 µg/ml, 37.5 µg/ml, 18.75 µg/ml, 9.375 µg/ml and 4.687 µg/ml with 1500 µl 10 % Folin-Ciocalteu reagent in each test tube. Upon mixing extract or Garlic acid with Folin-Ciocalteu (Loba chemie) reagent and incubating for five minutes, 1500 µl of 7.5% sodium carbonate ($Na2CO_3$) was added, mixed by swirling and incubated in the dark

for 60 minutes under room temperature. Absorbance of the reaction mixture was read at 760nm against reagents without sample/standard as the blank using a UV-Vis spectrophotometer (Shimadzu 1601). The standard Garlic acid calibration curve was constructed using the absorbance of the various concentrations of Garlic acids used. Using the regression equation, the concentration of total phenol in the extracts was computed and expressed as milligram of Garlic acid equivalent per gram of dry weight (GAE, mg/g dw), (Saeed *et al.*, 2012).

### 3.12.2 Total flavonoid content of the water and methanolic extracts

The aluminum chloride calorimetric method was used to evaluate the total flavonoid content in leaves and stem bark of *F. angolensis* and *F. hildebrandtii* respectively. This briefly involved mixing 125 µl of the samples (concentration level of 1 g/ml) extract or catechin (Sigma Aldrich) at concentration levels of 200 µg/ml–12.5 µg/ml with 100 µl 5 % of sodium nitrate and incubated at room temperature for six minutes. Upon completion of the incubation period, exactly 75 µl of 4 % sodium hydroxide was added followed by 750 µl of 2% aluminum chloride to make a final volume of 1000 µl. Using distilled water, this volume was made up to 2500 µl and absorbance measured at 510 nm using UV-spectrophotometer double beam (Shimadzu 1601) against reagents without sample/standard as the blank. The catechin calibration curve was plotted from the absorbance versus concentration values and the regression equation obtained. Using the equation, the concentration of the total flavonoid was computed and expressed as milligrams of catechin equivalent per gram of dry weight (CE, mg/g dw), (Cosmulescu *et al.,* 2017).

## 3.13 Data analysis

The obtained raw data (appendix X) was tabulated in Microsoft excel 365 and then descriptive analysis was done using Minitab version 19.2 or GraphPad prism software

to obtain mean and standard deviation. One way or Two-way analysis of variance (ANOVA) was later done to compare group means with statistical significance set at $P \leq 0.05$. This was followed by Tukey's post hoc tests for multiple comparison. Analyzed data was presented by tables or graphs.

### 3.14 Ethical consideration

Permission for carrying out this study and ethical clearance was gotten from Masinde Muliro University of Science and Technology (Appendix I), particularly School of Postgraduate Studies (MMUST/IREC/119/2023). In addition, a research permit was obtained from The National Commission for Science, Technology and Innovation (NACOSTI) (Appendix II) before commencement of the research (NACOSTI/P/23/24581). Efforts were made to ensure reduction of animal suffering by adopting, organization for economic co-operation and development (OECD 423) guidelines, Animal Research; Reporting of *In vivo* Experiments (ARRIVE) guidelines, version 2.0 as well as the recommendation of the biosafety, animal ethics committee of Mount Kenya University.

# CHAPTER FOUR

# RESULTS

## 4.1 Introduction

This chapter presents figures and tables of the study findings. It shows graphical representation of antinociceptive, anti-inflammatory and antioxidant activities. Furthermore, tables and graphs were used to represent qualitative phytochemical composition and quantitative analysis, respectively.

## 4.2 Antinociceptive activity of *F. angolensis* leaves, water and methanol extracts

The antinociceptive activities of *F. angolensis* water and methanol extract at increasing dose level of 2 mg/kg-250 mg/kg was evaluated by calculating percentage inhibition of acetic acid-induced writhing in mice against aspirin 150mg/kg as standard. As figure 4.1 shows, the activity of methanol and water extracts increased in a dose dependent fashion reaching a maximum pain suppression of 81.95±10.76% and 83.80±6.73%, respectively, at 250 mg/Kg (Figure 4.1) against 84.99±3.26% for the standard aspirin. Notably, methanol leaf extract of *F. angolensis* at 50 mg/kg and 250 mg/kg, had comparable percentage writhing inhibition to standard aspirin 150 mg/kg ($p>0.05$; Figure 4.1 C and D). However, water extracts exhibited significantly lower percentage inhibition of writhing compared to aspirin 150 mg/kg at dose range of 2 mg/kg – 250 mg/kg, ($p<0.05$; one-way ANOVA, Tukey's test; Figure 4.1). The water extract as well exhibited significantly lower percentage writhing inhibition at all tested doses when compared to the methanolic extracts ($p<0.05$, one-way ANOVA, Tukey's test; Figure 4.1). Altogether, these data indicate potent antinociceptive activity of *F. angolensis* leaf extracts in mice, particularly methanol extracts at doses 50 mg/Kg and above.

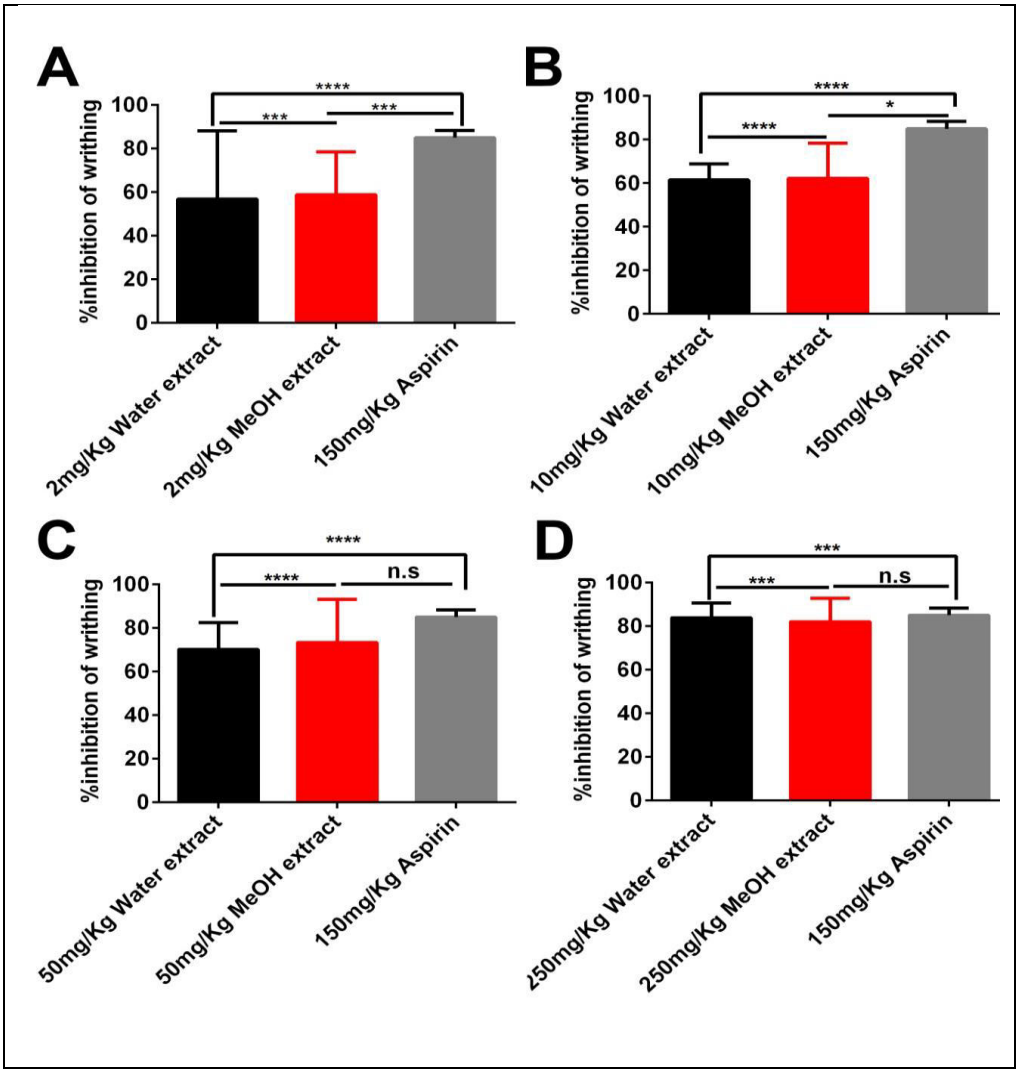



**Figure 4.1: Percentage inhibition of writhing by increasing doses of *F. angolensis* water and methanol leaf extracts**

*A). 2 mg/Kg, B) 10 mg/kg, C) 50 mg/Kg and D) 250 mg/Kg in comparison with aspirin standard (150 mg/Kg). Data was analyzed by one-way ANOVA followed by Tukey's post hoc test and presented as mean±SD. Abbreviation, ns; not significant, MeOH; methanol, Mg; milligram, Kg; kilogram, SD; standard deviation, *; p<0.05, ***; P<0.001, ****; p<0.0001.*

### 4.3 Antinociceptive activity of *F. hildebrandtii* stem bark, water and methanol extracts

The antinociceptive activities of *F. hildebrandtii* water and methanol extracts at increasing dose (2-250 mg/Kg, *p.o*) was evaluated by calculating the percentage inhibition of acetic acid-induced writhing in mice compared with 150 mg/kg of aspirin as standard drug. Water and methanol extracts of *F. hildebrandtii* stem bark exhibited a dose-dependent inhibition of acetic acid-induced writhing in mice.

Notably, the percentage inhibition of acetic acid-induced writhing by 50 mg/kg and 250 mg/kg of *F. hildebrandtii* methanolic extract was comparable to that of the standard aspirin at 150 mg ($P>0.05$, one-way ANOVA, Tukey's test; Figure 4.2 C and D). However, at 2 mg/kg and 10 mg/kg of *F. hildebrandtii* methanolic extract and *F. hildebrandtii* water extract at all the tested dose levels (2-250 mg/kg) exhibited significantly lower percentage inhibition of acetic acid-induced writhing compared to standard aspirin at 150 mg/kg ($P<0.05$, Tukey's test). Figure 4.2 shows that methanol extract of *F. hildebrandtii* exhibited significantly higher percentage inhibition of acetic acid-induced writhing compared to the water extract at all the tested concentrations ($P<0.05$, one-way ANOVA; Tukey's test).

