ANTI-INFLAMMATORY, ANALGESIC, AND CYTOTOXIC EFFECTS OF THE PHYTEXPONENT PREPARATION: A POLYHERBAL FORMULATION

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DEPARTMENT OF PUBLIC HEALTH, PHARMACOLOGY AND TOXICOLOGY

FACULTY OF VETERINARY MEDICINE

UNIVERSITY OF NAIROBI

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I declare that this research thesis is my original work and has not been previously presented in this University or any other Institution for the award of this or any other degree.

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DEDICATION

I dedicate this work to my mother (Loice) and my father (Tobias), who first taught me the value of education and critical thinking.

Also, I dedicate this thesis to all my classmates, lecturers, and supervisors for the moral support and scholarly guidance they have accorded me. Thank you!

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TABLE OF CONTENTS

DECLARATION i
DEDICATION ii
ACKNOWLEDGEMENTS iii
LIST OF TABLES vi
TABLE OF FIGURES vii
LIST OF APPENDICES viii
ACRONYMS AND ABBREVIATIONS ix
ABSTRACT x
CHAPTER ONE 1
1.0 INTRODUCTION 1
1.1 Background Information 1
1.2 Statement of the problem and Justification of the study 7
1.3 Study objectives 8
1.3.1 General Objective 8
1.3.2 Specific objectives 8
1.4 Research Questions 9
CHAPTER TWO 10
2.0 LITERATURE REVIEW 10
2.1 Biochemical Basis of Pain 10
2.2 Biochemical and molecular basis of inflammation 11
2.3 Neural Transmitters and Nociceptive Systems 13
2.4 Analgesic assays 14
2.5 Non-steroidal anti-inflammatory drugs 15
2.6 The role of medicinal plants in the management of pain and inflammation 21
2.7 Preparation and composition of the Phytexponent: A polyherbal 23
formulation used in this study 23
2.8 Toxicity of Herbal Products 26
3.0 MATERIALS AND METHODS 27
3.1 The source of the Phytexponent polyherbal formulation 27
3.2 Experimental animals 27
3.3 Determination of *in vivo* anti-inflammatory activity using Carrageenan- 27
induced paw oedema in mice technique 27
3.4 Determination of potental analgesic effects of the Phytexponent 29

3.5 Cell culture technique........31
3.5.1 Vero E6 cell line culture........31
3.5.2 Passaging technique........32
3.5.3 Trypsinisation and resuspension procedures........32
3.5.4 Determination of the *in vitro* cytotoxic effects of the Phytexponent........32
preparation........32
3.6 Data management and statistical analysis........34
3.7 Ethical Consideration........34
CHAPTER FOUR........36
4.0 RESULTS........36
4.1 Anti-inflammatory activity of the Phytexponent preparation in Swiss........36
albino mice........36
4.2 Analgesic activity of the phytexponent preparation........41
4.3 *In vitro* cytotoxic effects of the Phytexponent preparation........42
CHAPTER FIVE........43
5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS........43
5.1 Discussion........43
5.2 Conclusions........53
5.3 Recommendations for further studies........53
REFERENCES........54
APPENDICES........80

LIST OF TABLES

Table 3.1: Experimental design for the determination of anti-inflammatory activity of the Phytexponent........29

Table 3.2: Experimental design for the determination of analgesic activity of the Phytexponent.....30

Table 4.1:Anti-inflammatory activity of the Phytexponent preparation in Swiss albino mice 40

Table 4.2: *In vitro* cytotoxic effects of the Phytexponent on Vero cell line.......................................43

TABLE OF FIGURES

Figure 2.1: Structure of diclofenac 17

Figure 2.2: Structure of indomethacin 18

Figure 2.3: Structure of acetylsalicylic acid (aspirin) 19

Figure 4.1: Analgesic effects of the Phytexponent preparation of selected medicinal plants in acetic acid-induced writhing in mice 41

LIST OF APPENDICES

Appendix 1: Ethical Approval Letter 80

Appendix 3: Research Permit granted by the National Commision for Science, Technology, and Innovation 81

Appendix 4: Monograph of the Phytexponent formulation 82

Appendix 5: The researcher carrying out experiments in the laboratory 83

Appendix 6: *In vivo* anti-inflammatory activity data 84

Appendix 7: Analgesic activity data 87

Appendix 8: *In vitro* cytotoxicity data (MTT-Assay) 88

ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
GABA	Gamma Aminobutyric Acid
KEMRI	Kenya Medical Research Institute
LD_{50}	Median Lethal Dose
LPS	Lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NACOSTI	National Commission for Science, Technology, and Innovation
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NCI	National Cancer Institute
NF	Nuclear Factor
NMDA	N-Methyl-D-Aspartate
NSAIDs	Non-Steroidal Anti-inflammatory Drugs
OECD	Organization for Economic Co-operation and Development
PBS	Phosphate-Buffered Saline
PGHS	Prostaglandin Endoperoxide H Synthase
PH	Potential of Hydrogen
ROS	Reactive Oxygen Species
SDH	Succinate Dehydrogenase
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
WHO	World Health Organization

ABSTRACT

Pain and inflammation are the commonest manifestations of various pathologies, and are associated with high morbidities, debility, and economic strife globally, especially in underdeveloped regions of sub-Saharan Africa. The currently available conventional analgesic and anti-inflammatory drugs cause serious side effects, some of which are life threatening, are unaffordable, and unavailable to all patients, especially in low-income countries, hence the need for better alternatives.

In the current study, the *in vivo* anti-inflammatory, analgesic, and *in vitro* cytotoxic activities of the Phytexponent preparation comprising the ethanolic extracts of *Viola tricolor, Echinacea purpurea, Allium sativum, Matricaria chamomilla,* and *Triticum repens* were investigated. The carrageenan-induced paw oedema technique was adopted to investigate the anti-inflammatory activity of the Phytexponent in experimental mice, at doses of 15.625 mg/Kg BW, 31.25 mg/Kg BW, 62.5 mg/Kg BW, 125 mg/Kg BW, 250 mg/Kg BW and 500 mg/Kg BW, with Indomethacin (10 mg/Kg BW) as positive control drug. The paw sizes of respective animals were measured using a plethysmographic technique, and the values used to calculate the percentage reduction in oedematous paw size, as an indicator of anti-inflammatory activity of the Phytexponent.

The acetic acid-induced writhing technique was used to determine the analgesic activity of the Phytexponent in experimental Swiss albino mice at similar doses as those used for anti-inflammatory assay and indomethacin (4 mg/Kg BW) as the reference drug. Then, the number of wriths were recorded and expressed as the percentage inhibition of writhing.

The standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay technique was used to investigate the *in vitro* cytotoxic effects of the Phytexponent in Vero E6 cell line with cyclophosphamide as a positive cytotoxic agent.

The percentage inhibitions of cell proliferation (percentage cytotoxicity) were determined according to a standard procedure. The study findings revealed that the Phytexponent preparation exerted significant anti-inflammatory effects in carrageenan-induced paw oedema mouse model, which ranged from 1.117±0.193% at the first hour to 11.162±0.091% at the fourth hour, at a dose of 31.25 mg/Kg BW, 6.240±0.242 % at the first hour to 17.407±0.186% at the fourth hour at a dose of 62.60 mg/Kg BW, 9.645±0.020% at the first hour to 31.795±0.090% at the fourth hourat a dose of 125 ,g/Kg BW, and 14.000±0.102% at the first hour to 37.931±0.133% in the fourth hour, at a dose of 250 mg/Kg BW ($p<0.05$). Notably, the Phytexponent significantly inhibited inflammation in a dose- and time-dependent manner ($p<0.05$).

The Phytexponent preparation exhibited significant analgesic activity ($p<0.05$) in experimental mice as depicted by reduced writhing frequencies (high percentage inhibitions of acetic acid-induced writhing), which increased from 55.054±0.174% at a dose of 31.25 mg/Kg BW to 94.982±0.098% at a dose of 250 mg/Kg BW, in a dose-dependent manner ($p<0.05$). The Phytexponent exhibited significantly higher analgesic activity at doses of 125 mg/Kg BW (75.924±0.253%) and 250 mg/Kg BW (94.982±0.098%) than indomethacin (64.786±0.098%), indicating higher analgesic efficacy. The Phytexponent preparation was not cytotoxic to Vero E6 cells as indicated by high CC_{50} value (>1000 μg/ml) compared to cyclophosphamide (CC_{50}= 2.48μg/ml). The present study indicated that the Phytexponent formulation has significant *in vivo* anti-inflammatory and analgesic activities in mice models and is not cytotoxic to Vero E6 cell line. Therefore, based on the study findings, the Phytexponent formulation is a potential source of safe analgesic and anti-inflammatory associated phytocompounds. Further empirical studies, determination of mode(s) of anti-inflammatory and

analgesic efficacy, and safety of the Phytexponent and its bioactive phytochemicals should be undertaken.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Inflammation is a response of a tissue to a noxious stimulus, such as physical injury, irritant agents and pathogens (Chen *et al.*, 2018). It causes increased vascular permeability, changes in blood flow, and migration of leucocytes to the affected sites (Chen *et al.*, 2018). Pain refers to an unpleasant emotional and sensory experience that results from tissue damage and acts as a signal to warn against further disturbances (Raja *et al.*, 2020). The focus in pain management is to eliminate or remove its cause.

Pain, fever, and inflammation are associated with a myriad of pathological processes in the body (Cross, 1994; Ogoina, 2011; Walter *et al.*, 2016; Woessner, 2006). There are two forms of pain nociceptive that result from tissue injury: due to activation of specific nociceptors and the neuropathic pain that is caused by structural damage to the nerves (Marchand, 2008). Pain is a major health problem with profound debility in the afflicted subjects and persistent inflammation causes chronic diseases and promote tumour development (Olela *et al.*, 2020). On the other hand, fever is a sign of disease colonisation, which signals an inflammatory response aimed at limiting the spread of the microbes (Chen *et al.*, 2018; Pearlman, 1999).

There are various anti-inflammatory and antinociceptive drugs for the treatment of inflammation and pain (Giorno *et al.*, 2019; Herrero *et al.*, 2003; Newman and Agyare, 2017). However, there is an unending search for new therapeutic compounds to serve as alternatives because of the inaccessibility, unaffordability, adverse effects, and low efficacy of existing conventional medications (Herrero *et al.*, 2003; Olela *et al.*, 2020). In this regard, focus has shifted to investigating natural products, especially medicinal plants, as one of the most promising therapeutic agents for inflammatory diseases (Moriasi *et al.*, 2021b; Raisa *et al.*, 2018; Shojaii *et al.*, 2015; Wambugu *et al.*, 2011).

The most widely used anti-inflammatory agents are the non-steroidal anti-inflammatory drugs (NSAIDs) which act by inhibiting the cyclooxygenase (COX) enzymes, thereby prohibiting the production of prostaglandins (Monteiro and Steagall, 2019; Newman and Agyare, 2017; Ricciotti and Fitzgerald, 2011). However, they have been shown to cause serious side effects, such as liver damage, aseptic meningitis, and bone fractures (Felson, 2016).

In many African communities, especially in the rural areas, herbs are still used to manage various diseases because they are readily available and relatively less expensive compared to conventional medicines (Moriasi *et al.*, 2020a; Waiganjo *et al.*, 2020; World Health Organization (WHO), 2013). According to the World Health Organization, more than 85% of traditional medicine comes from plant

extracts (Ighodaro and Omole, 2012). In Kenya, there are various remedies for pain, fever, and inflammation, including some herbs, that are used in traditional medicine (Mukungu *et al.*, 2016; Nankaya *et al.*, 2020; {Ochwang} *et al.*, 2014). Traditionally, analgesic substances have been obtained from plants, with modes of action of some of them already extensively documented (Gwinnutt, 2007; Kumar *et al.*, 2010). Research data shows that plant-derived natural products are a bulwark of future drug discovery, especially for treatment of inflammation and pain (Calixto *et al.*, 2001; Fürst and Zündorf, 2014; Nunes *et al.*, 2020). This is encouraging, considering that more than 80% of the population in third world countries, especially in Africa, do not have access to modern medicine and entirely depend on traditional medicine for healthcare needs { World Health Organization (WHO), 2013].

In the last few years, ethnobotanical research has revisited traditional literature in the search for novel remedies for various ailments (Abreu *et al.*, 2012; Andrade-Cetto *et al.*, 2019; Moriasi *et al.*, 2020b). Plants hold assurance for discovery of new and effective drugs against pain and inflammation (Nunes *et al.*, 2020). Various inflammatory diseases such as ankylosing spondylitis, rheumatoid arthritis, systematic lupus erythematosus, rheumatic fever, and osteoarthritis are currently being managed by an array of synthetic drugs (Monteiro and Steagall, 2019). However, most of them are associated with adverse side effects, high costs, inaccessibility, which limit their usage (Felson, 2016).

Worldwide, drugs derived from plants offer a stable market and they serve as a source of novel drugs (Nunes *et al.*, 2020). In general, natural products, more especially plants, are novel sources of chemical substances with therapeutic capabilities (phytochemicals) (Abreu *et al.*, 2012; Moriasi, *et al.*, 2020c). Most of the anti-inflammatory, anti-malarial, analgesic, and antipyretic drugs have their origin in plants, including chloroquine, morphine, and aspirin (Patridge *et al.*, 2016; Veeresham, 2012). Therefore, there is a need to conduct more studies on plants to discover potent, accessible, affordable and safe products for the alleviation of pain and inflammation, and associated disorders.

Even though medicinal plants have extensive and longstanding utilization in alternative and complementary therapy, various concerns regarding their safety have been raised (George, 2011). For instance, there are no clear guidelines which govern traditional medicine, thus allowing unscrupulous practisoners to thrive (Arora, 2015). Additionally, there is scanty data on herb-herb and herb-drug interactions and associated effects to effectively guide prescriptions (Kaur *et al.*, 2013).

Moreover, in traditional medicine practice, there are no clearly outlined dosage forms for specific diseases and expected side effects (Kaur *et al.*, 2013). Furthermore, the lack of safety and toxicity profiles of many medicinal plants further cripples the confidence accorded to herbal medicine. As a result, it is

imperative to evaluate toxicity and safety of herbal preparations used to manage various diseases to avert the development of undesirable effects and fatalities (Arora, 2015; George, 2011; Kaur *et al.*, 2013).

Herbal remedies, such as the Phytexponent preparation containing ethanolic extracts of *Viola tricolor Echinacea purpurea, Allium sativum, Matricaria chamomilla,* and *Triticum repens* have been used in complementary and alternative medicine to manage inflammation and pain, and associated syndromes, and has demonstrated appreciable level of efficacy (Moriasi *et al.*, 2021). Polyherbal preparations, such as the Phytexponent are relatively cheap, readily available, cause fewer side effects, and are easy to administer (Atawodi, 2001; Girish *et al.*, 2004; Jangle, 2012). The plants used to formulate the Phytexponent are used in traditional medicine since they possess various pharmacologic activities against a variety of disease conditions. For instance, *Viola tricolor* has been traditionally used for treatment of inflammatory lung and skin ailments, such as ulcers, itching, scabs, psoriasis, and eczema (Hellinger *et al.*, 2014). Besides, *Echinacea purpurea,* which is indigenous to North America, is the most widely cultivated medicinal plant for use in chemotherapy, and is commonly used to alleviate cold symptoms. Manayi *et al.* (2015) noted that the herb has anti-inflammatory and immunostimulatory properties.

Additionally, *Allium sativum* (Garlic) is widely used as a food ingredient, and as an aphrodisiac to cause sexual arousal,pleasure and performance (Jayanthi and Dhar, 2011). Garlic extracts have more than 200 chemicals that have been identified to date, and determonstrated to be effective in treating various conditions, including some types of cancer (Martins *et al.*, 2016). (Arreola *et al.* (2015) reported that garlic products can be prepared in liquid or solid forms. The plant has many anti-inflammatory effects, including anticancer, antingiogenic, and free radical-mediated anti-inflammatory effects, antiobesity, among others (Yang *et al.*, 2018; Moriasi, *et al.*, 2021a).

Recently Moriasi *et al.* (2021a) investigated the *in vitro* anti-inflammatory, antioxidant activities of the Phytexponent and observed significant efficacy. Furthermore, qualitative phytochemistry of the Phytexponent revealed the presence of bioactive phytochemicals with diverse pharmacologic effects, including anti-inflammation (Moriasi *et al.*, 2021a) . However, there is a scarcity of documented studies on the *in vivo* efficacy of this polyherbal product, its mode(s) of action in various disease states, its toxicity, and safety. Therefore, this study was designed to investigate the *in vivo* anti-inflammatory, analgesic, and cytotoxic effects of the Phytexponent preparation of *Viola tricolor, Echinacea purpurea, Allium sativum, Matricaria chamomilla,* and *Triticum repens*, as a potential alternative source of affordable, accessible, potent, and safe analgesic and anti-inflammatory lead compounds for drug discovery and development.

1.2 Statement of the problem and Justification of the study

Fever, inflammation, and pain are critical signs manifesting in many diseases affecting humans and other animals, and lead to poor quality of life, disability, depression, mortality, and financial loss (Ricciotti and Fitzgerald, 2011; Taylor *et al.*, 2011; Khandaker *et al.*, 2015; Réus *et al.*, 2015; Walter *et al.*, 2016; Sahlmann and Ströbel, 2016; Sommer *et al.*, 2018).

Unfortunately, the management of pain, and inflammation is expensive, and it typically entails the administration of different classes of drugs which are associated with various insufficiencies (Felson, 2016). Most of these drugs have serious side effects, such as gastric ulcers, hepatotoxicity, nephrotoxicity, cardiotoxicity, among others, caused by non-steroidal anti-inflammatory drugs like aspirin, diclofenac, among others (Fokunang, 2018; Harirforoosh *et al.*, 2013; Sylvester, 2019). Research has established that herbal remedies are cheap, easily available, effective, and elicit fewer side effects (Azab *et al.*, 2016; Nasri and Shirzad, 2013; Olela *et al.*, 2020). However, many of the plant-based remedies have not been scrutinised with scientific precision to determine their efficacy, composition, mode of action, toxicity profile and safety.

Herbal remedies, such as the Phytexponent preparation composed of ethanolic extracts of *Viola tricolor Echinacea purpurea, Allium sativum, Matricaria chamomilla,* and *Triticum repens*, have been used to manage pain and

inflammatory conditions with demonstrable degree of efficacy (Moriasi *et al.*, 2021a). Additionally, herbal preparations are cost effective, readily available, with fewer side effects, and easy to administer. Therefore, scientific studies on their pharmacologic efficacy, toxicity, safety is a worthy undertaking as they present a viable alternative source of potent therapies for various maladies, including pain and inflammation.

1.3 Study objectives

1.3.1 General Objective

The main objective of the study was to investigate the *in vivo* anti-inflammatory, analgesic, and cytotoxic effects of the Phytexponent preparation: A polyherbal formulation.

1.3.2 Specific objectives

i. To determine the *in vivo* anti-inflammatory activity of the Phytexponent preparation in Swiss albino mice.

ii. To investigate the analgesic effects of the Phytexponent preparation in Swiss albino mice.

iii. To evaluate the cytotoxic effects of the Phytexponent preparation in Vero E6 cell line from the green monkey kidney cells.

1.4 Research Questions

This study was guided by the following research questions:

i. Does the Phytexponent preparation have anti-inflammatory activity in Swiss albino mice?

ii. Does the Phytexponent preparation have analgesic activity in Swiss albino mice?

iii. What are the cytotoxic effects of the Phytexponent preparation in Vero E6 cell line?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biochemical Basis of Pain

Experiencing pain is important for the survival of mammals, following physical injury, toxicity or pathogenic assault, with the aim of warning the organism to escape the stimuli, and averting tissue damage (Gitahi et al, 2015; Kumar and Elavarasi, 2016). For humanity, pain is a universal experience that everyone is accustomed to. The unpleasant subjective experience can affect all areas of life as it involves neocortical, psychological, physiological, and biochemical processes (Almeida *et al.*, 2001; Camussi *et al.*, 1981; Kumar and Elavarasi, 2016; Omoigui, 2009).

The pain threshold is the first perceptive pain to appear under a given condition or stimulation (Réus *et al.*, 2015). In scholarly literature, there are two types of pain: superficial and deep pain. Superficial pain results from intense stimulation of the skin while deep pain comes about from skeletal muscles, tendons, joints, and periosteum (Ji *et al.*, 2013; Lester, 2016; Swieboda *et al.*, 2013a).

Previous studies have established that one nociceptive stimulus creates a double pain sensation, with the second one being more diffuse (Abdo *et al.*, 2019; Craig *et al.*, 1994; Dai *et al.*, 2007; Yam *et al.*, 2018). Some scholars have proposed that the first and second pain is caused by the activation of A and C fibres (Frias and

Merighi, 2016; Sneddon, 2018; Tracey and Dickenson, 2012). Notably, the muscle, skin, and visceral (internal) nociceptors terminate as free nerve endings; hence can be easily activated by strong chemical, thermal, or mechanical stimulation. Furthermore, sensitisation by low PH, ischemia, inflammation, tissue injury can activate them (Baliki and Apkarian, 2015; Nickel *et al.*, 2012; Nijs *et al.*, 2012; Tracey, 2017).

It is well documented that nociceptor sensitisation is mediated by messenger systems, which lead to the production and release of histamines, serotonin, bradykinin, and prostaglandins (Cairns *et al.*, 2015; Clifford *et al.*, 2012; Deitos *et al.*, 2015; Henry, 2008). These chemicals have receptors on the surfaces of most nociceptive afferents, in association with the receptors for γ-amino butyric acid (GABA), opiates, and capsaicin (Frias and Merighi, 2016; Hung and Tan, 2018; Kidd *et al.*, 2004; Risch *et al.*, 2017). Activation of nociceptors triggers a cascade of processes which lead to modification of responses to stimuli (Sneddon, 2018; Tracey, 2017).

2.2 Biochemical and molecular basis of inflammation

Inflammation can be defined as a generalised, non-specific, yet beneficial response of body tissues to injury (Medzhitov, 2008; Nathan and Ding, 2010), associated with migration of several cell types, increased capillary permeability, growth of new tissue, and cell apoptosis (Moriasi *et al.*, 2021a). It is, therefore, a

basic mechanism for tissue repair and protection against infections and antigens, thereby averting further tissue damage. As such, it is a fundamental biological process and one of the most notable signs of disease (Sahlmann and Ströbel, 2016).

Infections, and the secretion of cytokines by macrophages, often cause endothelial cells to rapidly upregulate the expression of selectins, a type of surface proteins that bind mucin-like adhesion molecules. Furthermore, inflammatory response comes about as a way of repairing the system processes. This is the reason why it is associated with increased capillary permeability and migration of cell types. According to Stankov (2015), inflammation can be caused by hypoxia, hypersensitivity states, injury, and infection. In its acute phase, it manifests in fever, pain, and oedema (Chen *et al.*, 2018).

In the body, there are several chemicals that serve as inflammatory mediators, but they can be grouped broadly into lipid derivatives, cytokines, vasoactive amines, chemokines, complement, and proteases (Medzhitov, 2008). Examples of the substances include serotonin, histamine, bradykinin, nitric oxide, interleukins, leukotrienes, and tumour necrotic factors (TNFs). Etiological factors for inflammation include bacterial degradation products such as lipopolysaccharides, lipopeptides, peptidoglycans, formylmethionyl peptides, flagellin, fungi and virus products, as well as microbial DNA (Maina *et al.*, 2015).

In most cases, inflammatory responses are controlled, and they are beneficial to the body. However, sometimes they may be detrimental, especially when they are not well regulated, such as in the case of septic shock. Some instances of inflammation are associated with depressive illness, which increases with the extent of acute inflammation (Lordan *et al.*, 2019; Taylor *et al.*, 2011).

Management of patients with inflammatory disorders, including psoriasis and atopic dermatitis is still a challenge (Moriasi *et al.*, 2021b). There are various environmental, genetic, and immunological factors that contribute to the conditions. In particular, the adaptive immune system plays a critical role in their pathogenesis by causing the accumulation of inflammatory cells, such as the T-cells in the affected area. The T-cells cause cell-mediated inflammation by maintaining the activation of macrophages and dendritic cells, thereby transforming them into tissue destructive effector cells (Anoop. and Anoop, 2013; Chen *et al.*, 2018; Lordan *et al.*, 2019).

2.3 Neural Transmitters and Nociceptive Systems

There are several neural transmitters that sub-serve the pain states, including opioids, acetylcholine, glutamate, gamma aminobutyric acid (GABA), and neurokinins (Yam *et al.*, 2018). Acetylcholine works by imparting antinociceptive effects through various receptors such as the peripheral muscarinic cholinergic and central nicotinic receptors. Endogenous opioids have antinociceptive effects

and they are categorised into three as enkephalins, endorphins, and dynorphins. All of these substances cause an analgesic impact through opioid receptors liked with a G-protein that is classified as μ δ, κ opioid receptors (Yam *et al.*, 2018).

2.4 Analgesic assays

There are various tests that scientists use to assess nociception in laboratory animals. The most common ones are the writhing (abdominal constriction test), formalin test, tail flick, hot plate, and paw pressure tests (Deshmukh *et al.*, 2014). Writhing test is the most commonly used, especially when investigating visceral pain (Gawade, 2012; Koster *et al.*, 1959). It is an induction of abdominal constriction through the injection of acetic acid, which irritates the animal in the peritoneum, thus activating peripheral nociceptors (Gawade, 2012).