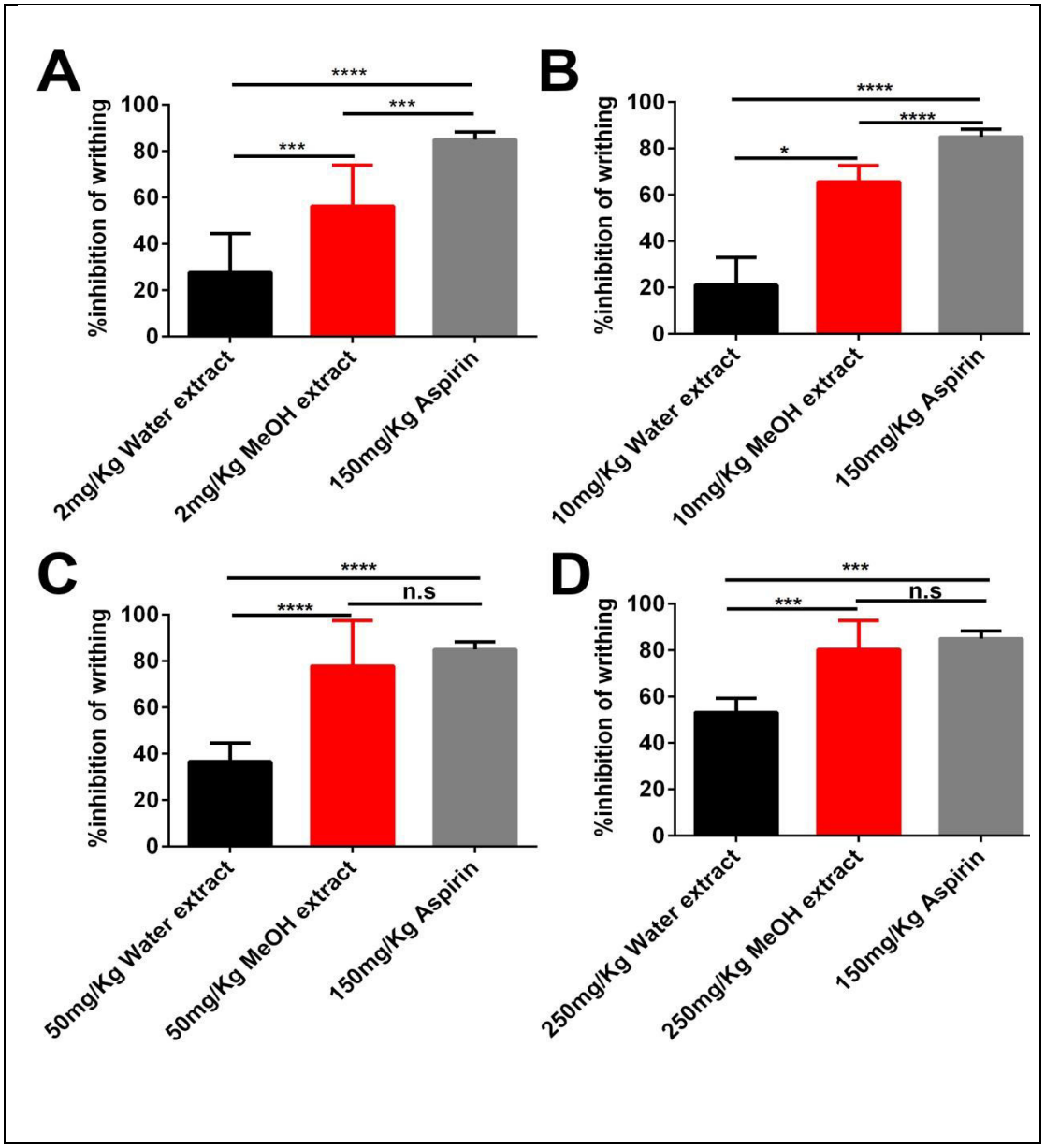



**Figure 4.2: Percentage inhibition of writhing by increasing doses of *F. hildebrandtii* stem bark, water, and methanol extracts**

*A). 2 mg/Kg, B) 10 mg/kg, C) 50 mg/Kg and D) 250 mg/Kg in comparison with aspirin standard (150 mg/Kg). Data was analyzed by one-way ANOVA followed by Tukey's post hoc test and presented as mean±SD. Abbreviation, ns; not significant, MeOH; methanol, Mg; milligram, Kg; kilogram, SD; standard deviation, *; p<0.05, ***; P<0.001, ****; p<0.0001.*

## 4.4 The anti-inflammatory activity *F. angolensis* leaf, water and methanolic extracts

The ability of the water and methanolic leaf extracts of *F. angolensis* in inhibiting carrageenan-induced oedema, was categorized as an appraisal of their anti-inflammatory activity. During the first hour, the results showed no significant difference in percentage inhibition of oedema in experimental animals treated with methanolic leaf extracts of *F. angolensis* at all the doses and those treated with the reference drug (Dexamethasone) ($P>0.05$; Figure 4.3). Notably, during the first hour, there was significantly higher activity of water extract than methanol extract ($P<0.05$; Figure 4.3). The same trend was observed in oedematose animals treated with the water and methanolic leaf extract of *F. angolensis* at a dose level of 2 mg/kg and 10 mg/kg during the second hour. Still at the second hour, *F. angolensis* water, leaf extract at 10 mg/kg recorded similar activity to the standard dexamethasone, at the fifth hour as shown in Figure 4.3($P>0.05$).

At the third hour, methanol extract had significantly greater activity than water extract except at 10 mg/kg ($P<0.05$; Figure 4.3). *F. angoleneis* water extract at 2 mg/kg and 10 mg/kg recorded similar activity to the standard dexamethasone at the fifth hour ($P>0.05$; Figure 4.3). Likewise, at the fourth hour, the percentage inhibition of carrageenan-induced oedema in experimental animals treated with methanol extract at 2 mg/kg of *F. angolensis* and those administered with dexamethasone was not significantly different ($P>0.05$; Figure 4.3). Notably, methanol extract exhibited significantly higher activity than water extract except at 10 mg/kg dose level. The results showed that the percentage inhibition of carrageenan-induced paw oedema in animals treated with the water and methanol leaf extract of *F. angolensis* at a

concentration of 2 mg/kg and 10 mg/kg was similar to each other at the fourth hour ($P$>0.05; Figure 4.3).

There was no significant difference between the percentage inhibitions of carrageenan-induced paw oedema in animals treated with 2 mg/Kg, 10 mg/kg, 50mg/kg, 250 mg/kg of the water leaf extract of *F. angolensis* and those treated with 2 mg/kg, 50 mg/Kg, 250 mg/kg of the methanolic leaf extract of *F. angolensis* at the fifth hour compared to the standard dexamethasone 10mg/kg ($P$>0.05; Figure 4.3). The activity of *F. angolensis* methanolic leaf extract at 50 mg/kg was similar to the activity of the same extract at 250 mg/kg dose level at the fifth hour ($P$>0.05). Methanol extract had significantly greater activity than water extract at all dose levels except at 10mg/kg.

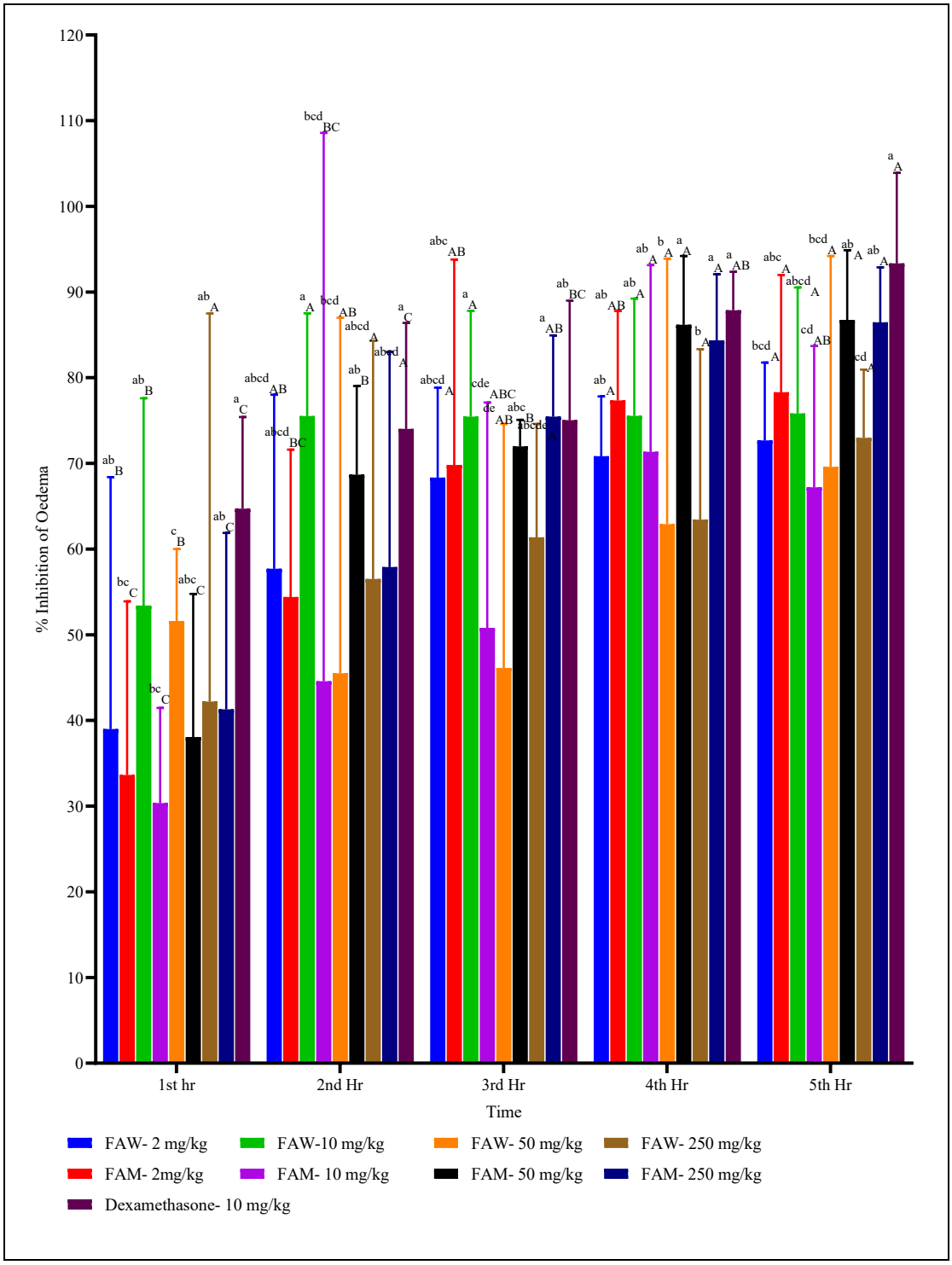



**Figure 4.3: Percentage inhibition of carrageenan-induced paw edema in experimental animals treated with water and methanol leaf extracts of *F. angolensis***

*Values are expressed in the form of Mean ± SD (n=5). Values with the superscript lowercase letters within the same hour and those with uppercase subscript letters*

*across the five-hour period are not significantly different ($p<0.05$; Two-Way ANOVA with Tukey's post hoc). FAW: Water extract of F. angolensis; FAM: Methanolic extract of F. angolensis.*

### 4.5 The anti-inflammatory activity of *F. hildebrandtii* stem bark, water and methanolic extract

This study also investigated the anti-inflammatory effects of the water and methanolic stem bark extracts of *F. hildebrandtii.* The results showed no significant difference in percentage inhibitions of edema in experimental animals treated with the water, stem bark extract of *F. hildebrandtii* at doses of 2 mg/kg and 50 mg/kg across the five-hour period ($P>0.05$; Figure 4.4). The results indicates that at 250 mg/kg, *F. hildebrandtii* methanol extract had similar activity to that of the standard dexamethasone 10 mg/kg but significantly greater in activity compared to water extract at first hour. In all dose levels at one hour, methanol extract had greater activity than water extract ($P<0.05$; Figure 4.4). Compared to other time periods, 2 mg/kg water extract, 10 mg/kg methanol extract and 50 mg/kg water extract had similar activity to the standard drug at the fifth hour ($P>0.05$; Figure 4.4).

During the second hour, there was no significant difference in activity between animals administered with 2 mg/kg, 50 mg/kg water extract and the standard drug dexamethasone at the fifth hour ($P>0.05$; Figure 4.4). Methanol extract exhibited significantly higher activity compared to water extract during this hour ($P<0.05$; Figure 4.4). However, it was observed that the percentage inhibitions of edema in dexamethasone-treated animals was significantly different from that observed in animals treated with the water, stem bark extracts of *F. hildebrandtii* at dose levels of 2 mg/kg and 50 mg/kg ($P<0.05$; Figure 4.4).

In the third hour, there was a significant difference in activity observed in mice treated with methanol extract at 2 mg/Kg, 10 mg/Kg, 50 mg/Kg, 250 mg/Kg body weight, and those administered with 10 mg/Kg body weight of dexamethasone ($P$<0.05; Figure 4.4). Water extract at 2 mg/kg and 50 mg/kg had similar activity to dexamethasone 10mg/kg at fifth hour. The activity of methanol extract was significantly greater than water extract at all dose levels ($P$<0.05; Figure 4.4).

Similarly, at the fourth hour, the results showed significant difference between the percentage inhibitions of paw oedema of methanolic stem bark extracts of *F. hildebrandtii* at concentrations of 2 mg/Kg, 10 mg/Kg, 250 mg/Kg and dexamethasone 10mg/kg standard ($P$<0.05; Figure 4.4). Notably, no significant difference was observed between water extract at 10 mg/kg and standard drug dexamethasone at the fourth hour ($P$>0.05; Figure 4.4). Activity of methanol extract was significantly greater than the activity of water extract in all dose levels at fourth hour.

In the fifth hour, there was no significant difference in activity of water and methanolic extracts of *F. hildebrandtii* at doses of 2 mg/Kg, 10 mg/Kg, 50mg/kg, 250 mg/kg and dexamethasone 10 mg/kg ($P$>0.05; Figure 4.4). Methanol extract had significantly greater activity than water extracts except at dose level of 10 mg/kg. The activity of the standard dexamethasone 10 mg/kg was similar to the activity of methanolic extract at 250 mg/kg ($P$>0.05; Figure 4.4).

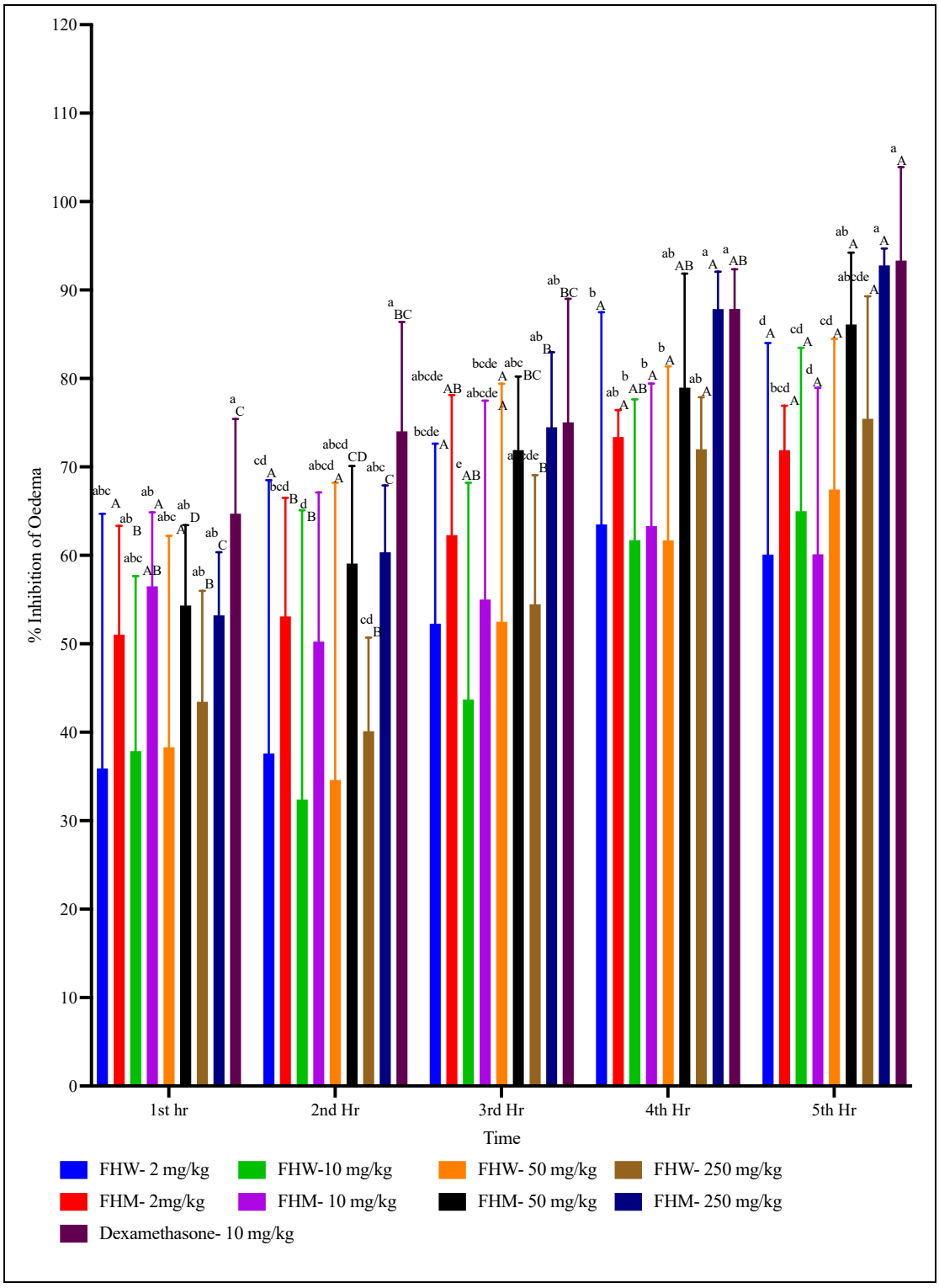



**Figure 4.4: Percentage inhibition of carrageenan-induced paw edema in experimental animals treated with water and methanol stem bark extracts of *F. hildebrandtii***

*Values are expressed in the form of Mean ± SD (n=5). Values with the superscript lowercase letters within the same hour and those with uppercase subscript letters across the five-hour period are not significantly different ($p<0.05$; Two-Way ANOVA with Tukey's post hoc). FHW: Water extract of F. hildebrandtii; FHM: Methanolic extract of F. hildebrandtii*.

### 4.6 Antioxidant activity of *F. angolensis* leaves, water and methanol extracts

*F. angolensis* water extract at concentrations ranging from 0.01-1000 µg/ml showed free radical scavenging activity of between 53.25±7.58 % - 86.56±3.25 %. Methanol extract at concentrations ranging from 0.01-1000 µg/ml showed free radical scavenging activity of between 47.94±3.85 % - 91.85±0.56 %. Ascorbic acid at the same concentrations range of 0.01-1000 µg/ml showed free radical scavenging activity of between 26.51±6.77 % - 96.38±0.89 %.

Overall, the leaf extracts and ascorbic acid exhibited a concentration dependent increase in % RSA ($p<0.0001$, two-way ANOVA, Figure 4.5). Post hoc analysis revealed that at lower concentrations range of 0.01 to 1 µg/ml, *F. angolensis* methanolic and water extracts had significantly higher % RSA than the standard ascorbic acid ($p<0.0001$, Tukey's test), with no significant differences between the water and methanol extracts. At the 10 µg/ml, ascorbic acid had significantly higher % RSA than both the methanolic and water extracts ($p<0.0001$, Tukey's test). However, at a much higher concentration of 100 µg/ml and 1000 µg/ml, the % RSA of ascorbic acid standard was only significantly higher than that of water extract $p<0.001$ and $p<0.005$, respectively (Figure 4.5), but not the methanolic extract ($p>0.05$, Tukey's test). The $IC_{50}$ for the water extract, methanol extract and ascorbic acid were 0.01 µg/ml, 0.90 µg/ml and 5.674 µg/ml, respectively. Collectively, figure 4.5 indicates that *F. angolensis* water

and methanolic extracts have high potency at lower concentration but lower potency at high concentration compared to ascorbic acid. Methanol extract had higher efficacy compared to water extract.

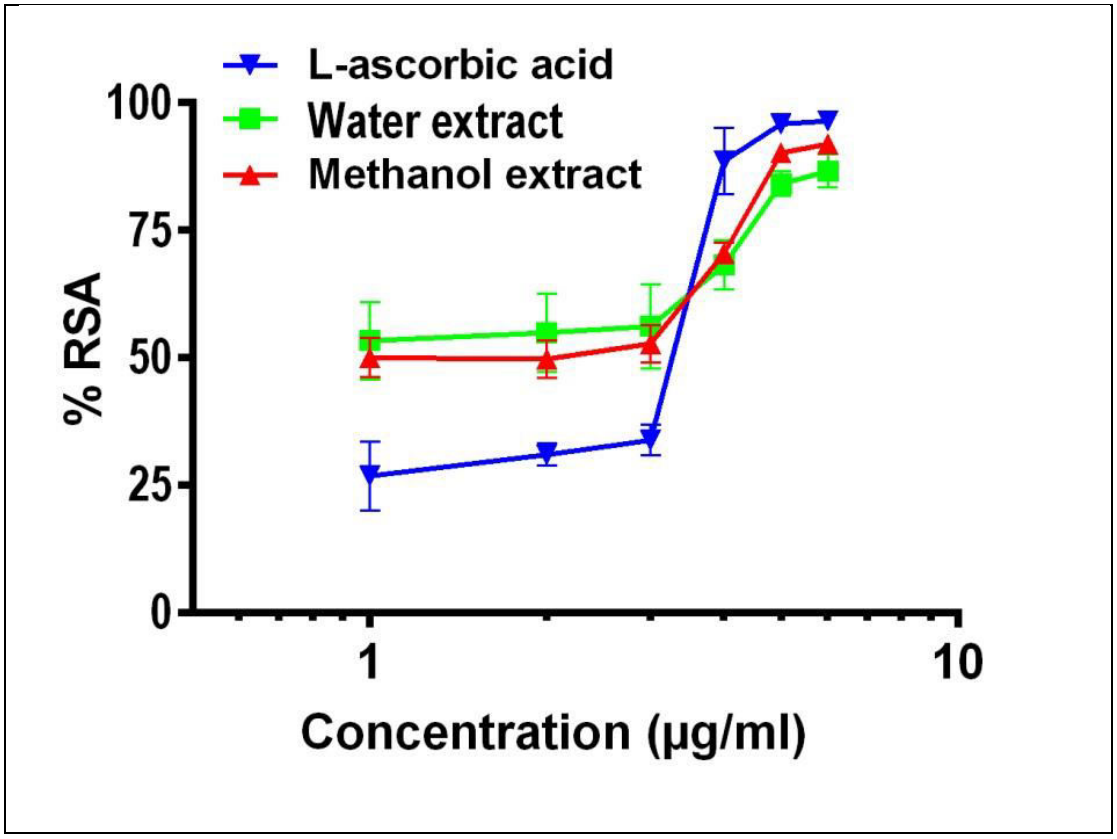



**Figure 4.5: Antioxidant activity of *F. angolensis* leaves, water and methanol extracts**

*Y-axis represents % RSA while the x-axis represents the log of concentration from 0.01 µg/ml - 1000 µg/ml. Abbreviation, % RSA; percentage radical scavenging activity, µg/ml; micro gram per milliliter.*

### 4.7 Antioxidant activity of *F. hildebrandtii* stem bark, water and methanol extracts

*F. hildebrandtii* water extract at concentrations ranging from 0.01-1000 µg/ml showed free radical scavenging activity of between 48.81±5.59 % - 93.64±1.39%. Methanol extract at concentrations ranging from 0.01-1000 µg/ml showed free radical scavenging

activity of between 49.26±3.63 % - 93.56±0.90 %. *F. hildebrandtii* water and methanol extracts exhibited concentration-dependent increase in % RSA reaching a maximum of 93.64±1.39 and 93.56±0.9, respectively (Figure 4.6, $P>0.05$; two-way ANOVA). Comparison with the standard ascorbic acid indicated that both extracts have a higher % RSA at lower concentration ranges of 0.01-1 µg/ml ($P<0.05$, Tukey's test). At concentration range of 10-100 µg/ml, *F. hildebrandtii* water and methanolic extracts exhibited lower % RSA compared to ascorbic acid (Figure 4.6, $P<0.05$, Tukey's test). However, there were no significant differences among the three treatments at 1000 µg/ml ($p>0.05$). The $IC_{50}$ for the water, methanol and ascorbic acid was 0.98 µg/ml, 0.987 µg/ml and 5.674 µg/ml respectively.