Animals react by arching their back, extending the hind limbs, and contracting their abdominal muscles. The response is more pronounced in rats and mice which exhibit a wave of constriction and elongation, a twisting of the trunk, and extension of hindlimbs. Substances that have analgesic effects reduce the frequency of the writhes, a feature that is used in the screening test (Moriasi *et al.*, 2021b). However, the method has a limitation since it cannot be used in the clinical testing for human subjects.

The formalin test is used when the focus is on the response to moderate pain stimuli that is caused by an injured tissue (Deshmukh *et al.*, 2014). Low concentrations of formalin are injected into the dorsal surface of the experimental animal's paw. The administration causes behaviours such as lifting, shaking, licking, and biting of the injected paw. Latency of nociception can be calculated using the length of time used in the responses. Although the test is easy, cheap, and sensitive, it has the disadvantage of exposing the animal to prolonged pain and tissue damage.

2.5 Non-steroidal anti-inflammatory drugs

Various drugs for treating pain, fever, and inflammation have been developed, whereby the most common ones are the non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenases, effectively reducing prostaglandin levels (Luca, 2015). The NSAIDs are therapeutic agents with diverse structural and pharmacodynamic properties but have similar mechanisms of action to alleviate pain and inflammation (Bacchi *et al.*, 2012; Fokunang, 2018).

Despite the NSAIDs displaying similarities in terms of action and toxicity profiles, they differ significantly in the manner they interact with the cyclooxygenase enzyme in the body (Newman and Agyare, 2017). As a result, they are popularly classified as salicylates (aspirin), profens or 2-arylpropionic acids (Ketoprofen, naprofen, ibuprofen and flurbiprofen), aryl alkanoic acids

(nabumetone, diclofenac, sulindac and indomethacin), sulfonamides (nimesulide), pyrazolidine derivates (phenylbutazone), fenamic acids or n-aryl anthranilic acids (meclofenamic acid, and mefenamic acid), and oxicams (meloxicam and piroxicam) (Soriano *et al.*, 2019).

The most prominently used NSAIDs include paracetamol, diclofenac, aspirin and indomethacin for alleviating pain and inflammation (Moriasi *et al.*, 2021b). Paracetamol is clinically used to effectively manage pain and pyrexia associated with mild to moderate inflammation; however, it cannot be used to treat severe or chronic inflammation, such as that associated with rheumatoid arthritis (van Rensburg and Reuter, 2019). Research indicates that paracetamol indirectly inhibits the COX enzyme, by inhibiting its POX binding site, which reduces the active site's activity. This is in contrast with other NSAIDs and coxibs which directly inhibit the activity of the COX enzyme, thereby inhibiting prostaglandin synthesis (Fokunang, 2018).

Diclofenac is a monocarboxylic acid consisting of phenylacetic acid derivative (2- [2,6- dichloranilino] phenylacetic acid) (Figure 2.1), whose main mode of action is via the inhibition of both COX-1 and COX-2 enzymes and prostaglandin synthesis (Gan, 2010). Research has shown that diclofenac also inhibits leukotriene synthesis, phospholipase A_2 activity, and modulates arachidonic acid levels. Just like other NSAIDs, the specific mechanism of diclofenac's anti-

inflammatory and analgesic activities, is unknown (Gan, 2010). Due to its non-selective inhibition of the COX enzyme, it is associated with gastric ulcers, bleeding, among other adverse effects, which limit its usage (Muchonjo *et al.*, 2021).

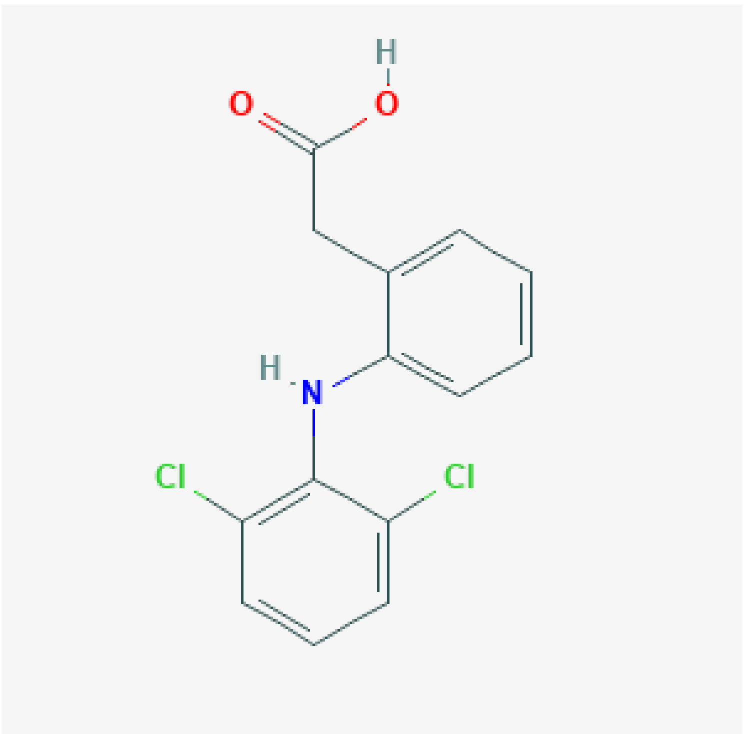

Figure 2.1: Structure of diclofenac

Indomethacin is a synthetic nonsteroidal indole-acetic acid derivative (1- (p-chlorobenzoyl)25-methoxy-2-methylindole-3-acetic acid) (Figure 2.2), which non-selectively inhibits the activity of both isozymes of the COX enzyme, preventing prostaglandin synthesis (Lucas, 2016). It is indicated to effectively control pyrexia, algesia-including migraines, and inflammation in the clinical setup, with higher potency compared to naproxen, ibuprofen, among others

NSAIDs. It is associated with adverse cardiovascular effects, nausea, dyspepsia, headache, hepatitis, jaundice, necrotizing fasciitis among other side effects (Lucas, 2016).

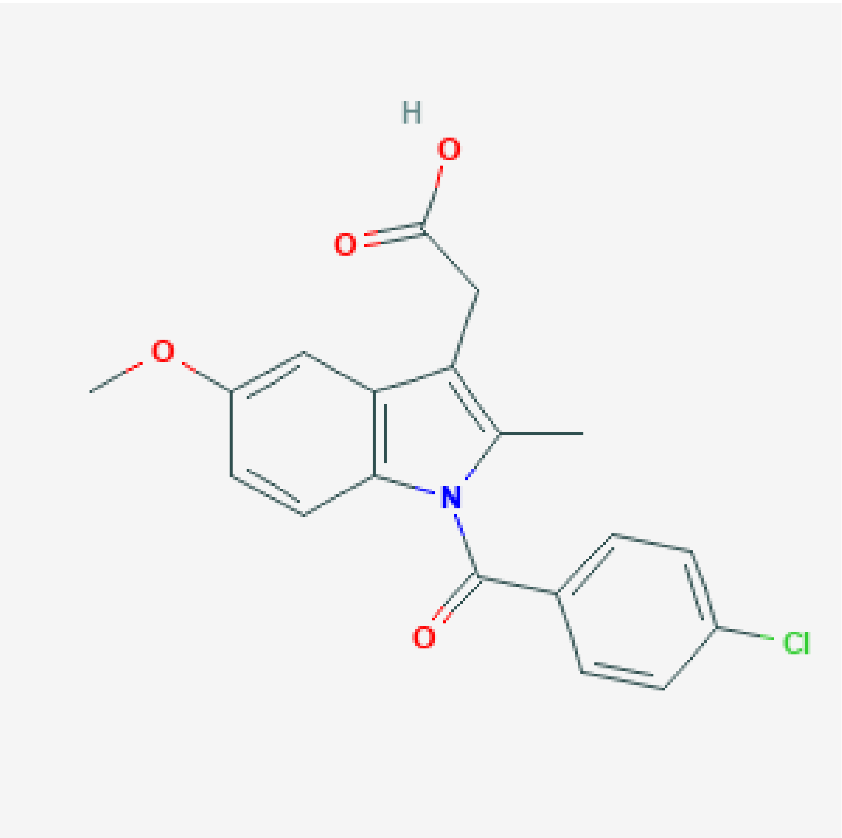

Figure 2.2: Structure of indomethacin

Acetylsalicylic (Aspirin) (Figure 2.3), non-selectively inhibits both COX-1 and COX-2, impeding prostaglandin synthesis, thereby inhibiting nociception and inflammation (Holstege, 2016). Despite its marked usage, aspirin has been associated with adverse effects including hepatotoxicity, nephrotoxicity, gastric

ulcerations, dyspepsia, cardiac effects, among others (Bacchi *et al.*, 2012; Fokunang, 2018; Harirforoosh *et al.*, 2013).

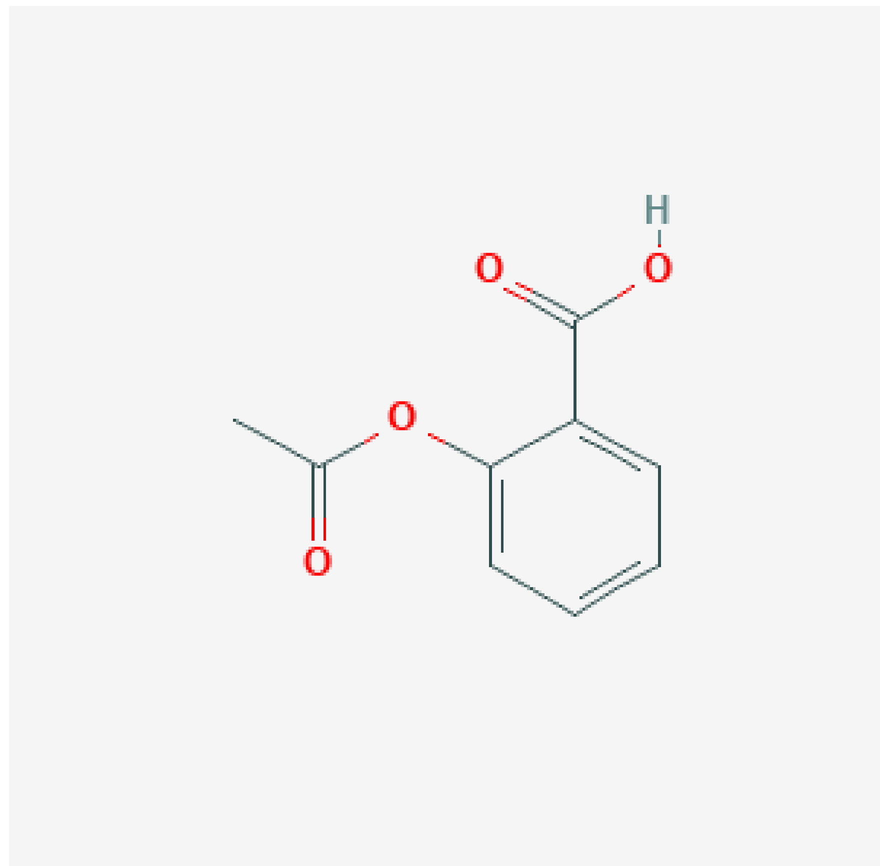

Figure 2.3: Structure of acetylsalicylic acid (aspirin)

Overwhelming scientific evidence has revealed that the inhibition of the cyclooxygenase enzyme is the main mechanism through which the NSAIDs exert their antipyretic, analgesic and anti-inflammatory activities (Newman and Agyare, 2017). The inhibition of the cyclooxygenase enzyme interferes with the synthesis of prostaglandins and other eicosanoids, which ameliorate fever, pain and inflammation in the body (Botting, 1988; Newman and Agyare, 2017).

The cyclooxygenase enzyme (COX) is also known as the prostaglandin endoperoxide H synthase (PGHS) and exists the COX-1 (PHGS-1) and COX-2 (PGHS-2) isoforms, respectively (Fitzpatrick, 2005). The two isoforms of the COX enzyme have a 60 % homology and exhibit significant structural differences. Both isoforms (COX-1 and COX-2) are encoded by different genes; however, both are membrane-bound glycoproteins which facilitate the synthesis of prostanoid from arachidonic acid involved in the mediation of fever, pain and inflammation in the body (Stolfi *et al.*, 2013).

Most of the mammalian cells like the seminal vesicle and endothelium constitutively express COX-1, which in quiescent conditions preform ‘housekeeping functions’ in the body (Attiq *et al.*, 2018). The COX-1 synthesises prostaglandins which have a protective role to the gastrointestinal tract, renal tract, modulate macrophage differentiation, mucus production and platelet aggregation. However, molecular studies have shown that COX-1 has a limited role in inflammation. Nevertheless, a nonselective inhibition of COX enzymes by some NSAIDs cause adverse effects in the gastro- and renal tracts, among others (Attiq *et al.*, 2018).