Collectively, this data indicates that *F. hildebrandtii* water and methanolic extracts are more potent at lower concentration but less potent at high concentration. Equally, the activity of both water and methanol extracts were the same at maximum concentration, as shown in figure 4.6 below. Comparatively, the activity of plant extract at maximum concentration was like that of the standard drug, ascorbic acid.

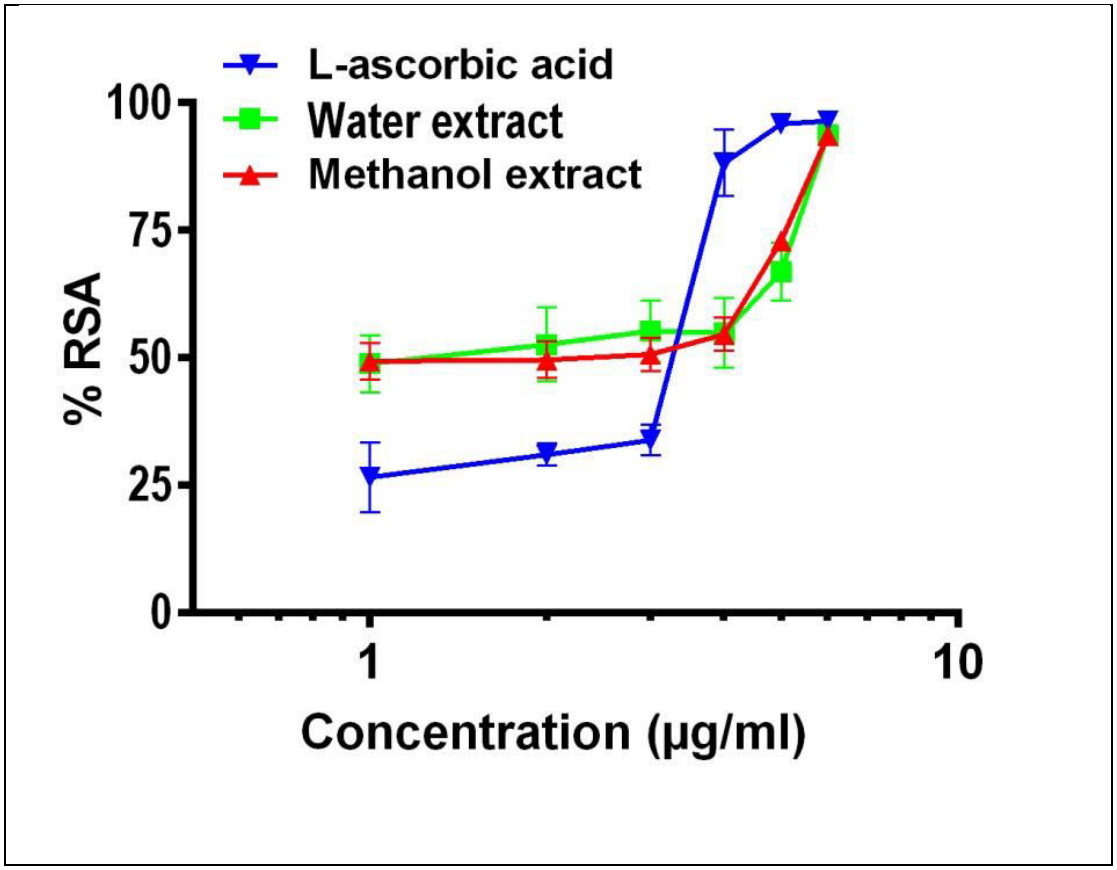



**Figure 4.6: Antioxidant activity of *F. hildebrandtii* stem bark, water, and methanol extracts**

*Y- axis represents % RSA while the x- axis represents the log of concentration from 0.01 µg/ml -1000 µg/ml. Abbreviations: % RSA; percentage radical scavenging activity, µg/ml; micro gram per milliliter.*

## 4.8 Phytochemical composition of *F. angolensis* leaves, water and methanol extracts

Qualitative phytochemical assay of *F. angolensis* water extract revealed the presence of phenols, steroids, alkaloids, glycosides, flavonoids, coumarins and anthraquinones. There was absence of terpenoids, saponins, tannins and anthocyanins. Methanol extract exhibited presence of steroids, phenols, flavonoids, coumarins, tannins, glycosides, alkaloids, terpenoids and anthraquinones but absence of anthocyanins and saponins (Table 4.5).

**Table 4.1: Phytochemical content of *F. angolensis* leaves, water and methanol extracts**

| Phytochemical | Test | Inference | |
|---|---|---|---|
| | | Water extract | Methanol extract |
| Steroids | Liebermann burchard test | + | + |
| Phenols | Ferric chloride test | + | + |
| Saponins | Frothing test | - | - |
| Tannins | Ferric chloride test | - | + |
| Cardiac glycosides | Keller kiliani test | + | + |
| Alkaloids | Dragendoff's test | + | + |
| | Mayer's test | + | + |
| | Wagner's test | + | + |
| Flavonoids | Alkaline reagent test | + | + |
| Terpenoids | Salkowski's test | - | + |
| Coumarins | Sodium hydroxide test | + | + |
| Anthocyanins | Hydrochloric acid (2N) test | - | - |
| Anthraquinones | Borntragger's test | + | + |

*(+) presence; (-) absence*

### 4.8.1 Total phenolic and total flavonoid content of *F. angolensis* leaf extracts

The total phenol content of the methanolic leaf extract was 55.52±3.05 mgGAE/g, which was significantly higher than 48±0.19 mgGAE/g of the water extract ($p$<0.05; t-test; Figure 4.7). Similarly, the methanolic leaf extract had significantly higher levels of total flavonoids than the water extracts (172.53±7.09 mgCE/g vs. 42.22±0.1 mgCE/g; $p$<0.05; t-test; Figure 4.7).

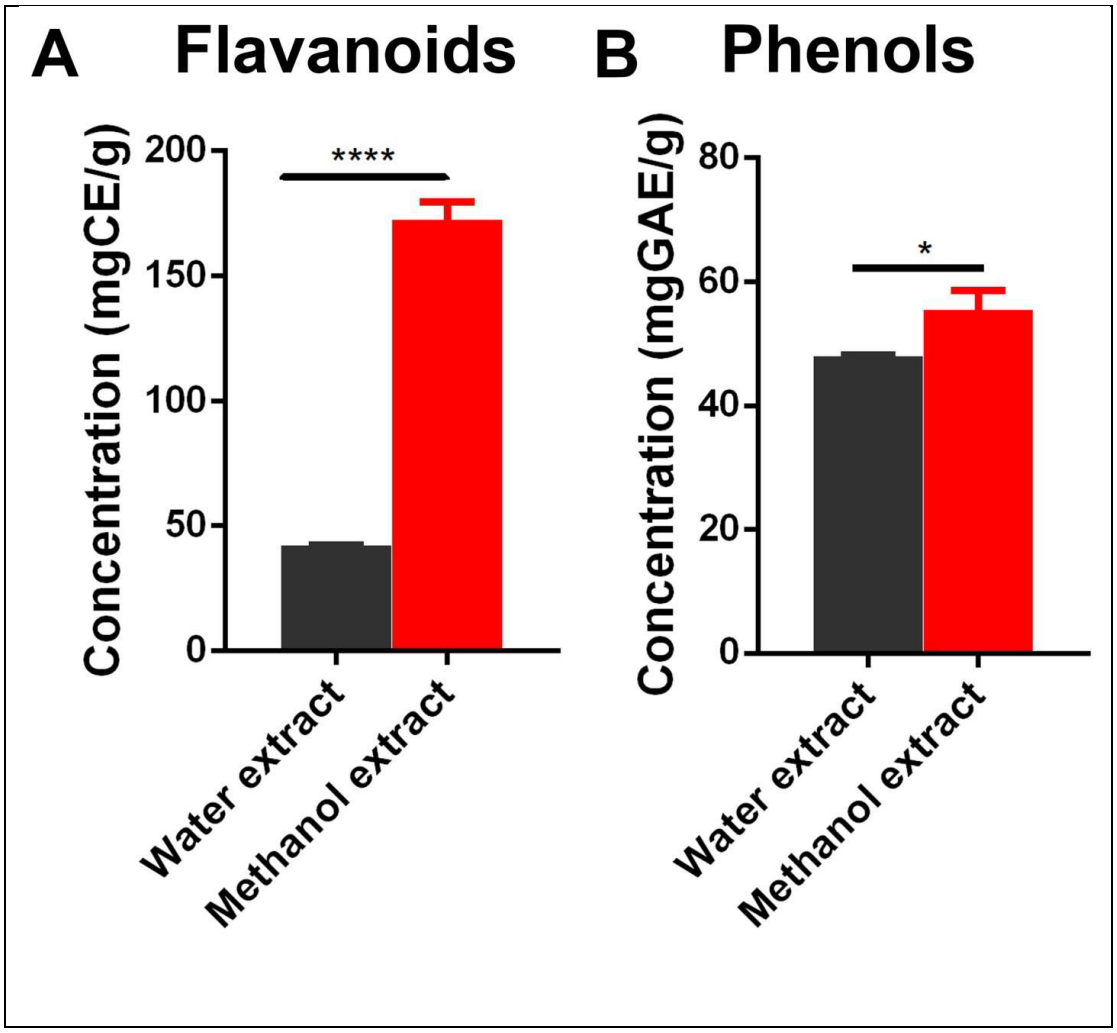



**Figure 4.7: Total phenolic and Total flavonoid content of *F. angolensis* water and methanol leaf extracts**

*Data was analyzed by student t-test followed by Tukey's post hoc test and presented as mean±SD. N= triplicate tests. Abbreviations: ns; not significant, CE; catechin equivalent, GAE; garlic acid equivalent, mg; milligram, g; gram, *; p<0.05, ***; P<0.001, ***; p<0.0001.*

### 4.9 Phytochemical composition of *F. hildebrandtii* stem bark, water and methanol extracts

Water and methanol extract of *F. hildebrandtii* exhibited presence of steroids, flavonoids, terpenoids, coumarins, flavonoids, alkaloids, tannin, saponins and glycosides, but anthocyanins and anthraquinones were absent (Table 4.7).

**Table 4.2: Phytochemical content of *F. hildebrandtii* stem bark, water and methanol extracts**

| Phytochemical | Test | Inference | |
|---|---|---|---|
| | | Water extract | Methanol extract |
| Steroids | Liebermann burchard test | + | + |
| Phenols | Ferric chloride test | + | + |
| Saponins | Frothing test | + | + |
| Tannins | Ferric chloride | + | + |
| Cardiac glycosides | Keller kiliani test | + | + |
| Alkaloids | Dragendoff's test | + | + |
| | Mayer's test | + | + |
| | Wagner's test | + | + |
| Flavonoids | Alkaline reagent test | + | + |
| Terpenoids | Salkowski's test | + | + |
| Coumarins | Sodium hydroxide test | + | + |
| Anthocyanins | Hydrochloric acid (2N) test | - | - |
| Anthraquinones | Borntragger's test | - | - |

*(+) presence; (-) absence*

### 4.9.1 Total phenolic and total flavonoid content of *F. hildebrandtii* stem bark extracts

As shown in figure 4.8, quantitative analysis revealed higher total flavonoid levels in *F. hildebrandtii* methanolic extract compared to water extract (132.18±0.26mgCE/g vs. 42.68±0.93 mgCE/g; $P<0.05$). *F. hildebrandtii* methanol extract also had higher total phenolic content compared to the water extract (34.590±2.49 mgGAE/g vs. 7.89±1.62 mgGAE/g**;** $P<0.05$). Notably, methanol extracts exhibited higher total flavonoid and total phenolic content compared to water extract ($P<0.0001$).