Upon tissue injury or trigger by some stimuli like the interleukin-1, tumour necrosis factor alpha (TNF-α) and lipopolysaccharide (LPS) induces the COX-2 enzyme in the injured site, vascular endothelium, among others, thereby

mediating, pain, inflammation, fever, among other associated responses in the body (Attiq *et al.*, 2018; Fitzpatrick, 2005). Besides, COX-2 has been shown to play housekeeping roles such as bone resorption, reproduction, neurotransmission, renal physiology, among others. Therefore, both the COX-1 and COX-2 isotypes can be both constitutive and inducible depending on the conditions and their inhibition can cause both beneficial and detrimental effects (Attiq *et al.*, 2018; Fitzpatrick, 2005; Fokunang, 2018).

2.6 The role of medicinal plants in the management of pain and inflammation

Nature is a good source of salvation for people's health problems because of the natural remedies that can be obtained from plant and animal products. Various research studies have reported on various plants that can be used to manage pain, inflammation, and fever (Maina *et al.*, 2015; Wanja, 2016; Cheruiyot, 2015; and Abu-Izneid, *et al.*, 2018).

The efficacy of various medicinal plants, including *Carissa edulis, Annona vepretorum, Acacia nilotica, Solanum incanum, Alhagi maurorum, Echinops echinatus, Panicum turgidum, Fagonia cretica, Lonchocarpus eriocalyx, Piliostigma thonningii, Mysthroxylon aethiopicum,* among others, against pain, inflammation, and pyrexia (Olela *et al.*, 2020; Mbiri *et al.*, 2016; Moriasi *et al.*, 2021b; Muchonjo *et al.*, 2021). Herbs are safer because they are natural; however, some of medicinal plant preparations have been shown to have adverse side

effects, such as allergic reactions, direct toxicity, and interaction with other drugs (Mensah *et al.*, 2019).

Since antiquity, plant-derived compounds have played a vital role in healthcare, more especially in remote areas not accessible for modern medicine (Vasanthi *et al.*, 2012). Various plants have phytochemicals with ability to fight diseases (Moriasi *et al.*, 2020a, 2020b). Some of these bioactive compounds are flavonoids, polyphenols, and catechins which are effective against cardiovascular disease, inflammatory bowel disease, and rheumatoid arthritis (Ibewuike *et al.*, 1997; Kamau *et al.*, 2016; Kurmukov, 2013; Wang *et al.*, 2014). The plant-based products have the advantage of showing few side effects unlike the synthetic products.

Various studies have demonstrated the use of medicinal plants, and some of them have validated their use for specific diseases (Vasundra and Divya, 2013; Sumithra *et al.*, 2011; Mukundi, *et al.*, 2015; Ishola *et al.*, 2014). There are general basic steps followed in development and use of plant products for medicinal purposes. First, the plant with the medicinal attributes is identified, its bioactive compounds are extracted and purified, and their effects validated *in vivo* using model animals (Gege-Adebayo *et al.*, 2013). Extraction of phytochemicals can be done using water or various organic solvents such as hexane, methanol, and ethanol.

2.7 Preparation and composition of the Phytexponent: A polyherbal formulation used in this study

The Phytexponent is a mixture of various plant extracts obtained from different plants, in a particular ratio, yielding a final product: A polyherbal formulation. The preparation procedure for the Phytexponent is as follows: (1) The appropriate plant materials are separately placed in a reaction vessel and extracted using alcohol (ethanol). (2) After two days, the respective mixtures are filtered, and the merc is squeezed to collect the extract. (3) The obtained extracts are then combined using a specific formula, based on dry weight: *Viola tricolor*- 3.77%, *Echinacea purpurea*- 26.42%, *Allium sativum*- 11.32%, *Triticum repens*- 26.42%, and *Matricaria chamomilla*- 32.08% (Swanstrom, 2007). The end product is a 62.1% ethanolic liquid concentrate of the mixed plant extracts.

Viola tricolor is a member of the Violaceae family, and commonly referred to as Heartsease, Johnny Jumpup, Call-me-to-you, or Bird's Eye (Lim, 2014). In Europe, the plant has been traditionally used to treat inflammatory lung and skin ailments, such as ulcers, itching, scabs, psoriasis, and eczema (Hellinger, et al., 2014). Previous research has revealed the presence of flavonoids, polysaccharides, phenylcarbonic acids, coumarins, catechins, and salicylic acid derivatives in *Viola tricolor* (Hellinger *et al*, 2014). It is also a rich source of macrocyclic peptides such as cyclotides, which act as immunosuppressive peptides that inhibit T-cell proliferation (Ravipati, 2016).

Besides, *Echinacea purpurea* is commonly known as eastern purple coneflower, or purple coneflower, and is indigenous to North America. It belongs to the Asteraceae family, and is the most widely cultivated medicinal plant for use in chemotherapy. Manayi *et al.* (2015) noted that the perennial medicinal herb has anti-inflammatory and immunostimulatory properties. It is commonly used for alleviating cold symptoms. In recent years, the herb has attracted the attention of many researchers, due to its many uses. Various studies have reported on its antimutagenicity, cytotoxicity, antidepression, virucidal, immunomodulation, and antianxiety effects of *Echinacea purpurea* (Gleeson, 2013; Markham and Dog, 2013; Signer *et al.*, 2020). However, it has been noted that the use of the plant leads to serious side effects such as urticaria, erythema, rash, pruritus, nausea, dyspnea, angioedema, and abdominal pain (Manayi *et al.*, 2015).

Some studies have reported that the *Echinacea* preparation can reverse inflammation induced by bacteria in a culture of epithelial cells through a reduction of cytokines (Sharma *et al.*, 2010). One study demonstrated that dried root powder of the herb inhibit edema in mice due to the inhibition of COX-1 and Cox-2 by alkylamides (Clifford *et al.,*2012).

Allium sativum (Garlic) is one of the most studied and best-selling herbal products in the world (Majewski, 2014). It is also widely used as a food ingredient, as an aphrodisiac and spice. It is a member of the onion family, Alliaceae (Moutia,

Habti, & Badou, 2018). Garlic extracts have more than 200 bioactive chemicals that have been identified so far, and most of them are effective in treating various conditions, including some types of cancer (Majewski, 2014). Arreola *et al.* (2015) reported that garlic products can be prepared in liquid or solid forms. The plant has many pharmacologic effects, including anticancer, antingiogenic, and antioxidant-mediated anti-inflammatory effects. In Swiss albino mice and Winstar rats, garlic has been shown to improve dyslipidemia, hyperglycemia, and allergy response (Arreola *et al.*, 2015). Furthermore, aqueous garlic extracts exert antioxidant activity by inhibiting excessive production of reactive oxygen species (ROS), or their quenching, and enhancing enzymes such as glutathione peroxidase, catalase, and superoxide dismutase (Arreola *et al.*, 2015).

Matricaria chamomilla is a well-known medicinal plant species of the Asteraceae family, used as a herbal remedy for various diseases, for many years (Singh *et al.*, 2011). Its products are ingredients in more than 26 drugs (Singh *et al.*, 2011). It is also an ingredient in several traditional homeopathy and unani medicinal preparations. Chamomile is used as an anti-inflammatory and antispasmodic drug, and to alleviate stomachache (Singh *et al.*, 2011). Moreover, it is extensively used as a tea drink or tonic, as well as for treating hysteria, anxiety, insomnia, and nightmares (Singh *et al.*, 2011).

Tricum repens is commonly referred to as couch grass, and is an invasive weed of the Gramineae family, commonly refered to as the wheat family (Sanguigno *et al.*, 2018). Its roots and leaves are used for making medicine for constipation, bladder swelling, cough, fever, hypertension, kidney stones, and inflammation (Sanguigno *et al.*, 2018). Notably, it is an important ingredient of some pharmaceutical formulations used to treat burns, skin lesions, and decubitus ulcers (Sanguigno *et al.*, 2018). In recent years, research focus has shifted to evaluating its anti-inflammatory properties (Sanguigno *et al.*, 2018).

2.8 Toxicity of Herbal Products

Studies have indicated that many people are now turning to herbal medicine, due to its presumed potency and safety (Saleem *et al.*, 2017). As such, there is a need to empirically investigate their efficacy and safety. Plants with medicinal activity should exhibit low toxicity since they are used for extended periods by humans (WHO, 2013). However, the toxicity and safety profiles of many plants remain unknown due to limited empirical data.

Because the phytexponent is a polyherbal mixture of five different medicinal plants, its antiinflmmatory and analgesic efficacy, toxicity and safety profiles are yet to be established despite its longstanding usage. Therefore, there is a need to investigate the cytotoxic effects of the phytoexponent preparation to lay an evidence-based framework towards its validation and further development.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 The source of the Phytexponent polyherbal formulation

The phytoexponent formulation (Pharmapath 27, Belgium; LOT NO:17E19) was purchased from a local pharmacy outlet in Nairobi and stored at room temperature according to the manufacturer's guidelines awaiting use.

3.2 Experimental animals

Swiss-albino mice weighing 24±1 g, and aged between four and five weeks old were purchased from the Kenya Medical Research Institute's animal breeding house. They were housed in standard conditions, in polypropylene rectangular cages measuring 30 cm × 20 cm × 13 cm in which soft wood shavings were added as bedding material. The mice were fed on standard laboratory rodent food (pellets) and clean water *ad-libitum* and maintained at natural day-night cycle. The experimental mice were acclimatised to the laboratory settings for 72 hours prior to experimentation.Proper handling and appropriate protocols for laboratory animal care and use were followed.

3.3 Determination of *in vivo* anti-inflammatory activity using Carrageenan-induced paw oedema in mice technique

In vivo anti-inflammatory activity of the phytoexponent was examined using the Carrageenan-induced paw edema technique in Swiss albino mice according to the

method of Winter *et al.* (1962), with minor modifications. Experimental mice were randomised into 8 groups comprising of 5 mice per group. Briefly, the normal control group [1] mice were orally administered with 10 ml/Kg BW of normal saline. The negative contron group [2] were given normal saline (10 ml/Kg BW) orally, and after 30 minutes, they were injected with 100 µl of 1 % Carrageenan (Sigma-Aldrich, Germany) at the subplantar region of the right hind paw (*s.p*). The positive control group [3] mice received 10 mg/Kg BW of indomethacin (Batch No. 20100, CarePlus Ltd, Kenya) orally and 100 µl of 1 % carrageenan through the subplantar region of the right hind paw after 30 minutes. Mice in groups 4 to 8 were administered orally with the Phytexponent preparation at dose levels of 15.625 mg/Kg BW, 31.25 mg/Kg BW, 62.5 mg/Kg BW, 125 mg/Kg BW, 250 mg/Kg BW and 500 mg/Kg BW, respectively, which were selected based on a pilot study, and 100 µl of 1 % Carrageenan via the subplantar route after 30 minutes.

The changes in paw diameter sizes were measured before induction of inflammation, and after 1 hour, 2 hours, 3 hours, and 4 hours, respectively, following the induction of inflammation, using a plethysmographic technique{the technique is evaluated to determine static lung volumes and airflow resistance}. Thereafter, the percentage changes in paw volumes were calculated and tabulated. Table 3.1 summarises this experimental design.

Table 3.1: Experimental design for the determination of anti-inflammatory activity of the Phytexponent preparation

Group	Treatment Adminstered
I: Normal Control	Normal Saline (10.00 ml/kg bw; *p.o.*) only
2:Negative Control	Normal Salne (10.00 mg/Kg BW; *p.o.*) + 1 % Carrageenan (*s.p.*)
3: Positive Control	Indomethacin (10.00 mg/Kg BW; *p.o.*) + 1 % Carrageenan (*s.p.*)
4: Test group [A]	Phytexpoent (15.625 mg/Kg BW; *p.o.*) + 1 % Carrageenan (*s.p.*)
5: Test group [B]	Phytexpoent (31.25 mg/Kg BW; *p.o.*) + 1 % Carrageenan (*s.p.*)
6: Test group [C]	Phytexpoent (62.50 mg/Kg BW; *p.o.*) + 1 % Carrageenan (*s.p.*)
7: Test group [D]	Phytexpoent (125.00 mg/Kg BW; *p.o.*) + 1 % Carrageenan (*s.p.*)
8: Test group [E]	Phytexpoent (250.00 mg/Kg BW; *p.o.*) + 1 % Carrageenan (*s.p.*)

Each group consisted of 5 mice; *p.o=per os (oral route); s.p*=subplantatar route; The volume of adminstration was 200µl.