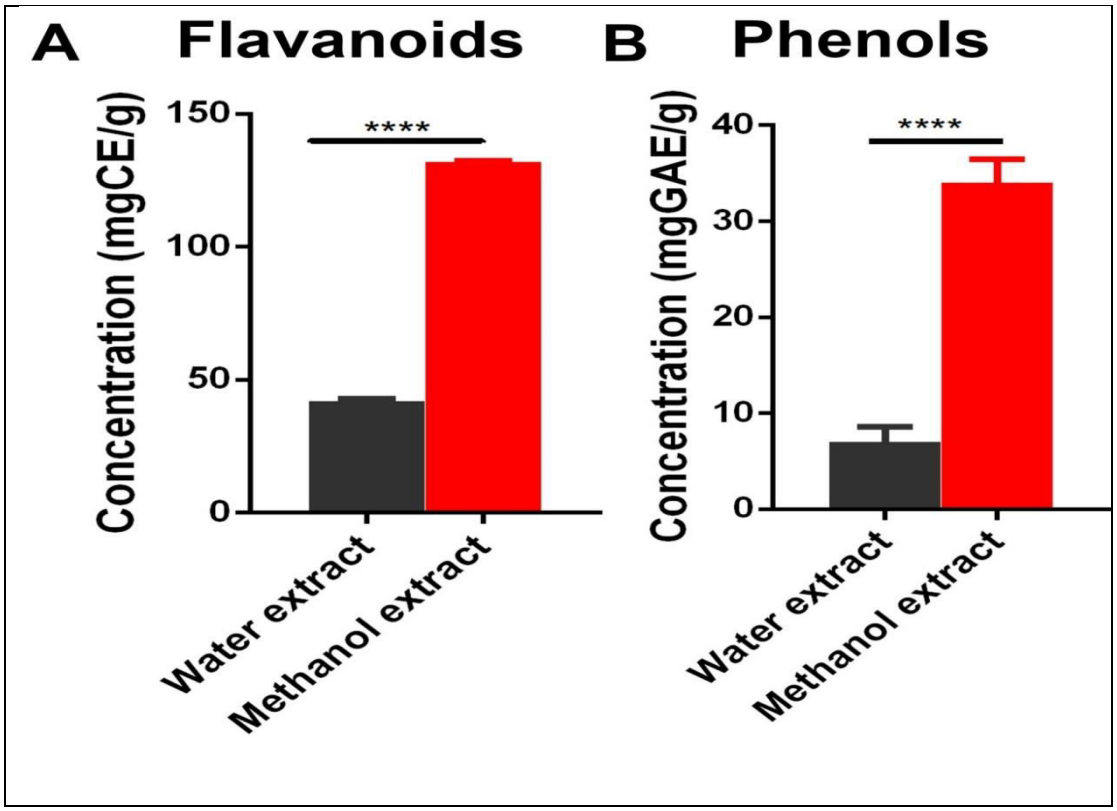



**Figure 4. 8: Total phenolic content and total flavonoid content of *F. hildebrandtii* stem bark, water and methanol extracts**

*Data was analyzed by student t-test followed by Tukey's post hoc test and presented as mean±SD. N= triplicate tests. Abbreviations: ns; not significant, CE; catechin equivalent, GAE; garlic acid equivalent, mg; milligram, g; gram, *; p<0.05, ***; P<0.001, ****; p<0.0001.*

# CHAPTER FIVE

# DISCUSSION OF THE RESULTS

## 5.1 Introduction

The results of the antinociceptive, anti-inflammatory, antioxidant and phytochemical constituents of *F. angolensis* leaves, and *F. hildebrandtii* stem bark were interpreted and discussed in this chapter in comparison with previous studies.

## 5.2 Antinociceptive activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water, and methanol extracts

*F. angolensis* methanol leaf extract at dose levels above 50 mg/kg exhibited similar writhing inhibition as the standard aspirin at a dose of 150 mg/kg (Figure 4.1). Similarly, the inhibition of writhing by the stem bark of *F. hildebrandtii* methanol extract at 50 mg/kg and 250 mg/kg was also comparable to that of standard aspirin at 150 mg/kg (Figure 4.2). This means that *F. angolensis* and *F. hildebrandtii* methanol extracts at doses above 50mg/kg are as effective as aspirin in ameliorating acetic acid-induced pain in mice. This could be attributable to various phytochemicals in *F. angolensis* and *F. hildebrandtii* methanol extracts (Table 4.1 and 4.2). Steroids isolated from *Phyllanthus corcovadensis* were shown to be responsible for analgesic effects (Santos *et al.*, 1995). Notably, steroids, phenols and flavonoids were found in the leaves of *F. angolensis* and stem bark of *F. hildebrandtii* extracts which might be acting synergistically against multiple targets as opposed to aspirin, which is a single pure agent. The study findings showed that the methanol extracts of *F. angolensis* and *F. hildebrandtii* exhibited higher writhing inhibition compared to the water extracts at all the dose levels (Figure 4.1; Figure 4.2; Tukey's test). This suggested higher antinociceptive potency of the methanol extracts which could be due to higher

flavonoids and phenolic content in methanolic extract compared to the water extract (Figure 4.7 and Figure 4.8).

Acetic acid causes release of endogenous mediators including prostaglandins, histamine, bradykinin and serotonin, which drive both inflammation and pain and consequently writhing response (Gawade, 2012; Bhuiyan *et al.,* 2020). Therefore, the antinociceptive effects of *F. angolensis* and *F. hildebrandtii* extracts is most likely linked to the observed anti-inflammatory activity as shown in figure 4.3 and 4.4. However, unlike the anti-inflammatory effects, the antinociceptive effects were dose-dependent, suggesting potential additional and direct antinociceptive mechanisms. This putative mechanism should be explored in future research. Furthermore, these results showed that *F. angolensis* and *F. hildebrandtii* could be like the conventional non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin which have both analgesics and anti-inflammatory effects. Altogether, the antinocipetive data of this study has validated the traditional use of these plants in management of pain and pain-related disorders (Mutinda *et al.,* 2022).

This is the first study done to investigate the pain suppression by the two species of *F. angolensis* and *F. hildebrandtii* extracts (Rutaceae). However, several other plants of the Rutaceae family have found much interest in the antinociceptive activity evaluation. Ethanolic extract of *Zanthoxylum riedelianum* leaves and stem bark (Rutaceae) has been reported to exhibit both antinociceptive and anti-inflammatory activities (Lima *et al.,* 2010). *Ruta graveolens*, popularly known as Rue of Rutaceae family was reported to exhibit antinociceptive activity in a tail immersion model. Hexane extract exhibited chronic antinociceptive effect that was partly related to the anti-inflammatory activity (Cunha *et al.*, 2015). The results of this study, on *Fagaropsis* species, are in agreement

with similar research done on plants from the same family. It is also in line with the findings of other work that demonstrated antinociceptive activity of herbal plants in a dose-dependent fashion (Yimer *et al*., 2020).

### 5.3 Anti-inflammatory activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, water and methanol extracts

Several human diseases are associated with acute or chronic inflammation, which can be managed by conventional anti-inflammatory drugs including cyclo-oxygenase (COX) inhibitors such as aspirin, ibuprofen, diclofenac and corticosteroid drugs for example, dexamethasone, prednisolone and triamcinolone (Slater *et al.,* 2010). Besides these conventional drugs, several medicinal herbs are also widely used in the management of inflammation and inflammation-associated diseases, particularly in Africa, where more than 80% of the population rely on herbal medicine (WH0, 2019). However, unlike the conventional drugs, very little pharmacological data is available about the anti-inflammatory efficacy of most of these commonly used herbal medicines. In this study, the anti-inflammatory effects of *F. angolensis* leaf extracts and *F. hildebrandtii* stem bark extracts were evaluated using carrageenan-induced paw edema in comparison with dexamethasone.

The findings indicates that 50 mg/kg and 250 mg/kg of *F. angolensis* methanolic and water extracts at all the experimental time points (1-5hours) inhibited paw edema to the same extent as 10 mg/kg of dexamethasone. Therefore, *F. angolensis* methanolic and water extracts at doses above 50 mg/kg have anti-inflammatory activities equivalent to the anti-inflammatory effects of 10 mg/kg of dexamethasone. However, dexamethasone was superior compared to the 2 mg/kg dose of the *F. angolensis* water extract in the first two hours, as well as compared to 10 mg/kg dose of methanol extract at 1-, 2- and

5-hour time points (Figure 4.3). This suggests that in doses below 50 mg/kg, the anti-inflammatory effects of *F. angolensis* water and methanol extracts are inconsistent. Therefore, this data provides evidence in support of *F. angolensis* leaves extracts having potent *in vivo* anti-inflammatory activity at doses above 50 mg/kg in mice. This consistent potent activity at doses above 50 mg/kg perhaps reflects the presence of optimal concentrations of anti-inflammatory bioactive molecules at such doses, but not at lower doses.

Similar to *F. angolensis* extracts, *F. hildebrandtii* stem bark extracts also exhibited potent inhibition of carrageenan-induced paw edema (Figure 4.4). Notably, the *F. hildebrandtii* stem bark methanolic extract in particularly, was as effective as 10 mg/kg of dexamethasone standard over the course of five hours. However, at the 2-hour time point, activity of 2 mg/kg dose was significantly lower as compared to dexamethasone 10 mg/kg (Figure 4.4). This lower activity at the 2-hour time point was also observed for the 2 mg/kg and 10 mg/Kg of *F. hildebrandtii* water extract (Figure 4.4). This may possibly be ascribed to low levels of anti-inflammatory phytochemicals at doses below 50 mg/Kg.

Carrageenan induces paw edema in a biphasic manner, with the first (early) phase occurring between 0-1 hour and the second (late) phase occurring at 1-4 hours post sub-plantar injection. The early phase is majorly driven by increased production of bradykinin, hydroxy tryptamine and histamine, while the late phase is predominantly due to COX-2 activation, which leads to increased release of prostanoids and production of free radicals (Afsar *et al*., 2015). Dexamethsone acts mainly by decreasing the amount of prostanoids and production of free radicals, which occurs in the late phase of carrageenan-induced inflammation. Therefore, the time-dependent

increase in paw edema inhibition in this study point to the possibility that *F. hildebrandtii* and *F. angolensis* extracts act in a similar manner to dexamethasone, that is; they inhibit production of prostanoids. However, future mechanistic studies should be done to confirm this potential mechanism.

The results of *in vivo* anti-inflammatory activities of *F. angolensis* leaves and *F. hildebradtii* stem bark in this study are consistent with and extend the findings of a previous *in vitro* study of *F. angolensis* root bark by Mukavi *et al*. (2020). In that *in vitro* study, *F. angolensis* root bark methanol-dichloromethane extract was shown to inhibit the lipopolysaccharide stimulated-synthesis of inflammatory cytokines in peripheral blood mononuclear cells. *V. nobilis* root bark (Rutaceae) exhibited formalin induced paw edema inhibition in mice just like in the aerial parts highlighting the pharmacological activity of plants from the family Rutaceae (Omujal *et al.,* 2020). Collectively, these findings provide support for potent anti-inflammatory effects of the genus *Fagaropsis* and explain the use of its plant parts in traditional management of various diseases associated with inflammation.