3.4 Determination of potental analgesic effects of the Phytexponent

The analgesic activity of the Phytexponent preparation was evaluated according to the method described by Koster *et al.* (1959) with slight modifications. Experimental mice were randomly allocated into 8 groups each consisting of 5 mice as shown in table 3.2. The normal control group mice [A] received normal

saline (10 mg/Kg BW; *p.o*) only. The negative control group [B] mice were orally admisntered with normal saline at 10 mg/Kg BW; *p.o* and acetic acid (0.6 % w/v; *ip*) (Lot#L148661503; Loba Chemie) after 30 minutes. On the other hand, the positive control group [C] mice received indomethacin (4 mg/Kg BW; *p.o*) and acetic acid (0.6 % w/v; *i.p*) after 30 minutes.

Besides, the experimental groups 4 to 8 of mice were orally administered with the Phytexponent preparation at dose levels of 15.625 mg/Kg BW, 31.25 mg/Kg BW, 62.5 mg/Kg BW, 125 mg/Kg BW, 250 mg/Kg BW, and 500 mg/Kg BW, respectively, 30 minutes before the intraperitoneal injection of 0.6 % v/v of acetic acid. The total number of writhes was recorded for each experimental mouse after 5 minutes of writhing induction for 15 minutes, and expressed as the percentage inhibition of writhing.

Table 3.2: Experimental design for the determination of analgesic activity of the Phytexponent

Group	**Treatment Adminstered**
A: Normal Control	Normal Saline (10.00 ml/kg bw; *p.o.*) only
B: Negative Control	Normal Salne (10.00 mg/Kg BW; *p.o.*) + 0.6 % w/v acetic acid (*i.p.*)
C: Positive Control	Indomethacin (10.00 mg/Kg BW; *p.o.*) + 0.6 % w/v acetic acid (*i.p.*)
D: Test group [1]	Phytexpoent (15.625 mg/Kg BW; *p.o.*) + 0.6 % w/v acetic acid (*i.p.*)
E: Test group [2]	Phytexpoent (31.25 mg/Kg BW; *p.o.*) + 0.6 % w/v acetic acid (*i.p.*)
F: Test group [3]	Phytexpoent (62.50 mg/Kg BW; *p.o.*) + 0.6 % w/v acetic acid (*i.p.*)

G: Test group [4]	Phytexpoent (125.00 mg/Kg BW; *p.o.*) + 0.6 % w/v acetic acid (*i.p.*)
H: Test group [5]	Phytexpoent (250.00 mg/Kg BW; *p.o.*) + 0.6 % w/v acetic acid (*i.p.*)

Each group consisted of 5 mice; *p.o=per os (oral route); i.p*=Intraperitoneal route; The volume of adminstration was 200µl.

3.5 Cell culture technique

3.5.1 Vero E6 cell line culture

The normal kidney epithelial cell line derived from the African green monkey (Vero E6) was obtained from the American Type Culture Collection (ATCC) (Rockville, USA), and preserved at the Centre for Virus Research, at the Kenya Medical Research Institute (KEMRI). The Vero cell line (Vero E6) was cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™, Sigma-Aldrich, Chem, St. Louis, MO), in an aseptic environment to avoid contamination.

The Vero E6 culture was supplemented with penicillin (100 units/ml)-streptomycin(100 µg/ml)(Sigma-Aldrich, St. Louis, MO, USA) to reduce extraneous bacterial contamination, and 10% foetal bovine serum (10 % FBS)(Bio Whittaker®, Verviers, Belgium). The culture was incubated at 37°C in an incubator (SHEL LAB™, Sheldon Mfg, Inc., OR, USA) with 5% CO_2 in air and 65 % humidity. The T75 culture flasks were used to culture the studied cells. The growth of cells was controlled thrice a week, on Monday, Wednesday, and Friday, respectively. The modified procedure of (Bibi *et al.*, 2012) was adapted in this study.

3.5.2 Passaging technique

The passaging of Vero E6 cells that had attained a 90%-100% confluence was performed in this study. The procedure involved the removal of old media, and washing the cells twice with Phosphate Buffered Saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA).

3.5.3 Trypsinisation and resuspension procedures

During cell passaging or transfer, 200 µl of trypsin (Batch No. G507308; LobaChemie) was carefully added through the side of the culture flask, swirled and then incubated for 10 minutes to detach the cells from the base of the culture flask. After that, the cell culture flasks were observed under an inverted microscope (Olympus, Tokyo, Japan) (×40 magnification) to confirm the detachment of cells. Thereafter, 5 ml of fresh media were added into the flask to suspend the detached cells and stop further trypsin activity. The cells that had detached as clamps were resuspended in 5ml of trypsin by gently purging to obtain a homogenous suspension.

3.5.4 Determination of the *in vitro* cytotoxic effects of the Phytexponent preparation

The standard 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay technique (Bibi *et al.*, 2012; Van Meerloo *et al.*, 2011) was used to determine the viability of Vero E6 cells in the presence and absence of the

Phytexponent preparation. In this assay, 100 µl of the growth medium was transferred into each well of the 96-multiwell plate and then seeded with 20,000 Vero E6 cells and allowed to attach overnight. Various serial concentrations of the Phytexponent and Cyclophosphamide (Sigma-Aldrich, St. Louis, MO, USA) (positive control) were added to respective wells in triplicate. After that, the multiwell plates were incubated for 48 hours at 37 ^{o}C, 5 % CO_2 and 95 % relative humidity in an incubator.

Following culturing, 10 µl of freshly prepared MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the plates were further incubated for 4 hours. With the help of a micropipette, therespective supernatants were aspirated followed by addition of 100 µl of DMSO (Lot#A218101702, Loba Chemie) to solubilise the MTT crystals. The plates were then agitated and optical densities of eachwell were measured using an ELISA scanning multiwell spectrophotometer (Multiskan Ex lab-systems) at 562 nm. The percentage inhibitions of cell proliferation (percentage cytotoxicity) was calculated using the following formula described by Fatemeh and Khosro, (2013).

$\% \, Cell\ inhibition = 1 - \left(\frac{Optical\ density\ of\ treated\ cells}{Optical\ density\ of\ control}\right) \times 100$ inhibition=1-Optical density of treated cells/Optical density of control×100

3.6 Data management and statistical analysis

The obtained data from anti-inflammatory, analgesic, and cytotoxicity assays were first tabulated on Excel (Microsoft 365) spreadsheet and then exported to GraphPad prism version 8.4.3 for analysis. The data were subjected to descriptive statistics and the results were expressed as $\bar{x} \pm SEM$ of independent replicate experiments. Then, One-Way ANOVA was done to determine significant differences among means of independent treatement groups followed by Tukey's *post hoc* test for pairwise comparisons and separations of means at α=0.05.

Unpaired student *t*-test statistic was performed to compare between the cytotoxic effects of the Phytexponent and cylophosphamide at 95% confidence level. The median cytotoxic concentrations (CC_{50}) of the Phytexponent and cyclophosphamide were also determined in this study. The findings of this study were presented in graphs and tables.

3.7 Ethical Consideration

The experimental mice were used and disposed of as per the guidelines set out by the University of Nairobi ethical review committee and the OECD (2008). The cell line (Vero E6) was used and disposed of according to the protocols set out by the Scientific Ethical Review Unit (SERU) of the Kenya Medical Research Institute (KEMRI/RES/7/5/2). Permission to conduct this study was obtained from

the University of Nairobi biosafety, animal use, and ethics committee (BAUEC)(FVM BAUEC/2020/265).

CHAPTER FOUR

4.0 RESULTS

4.1 Anti-inflammatory activity of the Phytexponent preparation in Swiss albino mice

There were significant inhibitions ($p<0.05$) in carrageenan-induced paw oedema in experimental mice, which were administered with the Phytexponent preparation, in a dose-dependent manner in the first hour (Table 4.1). Notably, no significant differences in percentage inhibition of oedema in mice were observed between the mice which received the Phytexponent preparation at a dose of 125 mg/Kg BW, and and those in the positive control group which were administered with indomethacin at a dose of 4 mg/Kg BW ($p>0.05$; Table 4.1). However, a significantly higher percentage inhibition of paw edema was observed experimental mice, which received 250 mg/Kg BW of the Phytexponent preparation compared with the other treatments ($p<0.05$; Table 4.1). Besides, the negative control mice had a significantly lower inhibition of oedema than all the other experimental mice ($p<0.05$; Table 4.1).

In the second hour, the negative control mice showed a significantly lower percentage inhibition of carragenaan-induced paw oedema in mice compared with all the other expermental mice ($p<0.05$; Table 4.1). Significant dose-dependent inhibitions of paw oedema in mice, which were treated with the Phytexponent at

the studied dose levels, were observed in this study ($p<0.05$). The mice, which were administered with 250 mg/Kg BW of the Phytexponent preparation, had a significantly higher percentage inhibtion of paw oedema compared with the percentage inhibitions of paw oedema recorded in mice in all the other treatment groups in the second hour ($p<0.05$; Table 4.1). A significantly low percentage inhibition of Carrageenan-induced paw oedema was observed in the negative control mice, compared with the inhibitions in all the other experimental groups ($p<0.05$; Table 4.1).

Generally, there was a significant dose-dependent percentage inhibitions of Carrageenan-induced paw oedema in mice which were administered with the Phytexponent preparation at the studied dose levels in the third hour ($p<0.05$; Table 4.1). The percentage inhibitions of carragenaan-induced paw oedema in mice which were treated with the Phytexponent preparation at a dose of 125 mgkKg bw and the reference drug (indomethacin 4mg/Kg BW) were not significantly different ($p>0.05$; Table 4.1). However, the negative control group mice exhibited a significantly lower percentage inhibition of paw oedema compared with all the other experimental mice ($p<0.05$; Table 4.1). Moreover, in the third hour, the mice that were treated with the Phytexponent preparation at a dose of 250 mg/Kg BW, had the highest inhibition of Carrageenan-induced paw oedema than all the other mice ($p<0.05$; Table 4.1).

In the fourth hour, a dose dependent percentage inhibition of Carrageenan-induced paw oedema was observed in mice that received the Phytexponent preparation at the studied dose levels ($p<0.05$; Table 4.1). The highest percentage inhibition of paw oedema was observed in mice that were administered with 250 mg/Kg BW of the Phytexponent, compared with all the other mice ($p<0.05$; Table 4.1). Generally, a dose dependent increase in percentage inhibitions of paw oedema was observed in mice that were treated with the Phytexpoent (Table 4.1). Besides, a significantly lower percentage inhibition of oedema was recorded in the negative control mice, than the inhibitions in all the other mice ($p<0.05$; Table 4.1).

A comparison of the percentage changes in carragenaan-induced paw oedema across time was also done in this study (Table 4.1). The observations revealed that the differences in percentage inhibition of Carrageenan-induced paw oedema in the normal control group micewere not signficiant across the four-hour period ($p>0.05$; Table 4.1). The findings further showed that the percentage inhibition of paw Carrageenan-induced paw oedema in the positive control mice increased significantly from the first hour through to the fourth hour ($p<0.05$; Table 4.1). Conversely, the percentage inhibition of paw oedema in the negative control group mice decreased significantly from the first hour to the fourth hour ($p<0.05$; Table 4.1).

Generally, the percentage inhibitions of Carrageenan-induced paw oedema in experimental mice that received the Phytexponent preparation at all the studied doses increased significantly from the first hour through to the fourth hour in a time-dependent manner($p<0.05$; Table 4.1). Notably, a significantly higher percentage inhibition of paw oedema was observed in mice which received 250 mg/Kg BW of the Phytexponent preparation, compared with all the other mice ($p<0.05$; Table 4.1).

Study Group	Treatment	%Inhibition of Carrageenan-induced paw oedema ($\bar{x}$±SEM)			
		1stHr	**2ndHr**	**3rdHr**	**4thHr**
Normal control	Normal saline only	$-0.040 \pm 0.018^{e}_{a}$	$0.039 \pm 0.014^{f}_{a}$	$0.030 \pm 0.008^{e}_{a}$	$0.020 \pm 0.007^{f}_{a}$
Negative control	Carrageenan +Normal saline	$-23.70 \pm 0.103^{f}_{d}$	$-25.623 \pm 0.170^{g}_{c}$	$-26.221 \pm 0.117^{f}_{b}$	$-27.679 \pm 0.173^{g}_{a}$
Positive control	Carrageenan + Indomethacin (4 mg/Kg BW)	$9.580 \pm 0.199^{b}_{d}$	$17.000 \pm 0.058^{b}_{c}$	$24.989 \pm 0.057^{b}_{b}$	$37.250 \pm 0.341^{b}_{a}$
Experimental Group A	Carrageenan + Phytexponent (31.25 mg/Kg BW)	$1.117 \pm 0.193^{d}_{d}$	$3.088 \pm 0.140^{e}_{c}$	$5.192 \pm 0.180^{d}_{b}$	$11.162 \pm 0.091^{e}_{a}$
Experimental Group B	Carrageenan +Phytexponent (62.50 mg/Kg BW)	$6.240 \pm 0.242^{c}_{d}$	$8.368 \pm 0.216^{d}_{c}$	$10.768 \pm 0.080^{c}_{b}$	$17.407 \pm 0.186^{d}_{a}$
Experimental Group C	Carrageenan + Phytexponent (125 mg/Kg BW)	$9.645 \pm 0.020^{b}_{d}$	$12.645 \pm 0.031^{c}_{c}$	$24.851 \pm 0.010^{b}_{b}$	$31.795 \pm 0.090^{c}_{a}$
Experimental Group D	Carrageenan + Phytexponent (250 mg/Kg BW)	$14.000 \pm 0.102^{a}_{d}$	$18.097 \pm 0.043^{a}_{c}$	$29.946 \pm 0.128^{a}_{b}$	$37.931 \pm 0.133^{a}_{a}$

Table 4.1:Anti-inflammatory activity of the Phytexponent preparation in Swiss albino mice

Values are expressed as $\bar{x} \pm SEM$;Means with similar superscript letters within the same column and similar subscript letters within the same row are not significantly different (One-Way ANOVA followed by Tukey's test; $p>0.05$).