Notably, Alemu and Misganaw, (2020) reported that 2000 mg/kg of *F. angolensis* stem bark methanol extract did not cause behavioral change or mortality in mice within 24 hour of administration. Lethal dose ($LD_{50}$) of *F. angolensis* methanolic extract was established to be more than 2000 mg/kg. Muia *et al*. (2020) noted that *F. hildebrandtii* root extracts at doses of 300-2000 mg/kg was associated with histopathological changes in liver and kidney of mice if given beyond 14 days but short-term administration may not result in toxicity. The doses that exhibited good anti-inflammatory activities comparable to dexamethsone are therefore below the potential toxic ranges, however toxicity on long term administration remains to be explored in future studies.

## 5.4 Antioxidant activities of *F. angolensis* leaves and *F. hildebrandtii* stem bark, *water* and methanol extracts

*F. angolensis* and *F. hildebrandtii* water and methanol extracts exhibited dose dependent *in vitro* antioxidant activity as demonstrated by increasing *%* RSA (Figure 4.5 and Figure 4.6). The results also indicated that *F. angolensis* and *F. hildebrandtii* extracts exhibited higher potency as demonstrated by lower $IC_{50}$ compared to ascorbic acid (Figure 4.5 and Figure 4.6). The higher potency of the plant extracts may be attributed to the presence of phenols and flavonoids (Figure 4.7; Figure 4.8), which have been shown to have antioxidant activities (Saeed *et al*., 2012).

The evidence of potent antioxidant activity in this study was inconsistent with those of Alemu and Misganaw, (2020) which showed lower antioxidant activity of *F. angolensis* stem bark methanol extract relative to ascorbic acid. This may be due to the differences in the extraction methods used, as well as potential qualitative and quantitative differences in antioxidant phytochemicals of the leaves in this study versus the stem bark in their study. The marked antioxidant activity of the stem bark of *F. hildebrandtii* observed during this study agrees with those of *C. exavata* leaf extract (Rutaceae). In that study, methanolic extract was reported to reduce ferrous chloride in the ferrous reducing antioxidant power model and showed scavenging of DPPH at potency close to quercetin (Albaayit *et al*., 2014).

At low concentration, both extracts had a higher activity than the standard ascorbic acid. However, at higher doses of 10-100 µg/ml, ascorbic acid had higher activity than the plant extracts, which becomes comparable to the extracts at 1000 µg/ml. Therefore, *Fagaropsis* extracts, and the standard ascorbic acid have similar maximal antioxidant

effects (*Emax*), despite the extracts having higher potency at low concentration. This could be due to differences in free radical scavenging mechanisms of ascorbic acid and the *Fagaropsis* extracts.

Oxidative stress plays a role in inflammation and pain (Arulselvan *et al*., 2016). Therefore, free radical scavenging may be one of the explanation and potential mechanisms of the anti-inflammatory and antinociceptive activities of *Fagaropsis* extracts.

### 5.5 Phytochemical composition of *F. angolensis* leaves and *F. hildebrandtii* stem bark extracts

In this study, there was presence of alkaloids, steroids, phenols, flavonoids, terpenoids, tannins, coumarins, glycosides and anthraquinones in methanolic extract of *F. angolensis* (Table 4.1). Water extract of *F. angolensis* exhibited presence of flavonoids, alkaloids, glycosides, phenols, steroids, anthraquinolones and coumarin. This indicates that more secondary metabolites were found in methanolic extracts than in water extracts suggesting that methanol was more efficient in extraction than water as a solvent. This could be due to methanol being able to extract both hydrophilic and hydrophobic compounds unlike water which predominantly extracts hydrophilic compounds.

These findings are consistent with previous studies on preliminary screening of stem bark and other parts of *F. angolensis* which indicated absence of saponin but presence of alkaloids, flavonoids, glycosides, phenols and steroids (Yiaile *et al.,* 2018; Alemu and Misganaw, 2020). Furthermore, the water extract of the bark of *F. angolensis* from Kaptumo sub county in Nandi County indicated presence of terpenoids, phenols, alkaloids, anthraquinones and flavonoids but absence of saponin just like in this study,

apart from terpenoids which were absent in the leaves of *F. angolensis* water extract from Embu County. This variation could be as a result of difference in plant parts used and location of the plants (Kimutai *et al*., 2016). The close similarity in distribution of phytochemicals could be because both results are for the same species.

*F. hildebrandtii* stem bark extracts were rich in alkaloids, terpenoids, glycosides, saponins, tannins, coumarins, steroids, phenols and flavonoids (Table 4.2). These findings were consistent with previous phytochemical analyses of *F. hildebrandtii* root extracts even though plant parts used were different (Muia *et al.,* 2020). However, unlike in the current study, coumarins were not detected in the root bark of *F. hidebrandtii*. This means that phytochemical content of the roots and stem bark are distributed the same in both plant parts and both can be used interchangeably for extraction of phytochemicals with exception of coumarins.

Notably, figure 4.7 shows that there was significantly higher total flavonoid content in methanol extract of *F. angolensis* compared to the water extract. This could be due to high distribution of flavonoids in methanol solvent than in water solvent. Total phenolic content (figure 4.7) was also significantly higher in methanol than in water extract, indicating that phenols are extracted more efficiently by methanol than by water.

*F. hildebrandtii* water and methanol extracts had significantly higher total flavonoid content in methanol extract compared to water extract (figure 4.8). There was also a significantly higher presence of total phenols in methanol extract compared to water extract (figure 4.8). Generally, higher concentration of total flavonoids and total phenolic content in methanol extract might be due to higher solubility of *F. hildebrandtii* stem bark flavonoids and phenols in methanol than in water. In terms of objective three, phenolic compounds are known to play a greater role in oxidative stress

amelioration due to hydroxyl group(s) donation. Presence of these hydroxyl groups favorably makes phenols more soluble in organic solvents such as methanol (Kumar and Goel, 2019). While the choice of water as a solvent based on the ethnomedicinal practice cannot be under scored, methanol extract had the best activity. Although these findings suggested that methanol is a better solvent compared to water, future comparative studies of different solvents to determine the most optimal one should be done.

Previously, methanolic extract of *C. exavata* (Rutaceae) had the highest amount of phenolic compounds than chloroform, petroleum ether and ethyl acetate extracts (Albaayit *et al.*, 2014). The study was consistent with this study which also showed higher concentration of phenolic content in methanol extract. Ethanolic extract of the dried seed of *Z. budrunga* (Rutaceae) using Follin - Ciocalteu model, exhibited high phenolic content just like in the two species of *Fagaropsis* from Rutaceae family (Islam *et al*., 2014).

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATIONS

## 6.1 Conclusions

- There was presence of *in vivo* antinociceptive activities associated with the leaves of *F. angolensis* and stem bark of *F. hildebrandtii,* water and methanol extracts
- There was presence of *in vivo* anti-inflammatory activities associated with the leaves of *F. angolensis* and the stem bark of *F. hildebrandtii*, water and methanol extracts
- There was presence of *in vitro* antioxidant activities associated with the leaves of *F. angolensis* and stem bark of *F. hildebrandtii,* water and methanol extracts
- There was presence of phytochemicals associated with the leaves of *F. angolensis* and stem bark of *F. hildebrandtii*, water and methanol extracts

## 6.2 Recommendations

### 6.2.1 Recommendation for action

The water and methanol extract of the leaves of *F. angolensis* and stem bark of *F. hildebrandtii* may be utilized as alternative drug for management of pain as claimed by traditional medicine practitioners and this study findings. The water and methanol extract of the leaves of *F. angolensis* and stem bark of *F. hildebrandtii* may be utilized as alternative drug for management of inflammatory disorders as claimed by traditional medicine practitioners and this study findings.

The water and methanol extract of the leaves of *F. angolensis* and stem bark of *F. hildebrandtii* may be utilized as alternative drug for management of oxidative stress as claimed by traditional medicine practitioners and this study findings. There should be

preservation of these plants as they are sparsely distributed in Kenya. Some of the ways recommended for preservation include.

- Regulated harvesting of wild plants occurring in the parts of the country, where these plants are widely used in traditional medicine.
- Adequate sensitization of the communities and institutions in need to establish botanical gardens for plantation and preservation of medicinal plants.
- Research on propagation of *F. angolensis* and *F. hildebrandtii.*

### 6.2.2 Recommendation for further research

The research recommends identification and characterization of phytochemicals responsible for antinociceptive, anti-inflammatory and antioxidant activities of both leaves of *F. angolensis* and stem bark of *F. hildebrandtii* extracts. Further research should be done on *in vivo* antioxidant activity of both *F. angolensis* and *F. hildebrandtii* extracts. Additionally, research on antinociceptive and anti-inflammatory activities using specific disease models should be done. Furthermore, research should be done on the mechanism of action of the antinociceptive, anti-inflammatory and antioxidant, activities.

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# APPENDICES

## Appendix I: Approval letter from the school

**MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY**

Tel: 056-31375
Fax: 056-30153
E-mail: ierc@mmust.ac.ke
Website: www.mmust.ac.ke

P. O. Box 190,
50100.
Kakamega,
**KENYA**

**Institutional Scientific and Ethics Review Committee (ISERC)**

REF: MMU/COR: 403012 Vol 6 (01) Date: February 17th, 2023

To: Peter Maloba

Dear Mr.,

**RE: EVALUATION OF PHARMACOLOGICAL PROPERTIES AND PHYTOCHEMICAL COMPOSITION OF FAGAROPSIS ANGOLENSIS AND FACAROPSIS HILDEBRANDTII (RUTACEAE)**

This is to inform you that the ***Masinde Muliro University of Science and Technology Institutional Scientific and Ethics Review Committee (MMUST-ISERC)*** has reviewed and approved your above research proposal. Your application approval number is **MMUST/IERC/119/2023.** The approval covers for the period ***February 17th, 2023*** *to* ***February 17th, 2024.***

This approval is subject to compliance with the following requirements;

i. Only approved documents including informed consents, study instruments, MTA will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by ***MMUST-ISERC.***
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to ***MMUST-ISERC*** within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to ***MMUST-ISERC*** within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to ***MMUST-ISERC***.