4.2 Analgesic activity of the phytexponent preparation

The findings revealed a positive dose-dependent significant increase in the percentage inhibition of acetic-induced writhing in mice ($p<0.05$; Figure 4.1). Notably, at doses of 125 mg/Kg BW and 250 mg/Kg BW of the Phytexponent preparation.The percentage inhibitions of acetic acid-induced writhing were significantly higher than the percentage inhibitions caused by indomethacin (reference drug) ($p<0.05$; Figure 4.1). However, indomethacin exhibited a significantly higher inhibition of acetic acid-induced writhing in mice compared with the inhibitions caused by the Phytexponent at dose levels of 31.25 mg/Kg BW and 52.50 mg/Kg BW ($p<0.05$; Figure 4.1).

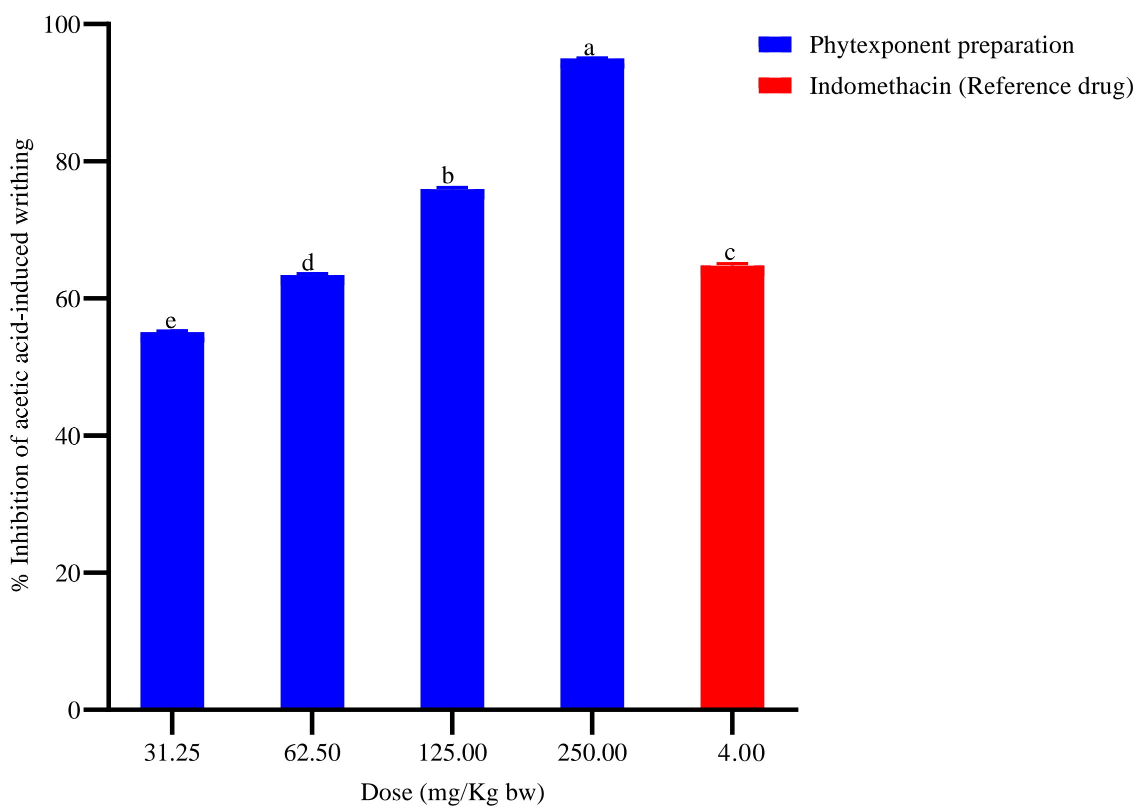

Figure 4.1: Analgesic effects of the Phytexponent preparation of selected medicinal plants in acetic acid-induced writhing in mice

Bars with dissimilar letters are significantly different (One-Way ANOVA followed by Tukey's test; $p<0.05$)

4.3 *In vitro* cytotoxic effects of the Phytexponent preparation

In this study, the results depicted a significantly positive dose-dependent increased percentage cytotoxicity of the Phytexponent on Vero cell line (normal cell line) ($p<0.05$; Table 4.2). Similarly, the reference drug (cyclophosphamide) caused a dose-dependent increase in cytotoxicity to Vero cell *in vitro* ($p<0.05$; Table 4.2).

A comparison between the cytotoxic effects of the Phytexponent and cyclophosphamide was also done. The results showed that at all the tested concentrations, the cytotoxicity of cyclophosphamide was significantly higher than that of the Phytexponent in Vero cells ($p<0.05$; Table 4.2). Furthermore, the median cytotoxic concentrations (CC_{50}) were >1000 µg/ml (1137.83µg/ml) for the Phytexponent and 2.48µg/ml for cyclophosphamide (Table 4.2).

Well	**Concentration (µg/ml)**	**% Cytotoxicity on Vero cell line**	
		Phytexponent	**Cyclophosphamide**
A	0.00	0.00±0.00	0.00±0.00
B	1.37	$2.82±1.41^{d}_{b}$	$46.54±0.34^{f}_{a}$
C	4.12	$3.57±1.47^{d}_{b}$	$55.52±1.27^{e}_{a}$
D	12.35	$5.34±1.47^{cd}_{b}$	$64.05±1.55^{d}_{a}$
E	37.04	$7.23±2.02^{cd}_{b}$	$68.46±2.51^{d}_{a}$
F	111.11	$8.66±1.93^{c}_{b}$	$73.73±3.13^{c}_{a}$
G	333.33	$18.88±2.00^{b}_{b}$	$85.73±0.99^{b}_{a}$
H	1000	$43.69±0.61^{a}_{b}$	$96.03±0.47^{a}_{a}$
	CC_{50}(µg/ml)	>1000 (Approx:1137.83)	2.48

Table 4.2: ***In vitro*** **cytotoxic effects of the Phytexponent on Vero cell line**

Values are expressed as $\bar{x} \pm SEM$; Means with similar superscript letter along the columns are not significantly different (One-Way ANOVA followed by Tukey's test; p<0.05); Means with different subscript letters across rows are significantly different (unpaired student t-test; p<0.05).

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Inflammation is the body's immune response to injurious stimuli like pathogens, irritants, trauma, chemicals, among other assaults (Chen *et al.*, 2018). Although inflammation's main goal is to eradicate infections and initiate tissue recovery, uncontrolled inflammation can cause deleterious effects that manifest in various chronic conditions (Garn *et al.*, 2016; Greenstein and Brook, 2011; Oike, 2011). Efforts have been made to understand the pathophysiology of inflammation and various molecules' role, including prooxidantsthat drive it (Baierle *et al.*, 2015; Reuter *et al.*, 2010; Solleiro-Villavicencio and Rivas-Arancibia, 2018). It is now clear that inflammation encompasses complex and diverse humoral and cellular mechanisms, including signalling molecules, immune cells, and gene regulatory molecules like the nuclear factor-kappa B (NF-kB)(Ahmed, 2011; Neher *et al.*, 2011).

Study has shown that ischemia, microbial infections, thermal and physical shocks, antigen-antibody interactions, and chemical irritants can initiate inflammation in the body (Stankov, 2015). At the tissue level, inflammation manifests in pain, swelling, redness, heat, and loss of function of the affected tissue, due to local immune, vascular and cellular responses to the offending stimuli(Hautz *et al.*, 2012; Sommer *et al.*, 2018). Altered permeability of the vasculature, recruitment of leukocytes, infiltration, the release of inflammatory mediators, and

the destruction of the affected and surrounding tissues culminate inflammatory responses(Sahlmann and Ströbel, 2016). Moreover, histamine, bradykinins, tumour necrosis factor α (TNF-α), serotonins, phospholipase A_2, interleukin-6, interleukin 1β, leukotrienes, nitric oxide (NO), cyclooxygenase 2 (COX-2), and lipoxygenases are the most important mediators of inflammation in the body (Abdulkhaleq *et al.*, 2018; Hautz *et al.*, 2012).

Inflammatory responses can be either acute or chronic(Sahlmann and Ströbel, 2016; Stankov, 2015). Acute inflammation is characterised by increased permeability of blood vessels, protein extravasation, and brief accumulation of leukocytes, which are mediated by COX-2, serotonin, and histamine and persists for a few minutes following tissue injury (Anoop and Anoop, 2013; Lordan *et al.*, 2019). Chronic inflammation results from the failure to resolve acute inflammation and autoimmune response to self-antigens, which are mediated by lipoxygenases, prostaglandin E_2(PEG_2), and NO. Chronic inflammation leads to rheumatoid arthritis, chronic periodontitis, systemic lupus erythematosus (SLE), chronic peptic ulcers, asthma, diabetes, cancer, among other complications (Briot *et al.*, 2017; Chen *et al.*, 2018).

Currently, the management of inflammation mostly utilises the non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, indomethacin, naproxen, ketoprofen, and ibuprofen, which inhibit the activity of COX-2 enzyme, which in turn deter the synthesis of prostaglandins like PGE_2 (Newman and Agyare, 2017).However, NSAID therapy causes dependence, is arguably unaffordable, inaccessible, and is often associated with adverse effects such as nephrotoxicity, cardiotoxicity, hepatotoxicity, intestinal bleeding, gastric ulcers, among other effects (Felson, 2016; Fokunang, 2018; Gan, 2010; Harirforoosh *et al.*, 2013). The drawbacks of conventional

inflammation management have reignited the search for alternative safer, efficacious, affordable, and accessible anti-inflammatory agents.

Herbal medicine has played a central role in meeting the primary healthcare needs of humankind since antiquity (Oliver, 2013; Unnikrishnan Payyappallimana, 2010). It is now estimated that over 80% of the people living in low- and middle-income countries, especially in Africa and Asian continents, entirely depend on medicinal plants for healthcare (WHO, 2013; 2018). Currently, there is a renewed research interest in natural products of plant origin due to their easy availability, accessibility, affordability, cultural acceptability, and fewer side effects (Haidan *et al.*, 2016; WHO, 2013). In light of this, this study was designed to investigate the anti-inflammatory, analgesic, and cytotoxic effects of the Phytexponent preparation of selected medicinal plants as a potential source of safer, efficacious, accessible, and affordable anti-inflammatory and analgesic molecules.

In this study, inflammation was induced in mice using carrageenan, a natural carbohydrate derived from edible red seaweed, widely used to screen plant extracts and molecules for anti-inflammatory efficacy (Winter *et al.*, 1962). Carrageenan induces a biphasic inflammatory response whereby distinct modulators are produced. In the early phase of carrageenan-inducedinflammation, cycloxygenase, histamine, and serotonin are produced, whereas in the late phase, which occurs after one hour, is characterised by PGE_2 synthesis, mediated by bradykinin and leukotrienes (Mansouri *et al.*, 2015; Necas and Bartosikova, 2013). In this case, the early and late phasesare characteristic of acute and chronic inflammation, respectively (Necas and Bartosikova, 2013). The upregulated synthesis of inflammatory mediators is due to the activation

and enhanced activity of the inducible nitric oxide synthase (iNOS) and COX-2 enzymes (Bukhari *et al.*, 2016; Necas and Bartosikova, 2013; Winter *et al.*, 1962).

Furthermore, carrageenan-induced inflammation increases the concentration and activity of pyrogenic cytokines, including the TNF-α, IL-1, and IL-6, among others (Necas and Bartosikova, 2013). Therefore, for an agent to be considered as having anti-inflammatory activity, it ought to alter the consequences of carrageenan-induced inflammation culminating inthe amelioration of its typical features such as oedema, pyrexia, redness, algesia, and tissue dysfunction (Adedapo and Ofuegbe, 2015; Coura *et al.*, 2015).

Studies have established that a solution of 1% carrageenan (prepared in physiologic saline), when injected at a volume of 50-150µl into the subplantar region, is sufficient to cause inflammation, which manifests in oedema (Amri *et al.*, 2017; Cai *et al.*, 2014). In this study, a subplantar injection of 100 µl of 1 % carrageenan into the right hind paw of experimental mice effectively induced inflammation, as evidenced by well-pronounced swelling around the injected site. The negative inhibitions of oedema are indiciative of progressive increase in oedema size, due to the inflammatory response to Carrageenan.

The findings revealed a progressive increase in oedematous paw size of the negative control mice throughout the treatment period, with significant progressive inhibitions,which indicates a successful induction of inflammation. Conversely, the reference drug (indomethacin) and the Phytexponent preparation effectively reduced oedema, in a dose- and time-dependent manner in mice as depicted by the percentage inhibtions of paw oedema, in a time- and dose-dependent

manner. Moreover, the findings indicated the Phytexponent successifully inhibited both the early and late phases of inflammation as depicted by the progresive increase in the percentage inhibitions of oedema. The time-dependent increase in percentage inhibition of oedema may be attributed to a higher bioavilability of the Phytexponent's active molecules, following metabolism and distribution to target sites (Capasso and Mannelli, 2020).

Indomethacin is a non-steroidal anti-inflammatory drug that interferes with the synthesis of prostaglandins from arachidonic acid by inhibiting the cyclooxygenase (COX) enzyme (Lucas, 2016; Summ and Evers, 2013). The COX enzyme exists in two isoforms: COX-1 and COX-2, respectively. Scientific evidence shows that COX-1 mainly catalyses the synthesis of prostaglandins, which are essential for maintaining the health and proper functioning of the gastrointestinal tract, platelet activity, renal functioning, and other vital physiological functions in the body (Fitzpatrick, 2005; Lucas, 2016).

On the other hand, COX-2 facilitates the synthesis of prostaglandins, which mediate pain, fever, and inflammation (Stolfi *et al.*, 2013). However, studies have shown that, in some instances,there is a crossover of the biological effects between COX-1 and COX-2 in the body (Attiq *et al.*, 2018). Just like other NSAIDs, indomethacin nonselectively inhibits both COX-1 and COX-2 to confer anti-inflammatory activity (Attiq *et al.*, 2018; Fitzpatrick, 2005; Fokunang, 2018). Even though the specific mode of action of the Phytexponent is yet to be established, the observations made herein partly suggest that its anti-inflammatory effects could be via the inhibition of the COX enzyme.

Pain is an unpleasant emotional and sensory experience resulting from tissue damage (Edu *et al.*, 2019). It acts as a warning signal to protect the body from actual or potential injury; however, it is associated with a disabling accompaniment of discomfort and adverse effects, characterising various medical conditions (Roizenblatt *et al.*, 2012; Treede *et al.*, 2019; Young Blood *et al.*, 2016). As a result, pain forms a critical component of disease diagnosis, and its management is among the most important therapeutic priorities in medical practice (Cox, 2010; Swieboda *et al.*, 2013b). Various analgesic agents are used to manage acute and chronic pain in patients (American Pain Society and The Joint Commission, 2010; Hylands-White *et al.*, 2017; Kumar, 2007). Currently, the most typical group of analgesic drugs used to manage pain comprises the NSAIDs, whose efficacy is based on the central and peripheral inhibition of prostaglandin synthesis. They interfere with the conversion of arachidonic acid to prostaglandins by inhibiting the COX enzyme's activity, thereby interfering with nociception (Bacchi *et al.*, 2012).

Since NSAIDs interfere with prostaglandins' normal synthesis and functioning, their side effects are predictable and include decreased homeostasis, renal dysfunction, hepatic dysfunction, peptic ulceration, intestinal bleeding, among others (Felson, 2016; Harirforoosh *et al.*, 2013). Empirical evidence shows that over 20% of patients under long-term NSAID therapy develop duodenal and gastric ulcers with profound consequences (Gan, 2010; Harirforoosh *et al.*, 2013). In light of these, the search for alternative, potent, safer, accessible, and affordable analgesics has attracted much attention in the realm of medical research.The acetic acid-induced writhing is an experimental reflex model of visceral pain that has been extensively utilised to screen drugs and chemicals for analgesic efficacy in laboratory animals (Koster *et al.*, 1959). In the present study, 0.6 % of acetic acid was intraperitoneally administered into experimental mice to induce pain by

activating chemosensitive nociceptors, which manifests in writhing (Gupta *et al.*, 2015). Writhing is described as the arching of the back, extension of limbs, and the abdominal musculature contraction (Gawade, 2012). In this experiment, the level of analgesia is indicated by the percentage reduction in abdominal writhing frequency.

This study showed a dose-dependent increase in the percentage inhibition of acetic acid-induced writhing by the Phytexponent preparation in mice, indicating its potential analgesic property. Similarly, indomethacin, the positive control drug, successfully inhibited the acetic acid-induced writhing in mice resulting in high percentage inhibitions. Moreover, the observationsof the present showed that the Phytexponent preparation at dose levels of 125 mg/Kg BW and 250 mg/Kg BW had significantly higher percentage inhibitions of writhingcomparedto indomethacin. These observations suggest that the Phytexpoent preparation is more potent at these doses than indomethacin. Partly, this observation could be attributable to the various phytoactive principles present in the Phytexponent preparation, which may have acted at different sites in a multitarget fashion to thwart pain as opposed to a single target effect (inhibition of the COX enzyme) of indomethacin.

Preliminary studies have demonstrated that each medicinal plant, which comprises the Phytoexponent preparation, has anti-inflammatory and analgesic properties (Arreola *et al.*, 2015; Asadi *et al.*, 2020; Jayanthi and Dhar, 2011; Manayi *et al.*, 2015; Nargesi *et al.*, 2018; Piana *et al.*, 2013). Additonaly, a recent study demonstrate significant *in vitro* anti-inflammatory and antioxidant efficacy of the Phytexponent formuation (Moriasi *et al.*, 2021a). Therefore, a combination of the analgesic- and anti-inflammatory-associated phytocompounds of individual

plants in the Phytexponent preparation may have synergistically conferred the bioactivities reported in the present study.

Moreover, studies have shown that chronic pain can be successfully managed by agents which modify the neurochemistry of the spinal cord dorsal horn, like anticonvulsants, local anaesthetic analogues, tricyclic antidepressants, γ-aminobutyric acid (GABA) agonists, and N-methyl-D-aspartate (NMDA) antagonists (Clifford *et al.*, 2012; Reitz *et al.*, 1995; True *et al.*, 2013). Opiates are useful in managing chronic pain; however, tolerance, dependence,and loss of efficacy limit their usefulness (Chau *et al.*, 2008; Chen and Ashburn, 2015; Halberstadt, 2017).

To this end, only NMDA antagonists and epidural morphine have consistently demonstrated preemptive analgesic efficacies (Price *et al.*, 2000; Sen *et al.*, 2006; Weinbroum *et al.*, 2001). Therefore, to adequately manage pain and inflammation, a multifaceted approach using multitarget agents is the most viable strategy to alleviating pain in affected patients (Honnappa and Kesavan, 2016; Hwang *et al.*, 2013). The results of this study, therefore, posit that the Phytexponent, by virtue of its analgesic and anti-inflammatory efficacy, could be a promising candidate for further development.

Even though the specific mode of action and the specific analgesic and anti-inflammatory bioactive molecules have not been elucidated, its is suggestive that the phytexpoent formulation targets various pathways associated with immunity, inflammation, and pain. Possibly, the phytocompounds present in this formulation could be maintaining the redox homeostasis, thereby preventing cell damage, and modulating immunity, modifying the inflammatory and pain

transduction pathways, which together ensure the proper functioning of cellular molecules and avert cellular damage (Baierle *et al.*, 2015; Moriasi *et al.*, 2021b; Reuter *et al.*, 2010).

Medicinal plants have longstanding usage in managing various diseases and play an integral role in meeting primary healthcare needs, especially in Sub-Saharan Africa (Mahomoodally, 2013; WHO, 2013; 2018). Indeed the world health organization estimates that over 80% of the global population depends on herbal medicine for their healthcare needs (World Health Organization, 2018). Despite the extensive utilisation of herbal products to manage various diseases, serious concerns regarding their efficacy and safety have been raised (George, 2011).

In the present study, the 3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide (MTT) colorimetric assay technique (Bibi *et al.*, 2012; Van Meerloo *et al.*, 2011) was employed to assess *in vitro* cytotoxicity and safety effects of the Phytexponent to assess its safety. This has was first described by Mosmann (1983), and has been extensively applied in the screening of anticancer potential of chemicals and plant extracts.

The MTT assay measures the activity of mitochondrial enzymes, especially the succinate dehydrogenase (SDH), whose function is impaired by toxic agents leading to mitochondrial collapse and cell death (Van Meerloo *et al.*, 2011). During the assay, the mitochondrial NADH reduced the MTT to a purple formazan product, which is determined calorimetrically at a specific wavelength (520 nm). The amount of formazan produced is directly proportional to the number of cells in a particular cell line (Van Meerloo *et al.*, 2011). This technique was selected

due to its high reproducibility, safety, sensitivity, and robustness in determining cell viability and cytotoxicity (Aslantürk, 2018).

According to the National Cancer Institute (NCI) criteria, plant extracts with CC_{50}< 30 µg/ml are considered to be cytotoxic after 48-72-hour exposure to cells (de Oliveira *et al.*, 2015). The observation from study revealed that the reference drug (cyclophosphamide) was a potent cytotoxic agent by its low CC_{50} value (CC_{50}=2.48 µg/ml) as consistently demonstrated in other studies. On the other hand, the Phytexponent preparation demonstrated low cytotoxic effects, as witnessed by its high CC_{50} (CC_{50}>1000 µg/ml predicted to be 1137.83 µg/ml). These results indicate that this polyherbal formulation might be safe and may be used to treat pain and inflammation without eliciting cytotoxic effects. However, extensive toxicity studies should be conducted to establish their safety profile.

In current medical practice, pharmaceutical drugs are designed to confer specific biological effects that are accompanied by specific side effects (Lucas, 2016; Zitvogel *et al.*, 2013). However, medicinal plants demonstrate a broad spectrum of bioactivities; thus, there are no defined toxic profiles (Singh and Sedha, 2018). This is attributable to the enormous phytoconstituents that act synergistically to affect various physiological functions in a non-specific manner (Hussein and El-Anssary, 2019; Altemimi *et al.*, 2017; Moriasi, *et al.*, 2020a). If a medicinal plant contains toxic compounds, the toxic effects elicited could be fatal; therefore, it is critical to validate medicinal plants' safety to avert potential fatalities. This study's findings demonstrate that the Phytexponent is non-toxic to Vero cell-lines-normal cells and is a potential source of safe analgesic and anti-inflammatory agents.

5.2 Conclusions

Based on this study's findings:

i. The phytexponent prepration showed dose-dependent *in vivo* anti-inflammatory activity in carrageenan-induced paw-oedema in Swiss albino mice.
ii. The phytexponent preparation showed remarkabale dose-dependent analgesic effects against acetic acid-induced writhing in experimental mice.
iii. The phytexponent preparation was non-toxic to Vero E6 cell line, indicating its safety.

5.3 Recommendations for further studies

i. Further studies aimed at establishing the specific mechanism(s) through which the Phytexponent confers the anti-inflammatory and analgesic effects should be done.
ii. Evaluation of the anti-inflammatory and antinociceptive activities of the Phytexponent in other experimental models and in the clinical settings after extensive safety appraissal are encouraged.
iii. Extensive toxicity and safety evaluation of this polyherbal product should be performed to give way for its further development.
iv. Additionally, the Phytexponent's bioactive phytoconstituents responsible for anti-inflammation and analgesia should be isolated, identified, and optimised as leads for drug discovery and development.

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APPENDICES

Appendix 1: Ethical Approval Letter

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,
Kenya.

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

REF: FVM BAUEC/2020/265

Dr. Halvince Omondi Odira,
University of Nairobi
Dept. PHP & Toxicology
24/02/2020

Dear Dr. Odira,

RE: Approval of proposal by Faculty Biosafety, Animal use and Ethics committee

Anti-Inflammatory, Anti-nociceptive and Toxic effects of a phytoexponent preparation of selected plant extracts.

Dr. Halvince Omondi Odira J56/11858/2018

We refer to your MSc. proposal submitted to our committee for review and your application letter dated February 2020. We have reviewed your application for ethical clearance for the study.

The number of mice, the anti-inflammatory, anti-nociceptive and toxicity protocols meets minimum standards of the Faculty of Veterinary medicine ethical regulation guidelines.

We have also noted that registered veterinary surgeons will supervise the work.

We hereby give approval for you to proceed with the project as outlined in the submitted proposal.