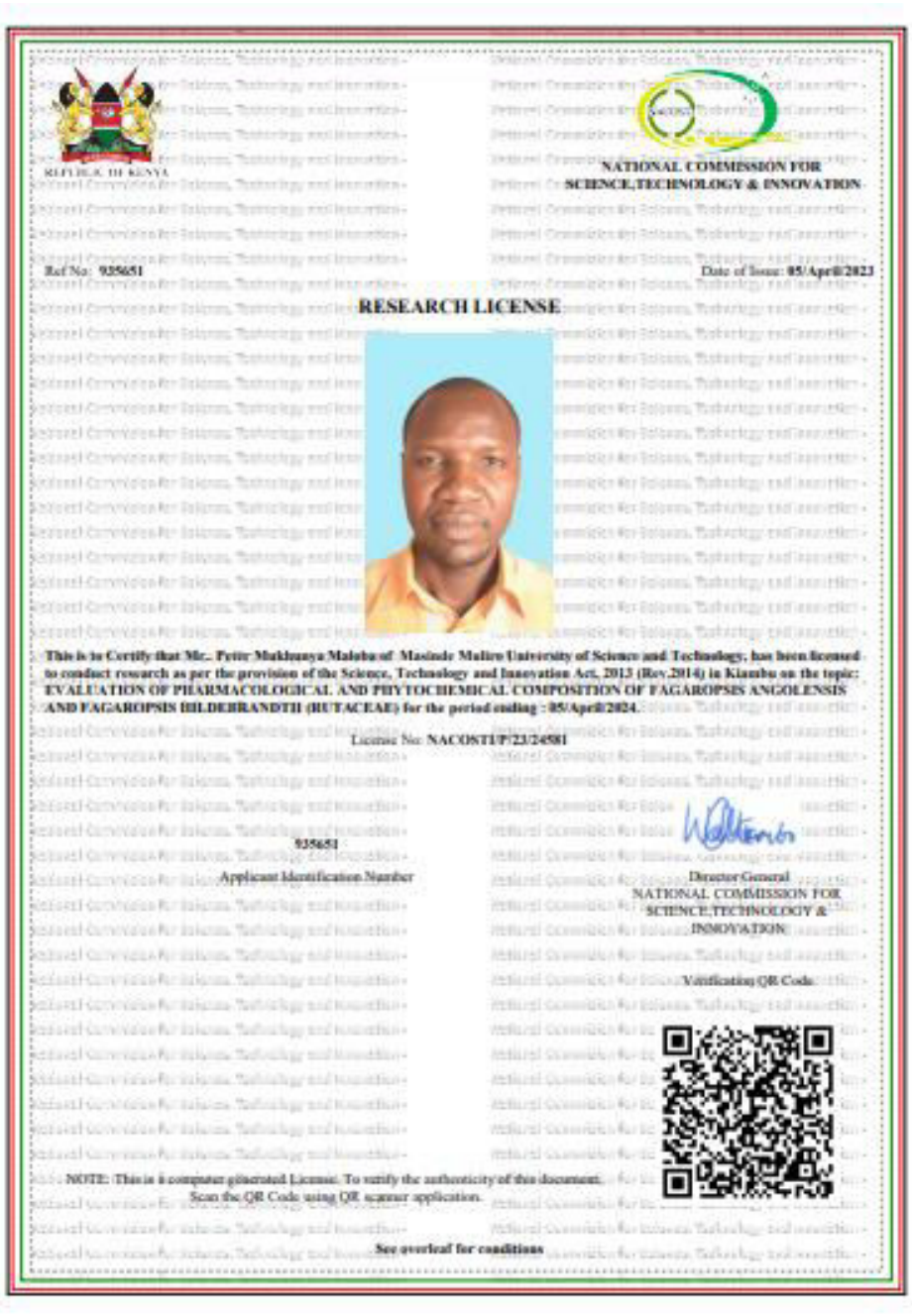
Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://research-portal.nacosti.go.ke and also obtain other clearances needed

Yours Sincerely,

Prof. Gordon Nguka (PhD)
**Chairperson, Institutional Scientific and Ethics Review Committee**

Copy to:
- The Secretary, National Bio-Ethics Committee
- Vice Chancellor
- DVC (PR&I)

## Appendix II: Approval letter from NACOSTI



REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Ref No: 935651

Date of Issue: 05/April/2023

RESEARCH LICENSE

This is to Certify that Mr.. Peter Mukhunya Maloba of Masinde Muliro University of Science and Technology, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Kiambu on the topic: EVALUATION OF PHARMACOLOGICAL AND PHYTOCHEMICAL COMPOSITION OF FAGAROPSIS ANGOLENSIS AND FAGAROPSIS HILDEBRANDTII (RUTACEAE) for the period ending : 05/April/2024.

License No: NACOSTI/P/23/24581

935651

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions

**Appendix III: Picture of *F. angolensis***



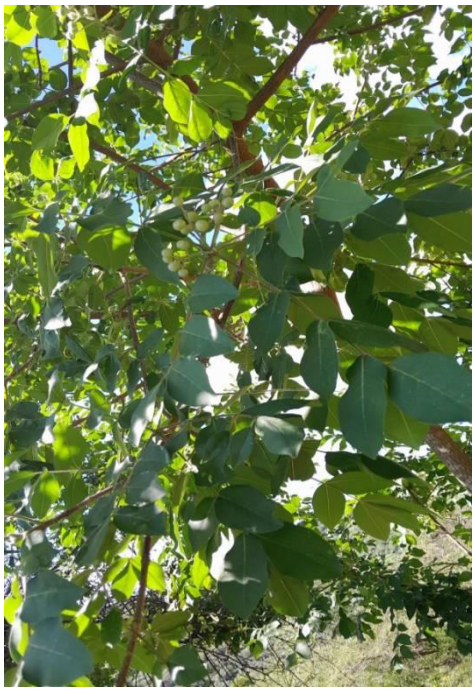
A B

(A) A photo of the whole mature tree (B) A photo of branches with leaves and fruits from the upper part of the tree.

**Appendix IV: Picture of *F. hildebrandtii***



A



B

(A) A photo of the whole mature tree (B) A photo of branches with leaves and fruits from the upper part of the tree.

**Appendix V: Voucher specimen authentication certificate**

NATIONAL MUSEUMS OF KENYA

WHERE HERITAGE LIVES ON

REF: NMK/BOT/CTX/3/4

27th January 2020

Jared Misonge Onyanja
Te.-0720-457787
**THIKA**

Dear Mr. Onyanja,

PLANT IDENTIFICATION

The plant specimens you brought to us for identification has been determined as follows:

GS001/2020 - *Hypericum revolutum* ssp. *Keniense* (Schweinf.) N. Robson.

**(Family: Hypericaceae)**

**Venacular name:** Muthathumwa (Kikuyu.)

Jm003/2015 – *Fagaropsis angolensis* (Engl.) Dale (**Family:** ~~Annonaceae~~ Rutaceae)

**Vernacular name:** Mukuria mbugu (Mbeere)

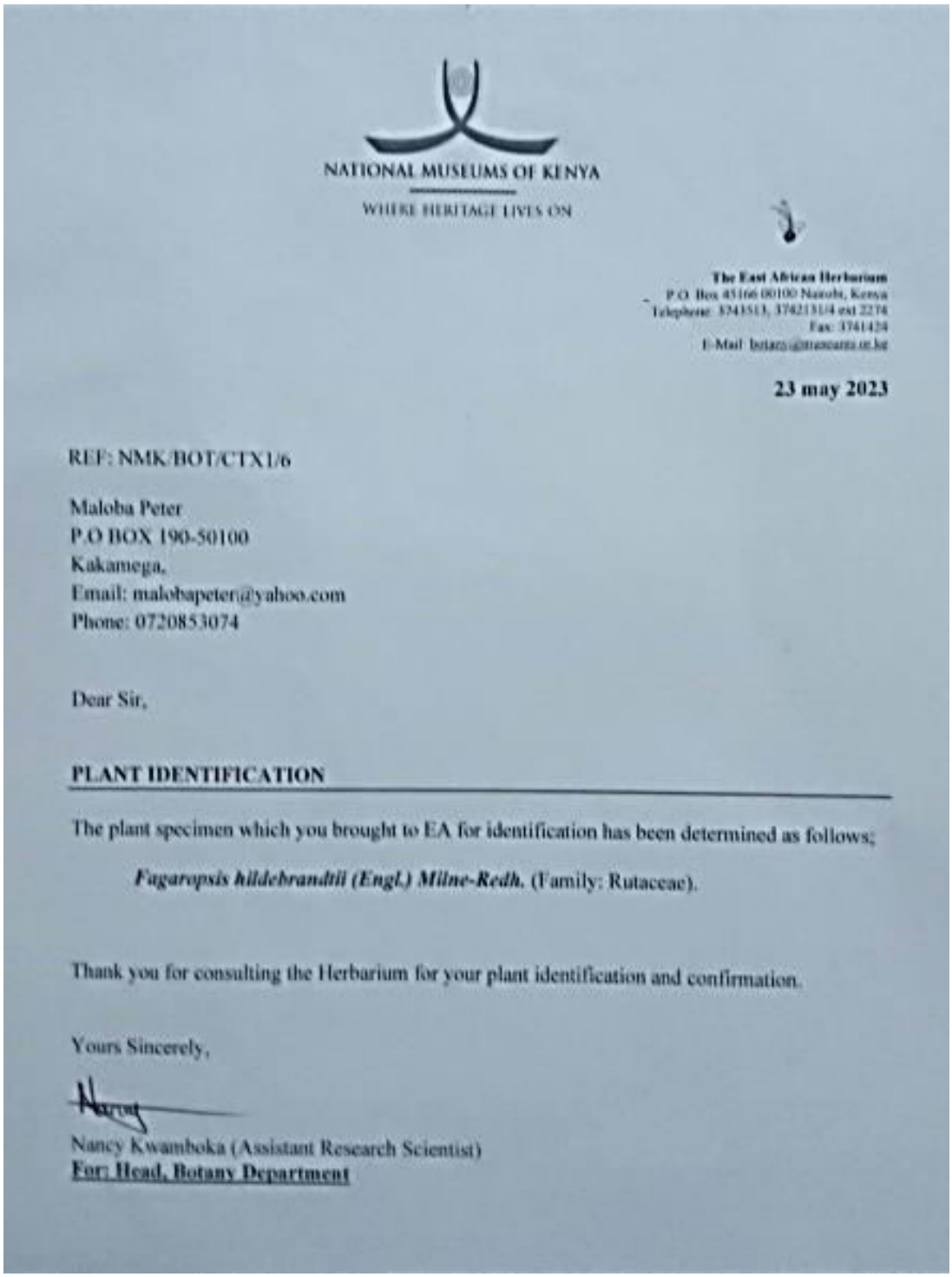
Thank you for consulting the EAH for plant identification and confirmation.

Yours sincerely,

NATIONAL MUSEUMS OF KENYA
05 MAR 2020
HERBARIUM

Mathias Mbale
**For: Head, Botany Department.**

**Appendix VI: Voucher specimen authentication certificate**



NATIONAL MUSEUMS OF KENYA

WHERE HERITAGE LIVES ON

**The East African Herbarium**
P.O. Box 45166 00100 Nairobi, Kenya
Telephone: 3743513, 3742131/4 ext 2274
Fax: 3741424
E-Mail: botany@museums.or.ke

**23 may 2023**

REF: NMK/BOT/CTX1/6

Maloba Peter
P.O BOX 190-50100
Kakamega,
Email: malobapeter@yahoo.com
Phone: 0720853074

Dear Sir,

**PLANT IDENTIFICATION**

The plant specimen which you brought to EA for identification has been determined as follows;

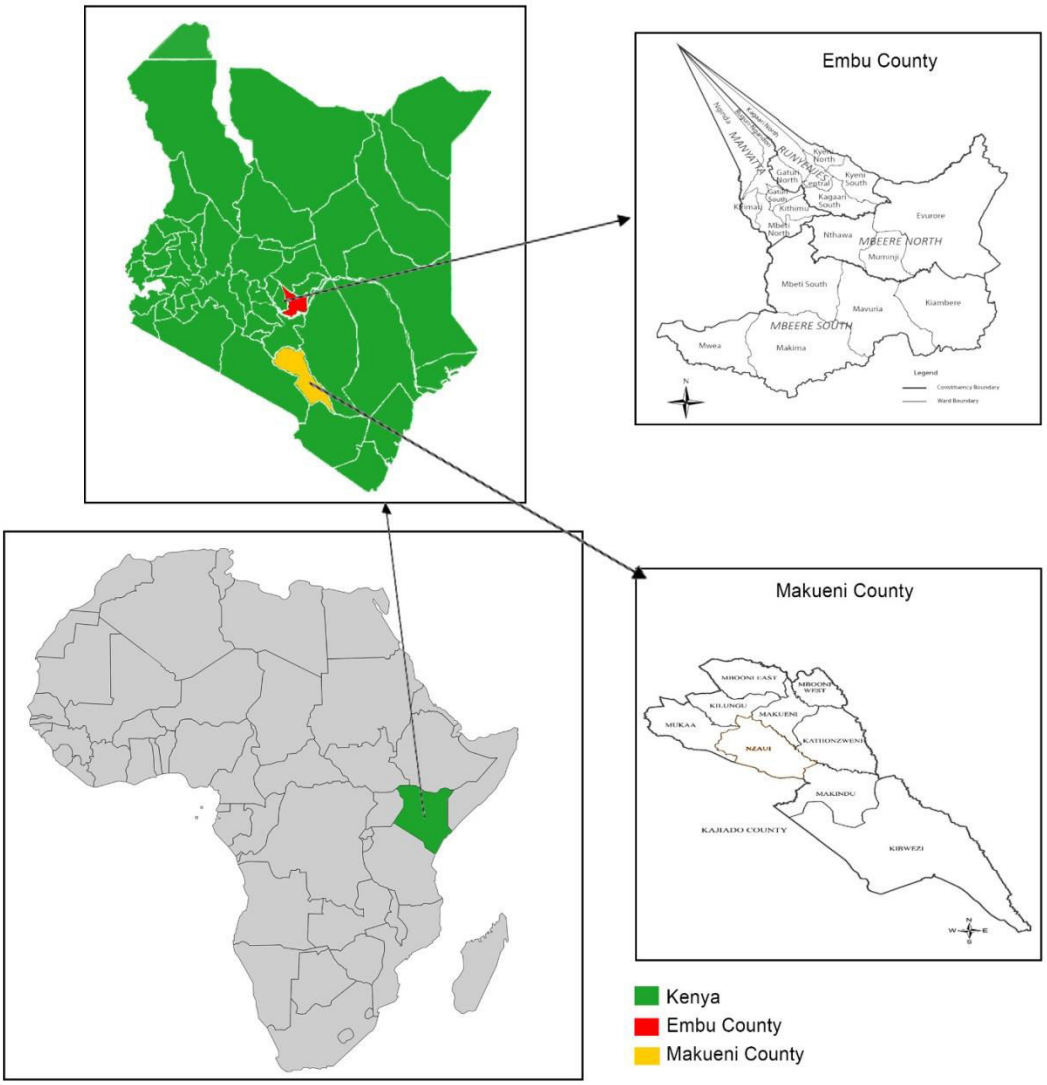
***Fagaropsis hildebrandtii (Engl.) Milne-Redh.*** (Family: Rutaceae).

Thank you for consulting the Herbarium for your plant identification and confirmation.

Yours Sincerely,

Nancy Kwamboka (Assistant Research Scientist)
**For: Head, Botany Department**

**Appendix VII: Study area; Embu and Makueni Counties**



**Appendix VIII: Dried stem bark of *F. hildebrandtii***



**Appendix IX: Experimental mice marked for easy identification**



## Appendix X: Raw data

## Table 1. Antinociceptive activity raw data showing number of writhing

| Analgesic activity of F.H water Extract | Mouse 1 | Mouse 2 | Mouse 3 | Mouse 4 | Mouse 5 | Total |
|---|---|---|---|---|---|---|
| Black Back 2mg/kg | 9 | 13 | 10 | 20 | 27 | 79 |
| Head Black 10mg/kg | 12 | 20 | 18 | 16 | 20 | 86 |
| Hind limb black 50mg/kg | 26 | 5 | 3 | 17 | 6 | 57 |
| Plain 250mg/kg | 4 | 18 | 3 | 10 | 18 | 53 |

| Analgesic activity of F. H Methanol extract | Mouse 1 | Mouse 2 | Mouse 3 | Mouse 4 | Mouse 5 | Total |
|---|---|---|---|---|---|---|
| | 20 | 10 | 10 | 21 | 52 | 113 |
| | 20 | 11 | 18 | 25 | 15 | 89 |
| | 25 | 12 | 4 | 15 | 10 | 66 |
| | 8 | 10 | 12 | 6 | 5 | 41 |

| Analgesic activity of F.A methanol Extract | Mouse 1 | Mouse 2 | Mouse 3 | Mouse 4 | Mouse 5 | Total |
|---|---|---|---|---|---|---|
| Red head 250mg/kg | 6 | 7 | 16 | 11 | 5 | 45 |
| Green, Black 50mg/kg | 5 | 11 | 9 | 12 | 33 | 70 |
| Plain 10mg/kg | 10 | 20 | 24 | 13 | 30 | 97 |
| Hind leg black 2mg/kg | 20 | 13 | 33 | 22 | 15 | 103 |

| Analgesic activity of F. A water extract. | Mouse 1 | Mouse 2 | Mouse 3 | Mouse 4 | Mouse 5 | Total |
|---|---|---|---|---|---|---|
| | 46 | 20 | 11 | 60 | 40 | 177 |
| | 36 | 38 | 20 | 25 | 65 | 184 |
| | 46 | 30 | 30 | 30 | 57 | 193 |
| | 45 | 3 | 15 | 122 | 15 | 200 |

| | Mouse 1 | Mouse 2 | Mouse 3 | Mouse 4 | Mouse 5 | Total |
|---|---|---|---|---|---|---|
| Negative control | 80 | 77 | 145 | 52 | 54 | 408 |
| Positive control | 6 | 11 | 7 | 6 | 9 | 39 |

**Table 2.Anti-inflammatory activity raw data showing paw circumference (mm)**

| Plant extract | Conc | 0 hr. | 1st hr. | 2nd hr. | 3rd hr. | 4th hr. | 5th hr. |
|---|---|---|---|---|---|---|---|
| F.A.M | 250 mg | 2.78 | 3.30 | 3.04 | 2.86 | 2.90 | 2.92 |
| F.H. W | | 2.48 | 3.94 | 3.64 | 3.30 | 3.14 | 3.00 |
| F.A. W | | 3.36 | 3.86 | 3.48 | 3.34 | 3.30 | 3.30 |
| F.H.M | | 2.54 | 3.48 | 3.06 | 3.00 | 2.90 | 2.84 |
| F.A.M | 50 mg | 2.84 | 3.40 | 3.14 | 2.90 | 2.82 | 2.86 |
| F.H. W | | 2.44 | 3.74 | 3.50 | 3.30 | 3.10 | 2.96 |
| F.A. W | | 3.44 | 4.16 | 3.74 | 3.70 | 3.34 | 3.30 |
| F.H.M | | 2.78 | 3.46 | 3.02 | 2.92 | 2.96 | 2.76 |
| F.A.M | 10 mg | 2.60 | 3.46 | 3.14 | 3.00 | 2.78 | 2.78 |
| F.H. W | | 2.48 | 3.60 | 3.44 | 3.34 | 3.16 | 2.98 |
| F.A. W | | 3.64 | 4.08 | 3.78 | 3.66 | 3.46 | 3.36 |
| F.H.M | | 2.30 | 3.02 | 3.04 | 3.00 | 2.94 | 2.92 |
| F.A.M | 2 mg | 2.86 | 3.62 | 3.26 | 3.04 | 2.84 | 2.80 |
| F.H. W | | 2.50 | 3.64 | 3.32 | 3.16 | 2.88 | 2.86 |
| F.A. W | | 3.46 | 4.20 | 3.60 | 3.56 | 3.52 | 3.38 |
| F.H.M | | 2.68 | 3.22 | 3.14 | 2.98 | 2.84 | 2.80 |
| Dexamethasone | 10 mg | 2.68 | 3.42 | 3.34 | 2.86 | 2.66 | 2.32 |
| Normal saline | | 2.82 | 3.68 | 3.28 | 3.06 | 2.90 | 3.22 |

**Table 3. Antioxidant activity raw data showing absorbance at 517nm**

F.H Methanol extract

| CONC µg/ml | T1 | T2 | T3 |
|---|---|---|---|
| 1000 | 0.074 | 0.0559 | 0.0577 |
| 100 | 0.2753 | 0.2678 | 0.2633 |
| 10 | 0.4438 | 0.4359 | 0.4461 |
| 1 | 0.4651 | 0.4243 | 0.4926 |
| 0.1 | 0.496 | 0.4902 | 0.4927 |
| 0.01 | 0.4999 | 0.4944 | 0.4941 |
| 0 | 0.9215 | 1.1367 | 0.9068 |

F.A Methanol extract

| CONC µg/ml | T1 | T2 | T3 |
|---|---|---|---|
| 1000 | 0.0786 | 0.08 | 0.0805 |
| 100 | 0.099 | 0.0993 | 0.0919 |
| 10 | 0.2785 | 0.2922 | 0.2962 |
| 1 | 0.4562 | 0.4576 | 0.4735 |
| 0.1 | 0.4856 | 0.4907 | 0.4998 |
| 0.01 | 0.5134 | 0.5042 | 0.5088 |
| 0 | 0.9215 | 1.1367 | 0.9068 |

F.H water extract

| CONC | T1 | T2 | T3 |
|---|---|---|---|
| 1000 | 0.0358 | 0.0239 | 0.0229 |
| 100 | 0.129 | 0.1521 | 0.1546 |
| 10 | 0.2138 | 0.1852 | 0.1944 |
| 1 | 0.2139 | 0.1767 | 0.2028 |
| 0.1 | 0.2142 | 0.2011 | 0.2073 |
| 0.01 | 0.2232 | 0.2319 | 0.2086 |
| 0 | 0.4241 | 0.346 | 0.6151 |

F.A Water extract

| CONC | T1 | T2 | T3 |
|---|---|---|---|
| 1000 | 0.0706 | 0.0579 | 0.0427 |
| 100 | 0.082 | 0.0591 | 0.0702 |
| 10 | 0.1386 | 0.1378 | 0.1427 |
| 1 | 0.1879 | 0.2005 | 0.1803 |
| 0.1 | 0.1944 | 0.2006 | 0.1948 |
| 0.01 | 0.2039 | 0.2047 | 0.2031 |
| 0 | 0.4241 | 0.346 | 0.6151 |

| standard ascorbic acid | | | |
|---|---|---|---|
| 1000 | 0.0284 | 0.0262 | 0.0272 |
| 100 | 0.0177 | 0.028 | 0.0245 |
| 10 | 0.0338 | 0.0848 | 0.109 |
| 1 | 0.5716 | 0.4298 | 0.425 |
| 0.1 | 0.4795 | 0.4381 | 0.4304 |
| 0.01 | 0.4642 | 0.4698 | 0.496 |
| 0 | 0.6822 | 0.6584 | 0.6118 |

**Table 4: Total phenolic and total flavonoid content**

| Extract | F.A.W | F.H.W | F.A.M | F.H.M |
|---|---|---|---|---|
| Total phenolic content | 47.68 | 9.91 | 50.98 | 37.07 |
| Total phenolic content | 48.32 | 4.69 | 61.32 | 29.61 |
| Total phenolic content | 48.01 | 9.08 | 54.27 | 37.09 |
| ABS | 1.2104 | 0.2887 | 1.2908 | 0.9514 |
| | 1.226 | 0.1614 | 1.5432 | 0.7696 |
| | 1.2182 | 0.2685 | 1.3711 | 0.9521 |

| Extract | F.H. W | F.A.W | F.A.M | F.H.M |
|---|---|---|---|---|
| Total Flavonoid content | 42.05 | 43.3 | 160.85 | 131.7 |
| Total Flavonoid content | 41.40 | 40.85 | 171.4 | 132.6 |
| Total Flavonoid content | 43.25 | 43.9 | 185.35 | 132.25 |
| ABS | 0.0945 | 0.087 | 0.3221 | 0.2638 |
| | 0.0952 | 0.0821 | 0.3432 | 0.2656 |
| | 0.09485 | 0.0882 | 0.3711 | 0.2649 |