Yours sincerely,

Dr. Catherine Kaluwa, Ph.D
Chairperson, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine,
University of Nairobi

Appendix 2: Research Permit granted by the National Commision for Science, Technology, and Innovation

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **537952**

Date of Issue: **26/November/2020**

RESEARCH LICENSE

This is to Certify that Dr.. Halvince Omondi Odira of University of Nairobi, has been licensed to conduct research in Nairobi on the topic: ANTIINFLAMMATORY, ANTINOCICEPTIVE AND TOXIC EFFECTS OF A PHYTEXPONENT PREPARATION OF SELECTED PLANT EXTRACTS for the period ending : 26/November/2021.

License No: **BAHAMAS ABS/P/20/7744**

537952

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.

Appendix 3: Monograph of the Phytexponent formulation

Exp
PHYT
Dietary Supplement

1. **Scheduling status**
A normal scheduling status is 1 droplet (50 µl) for one kg body weight / day. This dose is divided into three: morning, midday and evening. While the medicine contains an elevated amount of alcohol, it is advised to dilute the dose in three times its volume of cold beverage

2. **Proprietary name and dosage form**
PHYT^{EXPonent} (oral drops)

3. **Composition**
Each 10 drops / unit dosage contains:

- Matricaria chamomilla	85 µl MT
- Triticum repens	70 µl MT
- Viola tricolor	70 µl MT
- Echinecea purpurea	30 µl MT
- Allium sativum	10 µl MT
- Vit C	6.75 mcg
- Potassium	23.31 mcg
- Calcium	10.35 mcg

4. **Additives**
- Ethanol 283 µl

5. **Pharmacological classification**
Dietary Supplement

6. **Pharmacological action**
PHYT^{EXPonent} is a strong stimulant of the natural immunological system. The effect can be easily followed by clinical anamnesis and blood analysis.

7. **Indications**
Product based on plant extracts for oral use.
This is a natural product used to support the recovery of the immunity.

8. **Contra-indications**
Until yet no contra-indications none have been noted. Medical follow up is advised.

9. **Warnings**
It should be considered that PHYT^{EXPonent} contains elevated amounts of alcohol (62% v/v). Although the normal administered dose is low, extremely sensitive individuals can be adversely influenced in their driving ability or manipulating machinery.

10. **Interactions**
PHYT^{EXPonent} delays the therapeutically effect of tetracycline and causes the patient to vomit if taken together

11. **Pregnancy and lactation**
Only use after consultation of a medical doctor.

12. **Dosage and directions for use**
A normal scheduling status is 1 droplet (50ål) / kg body weight / day. The dose should be taken in three times: morning, midday and evening.

Dietary Supplement

Best is to dilute the dose in three times its volume of cold beverage. Warm beverages are able to decompose the active plant compounds. Caffeine containing beverages should be avoided 15 min before or after use.
Shake before

13. **Side effects and special precautions**
Until yet only beneficial side effects have been noted, as:
- a marked effect on acne and on several dermatological problems as skin mould infestations / juvenile acne
- a marked weight gain of the emaciated patient
- an amelioration of the fatigue of patients
- a mild form of diarrhea can occur in the beginning of the treatment as a result of the breakdown of viral material. It is never an indication to stop the medication.

No special precautions are necessary.

14. **Known symptoms of over dosage and particulars of its treatment**
For severely ill patients the dose can be doubled or even tripled, withou t signs of over dosage. However, the effects of a considerable alcohol administration should be considered.

15. **Identification**
Dark brown liquid with a typical plant odor.

16. **Presentation**
PHYT^{EXPonent} is supplied in brown plastic bottles of 100 ml with incorporated droplet counter and sealed plastic cap, packed in a cardboard box with a leaflet.

17. **Storage instructions**
Store below 30°C.
Avoid direct sunlight. Keep in a dark place.
KEEP OUT OF REACH OF CHILDREN

18. **Registration number**
Registry No: 001399

19. **Batch number and Expiry date**
On each product the batch number refers to the production date. The shelf life is four years from production date

20. **Name and business address of the holder of the certificate of registration**

Produced by PHARMAPATH SARL
27 Rue du commerce
8220 Mamer Luxembourg
www.pharmapath.lu
Made in BELGIUM

21. **Date of publication of the package insert**
August 2017
Review number 2017/01/1

Appendix 4: The researcher carrying out experiments in the laboratory

Appendix 5: ***In vivo*** **anti-inflammatory activity data**

Treatment	% Oedematous volume						Treatment	% Inhibition of carrageenan-induced paw oedema			
	0 Hr	1 Hr	2 Hr	3 Hr	4 Hr			1 Hr	2 Hr	3 Hr	4 Hr
Normal control	100	100.002	99.937	99.997	99.959		Normal control	-0.002	0.063	0.003	0.041
Normal control	100	100.1	99.97	99.52	99.877		Normal control	-0.1	0.03	0.48	0.123
Normal control	100	100.03	99.86	99.596	99.989		Normal control	-0.03	0.14	0.404	0.011
Normal control	100	100.01	99	99.658	99.996		Normal control	-0.01	1	0.342	0.004
Normal control	100	100.056	99.2	99.77	99.968		Normal control	-0.056	0.8	0.23	0.032
Negative control	100	123.64	125.979	126.126	127.763		Negative control	-23.64	-25.979	-26.126	-27.763
Negative control	100	123.5	125.689	126.597	127		Negative control	-23.5	-25.689	-26.597	-27
Negative control	100	124	125.854	126	126.969		Negative control	-24	-25.854	-26	-26.969
Negative control	100	123.975	124.997	126.38	127.795		Negative control	-23.975	-24.997	-26.38	-27.795
Negative control	100	123.586	125.598	126	127.868		Negative control	-23.586	-25.598	-26	-27.868
Positive control	100	90.28	84.14	75	63.8		Positive control	9.72	15.86	25	36.2
Positive control	100	91	82.98	75	62.98		Positive control	9	17.02	25	37.02
Positive control	100	89.956	82.8	74.99	62.97		Positive control	10.044	17.2	25.01	37.03
Positive control	100	90.0998	83	74.856	62		Positive control	9.9002	17	25.144	38
Positive control	100	90.765	83.08	75.21	62		Positive control	9.235	16.92	24.79	38
Phytexponent 250 mg/Kg BW	100	85.88727	81.8496	70.42881	62.29644		Phytexponent 250 mg/Kg BW	14.11273	18.1504	29.57119	37.70356
Phytexponent 250 mg/Kg BW	100	85.93295	81.89528	69.36243	61.66739		Phytexponent 250 mg/Kg BW	14.06706	18.10473	30.63757	38.33261

Phytexponent 250 mg/Kg BW	100	86.40797	82.3703	70.0764	62.09352		Phytexponent 250 mg/Kg BW	13.59204	17.62971	29.9236	37.90648
Phytexponent 250 mg/Kg BW	100	85.88636	81.84869	70.0764	61.8906		Phytexponent 250 mg/Kg BW	14.11364	18.15131	29.9236	38.1094
Phytexponent 250 mg/Kg BW	100	85.88727	81.8496	70.06523	62.3979		Phytexponent 250 mg/Kg BW	14.11273	18.1504	29.93477	37.6021
Phytexponent 125 mg/Kg BW	100	90.335	87.29	75.1544	67.9782		Phytexponent 125 mg/Kg BW	9.665	12.71	24.8456	32.0218
Phytexponent 125 mg/Kg BW	100	90.9034	87.64525	75.61142	68.7493		Phytexponent 125 mg/Kg BW	9.0966	12.35475	24.38858	31.2507
Phytexponent 125 mg/Kg BW	100	90.335	87.78735	75.34736	68.50579		Phytexponent 125 mg/Kg BW	9.665	12.21265	24.65264	31.49421
Phytexponent 125 mg/Kg BW	100	90.6395	87.3915	78.6176	68.21156		Phytexponent 125 mg/Kg BW	9.3605	12.6085	21.3824	31.78844
Phytexponent 125 mg/Kg BW	100	90.40605	87.29	75.1544	68.28258		Phytexponent 125 mg/Kg BW	9.59395	12.71	24.8456	31.71742
Phytexponent 62.50 mg/Kg BW	100	94.06005	91.35	91.03838	82.97399		Phytexponent 62.50 mg/Kg BW	5.93995	8.65	8.961616	17.02601
Phytexponent 62.50 mg/Kg BW	100	93.04505	91.23835	90.38738	82.16231		Phytexponent 62.50 mg/Kg BW	6.95495	8.76165	9.612616	17.83769
Phytexponent 62.50 mg/Kg BW	100	93.91795	91.88795	91.34306	82.14912		Phytexponent 62.50 mg/Kg BW	6.08205	8.11205	8.656936	17.85088
Phytexponent 62.50 mg/Kg BW	100	93.38	91.31955	91.03838	82.6899		Phytexponent 62.50 mg/Kg BW	6.62	8.68045	8.961616	17.3101
Phytexponent 62.50 mg/Kg BW	100	94.395	92.36297	91.35525	82.98921		Phytexponent 62.50 mg/Kg BW	5.605	7.63703	8.644749	17.01079
Phytexponent 31.25 mg/Kg BW	100	99.2873	97.2167	94.4508	89.18334		Phytexponent 31.25 mg/Kg BW	0.7127	2.7833	5.5492	10.81666

Phytexponent 31.25 mg/Kg BW	100	98.4347	96.9934	95.35468	88.72677		Phytexponent 31.25 mg/Kg BW	1.5653	3.0066	4.645316	11.27323
Phytexponent 31.25 mg/Kg BW	100	98.91175	96.425	94.69454	88.85867		Phytexponent 31.25 mg/Kg BW	1.08825	3.575	5.305456	11.14133
Phytexponent 31.25 mg/Kg BW	100	99.3279	97.12535	95.09063	88.2702		Phytexponent 31.25 mg/Kg BW	0.6721	2.87465	4.909372	11.7298
Phytexponent 31.25 mg/Kg BW	100	98.455	96.80055	94.4508	88.22048		Phytexponent 31.25 mg/Kg BW	1.545	3.19945	5.5492	11.77952

Appendix 6: Analgesic activity data

Analgesic Activity	
Treatment	% inhibition of writhing
Phytexponent 31.25 mg/Kg BW	55.7
Phytexponent 31.25 mg/Kg BW	54.86
Phytexponent 31.25 mg/Kg BW	55
Phytexponent 31.25 mg/Kg BW	54.67
Phytexponent 31.25 mg/Kg BW	55.04
Phytexponent 62.5 mg/Kg BW	62.94
Phytexponent 62.5 mg/Kg BW	63.02
Phytexponent 62.5 mg/Kg BW	63
Phytexponent 62.5 mg/Kg BW	64
Phytexponent 62.5 mg/Kg BW	64
Phytexponent 125 mg/Kg BW	76.32
Phytexponent 125 mg/Kg BW	75
Phytexponent 125 mg/Kg BW	76.4
Phytexponent 125 mg/Kg BW	75.8
Phytexponent 125 mg/Kg BW	76.1
Phytexponent 250 mg/Kg BW	94.67
Phytexponent 250 mg/Kg BW	95.25
Phytexponent 250 mg/Kg BW	95.1
Phytexponent 250 mg/Kg BW	94.89
Phytexponent 250 mg/Kg BW	95
Indomethacin 4 mg/Kg BW	65.19
Indomethacin 4 mg/Kg BW	64.3
Indomethacin 4 mg/Kg BW	64
Indomethacin 4 mg/Kg BW	65.48
Indomethacin 4 mg/Kg BW	64.96

Appendix 7: *In vitro* cytotoxicity data (MTT-Assay)

Cytotoxicity		
Concentration (μg/ml)	Cyclophosphamide	Phytexponent preparation
1.37	46.7632	0.252525
1.37	47.73	0.529101
1.37	45.59	0.761421
1.37	46.097561	6.097561
1.37	46.51613	6.451613
4.14	54.43	1.262626
4.14	58.57	0.793651
4.14	51.357	1.522843
4.14	57.560976	7.560976
4.14	55.699752	6.699752
12.35	65.75	1.515152
12.35	60.68	3.174603
12.35	64.356	4.568528
12.35	68.780488	8.780488
12.35	60.684864	8.684864
37.04	73.79	2.777778
37.04	61.66	3.174603
37.04	63.505	6.852792
37.04	73.41463	13.41463
37.04	69.925558	9.925558
111.11	73.84	3.030303
111.11	62.04	5.555556
111.11	75.962	9.898477
111.11	80.65854	13.65854
111.11	76.16625	11.16625
333.33	85.16	16.91919
333.33	84.91	17.46032
333.33	83.878	18.27411
333.33	89.58537	26.58537
333.33	85.13648	15.13648
1000	96.752	45.45455
1000	97.14	44.17989
1000	95.632	44.16244
1000	96.19512	42.19512
1000	94.43176	42.43176