PHYTOCHEMICAL SCREENING OF ANTI-INFLAMMATORY COMPOUNDS OF THE STINGING NETTLE (*Urtica* spp.)

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DECLARATION

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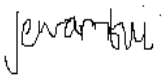
Signature …  …… Date……25/07/2023……

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DEDICATION

This work is dedicated to my mother, Ms. Njoroge, and my late aunt, Mrs. Ng'ang'a, who introduced me to the medicinal uses of the stinging nettle, Rest in Peace.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADAM 17	A Disintegrin and Metalloprotease 17
ANOVA	Analysis of Variance
AP	Activator Protein
BLASTN	Basic Local Alignment Search Tool Nucleotide
CD40	Cluster of differentiation 40
CEBIB	Center for Biotechnology and Bioinformatics
CTAB	Cetyl-Trimethyl-Ammonium Bromide
CFA	Complete Freund's adjuvant
CNS	Central Nervous System
COX	Cyclooxygenases
CRP	C - reactive protein
Cyclic AMP	cyclic Adenosine MonoPhosphate
DCM: M	Dichloromethane: Methanol
DHA	Docosahexaenoic Acid
DMARDs	Disease Modifying Anti-Rheumatic Drugs
DNA	Deoxyribo Nucleic Acid
dNTP	diNucleotide Triphosphate
EET	Epoxyeicosatrienoic acid
EMD	Empirical Mode Decomposition
ERK	Extracellular signal-Regulated protein Kinase

FLAP	Five Lipoxygenase Activating Protein
GAE	Gallic Acid Equivalent
GWAS	Genome Wide Association Studies
14-HDHA	14-HydroxyDocosahexaenoic Acid
HR	Histamine Receptor
HDL	High Density Lipoprotein
HLA-DR	Human Leukocyte Antigen- Death Receptor
5-HPETE	5-hydroperoxy eicosatetraenoic acid
HPLC-MS-MS	High Performance Liquid Chromatography – Mass Spectroscopy
IBS	Inflammatory Bowel Syndrome
IKKβ	IKB Kinase Beta
IL	Interleukin
IMN	Indomethacin
IP-10	Interferon-inducible Protein -10
ITS	Internal Transcribed Spacer
LT	Leukotriene- (A4, B4, C4, D4, E4)
LC-MS	Liquid Chromatography Mass Spectroscopy
LC-MS-ESI	Liquid Chromatography Mass Spectroscopy Electron Spray Ionization
LDL	Low Density Lipoprotein
LOX	15-lipoxygenase
LPS	Lipopolysaccharide

LTA4	Leukotriene A4
LTB4	Leukotriene B4
LTC4	Leukotriene C4
LTD4	Leukotriene D4
LTE4	Leukotriene E4
MACCS	Molecular Access System
MAPK	Mitogen Activated Protein Kinase
MHC	Major Histocompatibility Complex
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAG-1	NSAID Activated Gene-1
NCBI	National Center for Biotechnology Information
NCCAM	National Center for Complementary and Alternative Medicine
NECD	NSAIDs-exacerbated cutaneous disease
NEMO	Nuclear factor kappa-β Essential Modulator
NERD	NSAIDs-exacerbated respiratory disease
NF-kB	Nuclear Factor Kappa Beta
NIUA	NSAIDs-Induced Urticaria/Angioedema
NO	Nitric Oxide
NOD	Nodulation
eNOS	endothelial Nitric Oxide
iNOS	inducible Nitric Oxide

nNOS	Neuronal NOS
NSAIDS	Non-Steroidal Anti-Inflammatory drugs
NECD	NSAIDs-Exacerbated Cutaneous Disease
NERD	NSAIDs-Exacerbated Respiratory Disease
NIUA	NSAIDs- Induced Urticaria/Angiodema
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDB: 4COX	Protein Data Bank: Cyclo-oxygenase
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PGI2	Prostacyclin
PLA2	Phospholipases A_2
PPARs	Peroxisome proliferator activated receptors
PSA	Prostate Specific Antigen
PSORS1	Psoriasis-Susceptibility loci
PUFA	Polyunsaturated Fatty Acids
rbcL	ribulose bisphosphate carboxylase
rITS	ribosomal Internal transcribed spacer

ROS	Reactive Oxygen Species
RANKL	Receptor Activator of NFκB Ligand
RMSD	Root Mean Square Deviation
SNIDRs	Single NSAID-induced delayed reactions
SNIUAA	Single NSAID-induced urticaria/angioedema or anaphylaxis
SP-1	Specificity protein 1
STAT-3	Signal transducer and activators of transcription protein 3
TACE	TNF converting enzyme
TFC	Total Flavonoid Content
TGF-β	Transforming Growth Factor beta
TNF	Tumor Necrosis Factor
TNFR	TNF Receptors
TPA	12-O-tetradecanoylphorbol-13-acetate
TPC	Total Phenolic Content
TXA2	Thromboxane A2
UPLC-MS	Ultra Performance Liquid Chromatography Mass Spectroscopy
UPLC-UV-MS	Ultra Performance Liquid Chromatography Ultra Violet Mass Spectroscopy
VLDL	Very Low Density Lipoprotein
WE	Water extracts

ABSTRACT

The stinging nettle is an alimurgic plant known for its anti-inflammatory ethnomedical importance. However, there are no studies that have attempted to identify the specific anti-inflammatory compounds within the extracts of the stinging nettle leaves as well as their mechanism of action. Therefore, the objective of this study was to screen for bioactive compounds that target the cyclo-oxygenase pathway. The plant samples were identified by DNA (Deoxyribo Nucleic Acid) barcoding using ribulose bisphosphate carboxylase (*rbcL)* and Internal Transcribed Spacer (ITS) markers. The resulting consensus sequence was blasted to idebtify the sample species. Aqueous and methanol: dicholoromethane (1:1) plant extracts were prepared and analysed for their total phenolic and flavonoid content. *In vivo* carrageenan model was used to determine the anti-inflammatory capacity of these extracts. Further investigations in the form of Liquid Chromatography – Mass Spectroscopy (LC-MS) and Raman spectroscopy analysis were used for the identification of compounds. In addition to identification, the LC-MS performed the quantification of polyphenols. Molecular comparisons were done between indomethacin, a non-selective inhibitor, and the identified compounds prior to docking them with receptor Protein Databank cyclooxygenases-2 (PDB: 4COX). DNA barcoding identified *Urtica sp.* as the species used for this study. The total phenolic content of the aqueous and methanol: dichloromethane extracts were at 3.75 mg Gallic acid equivalent (GAE) /g dry sample and 6.26 mg GAE/g dry sample while total flavonoid content were at 0.3872 mg quercetin/g dry sample and 1.76 mg quercetin/g dry sample, respectively. An *in vivo* carrageenan model of inflammation based on aqueous extract administered at 750 mg/kg alleviated the carrageenan induced inflammation at 22.35% maximum inhibition rate. Raman spectroscopy identified phenolic acids (hydroxy-cinnamic acids) and flavonoids (flavan-3-ol). On the other hand, the LC-MS analysis yielded a wider range of phenolic acids specifically hydroxy-cinnamic and hydroxy-benzoic acids and flavonoids (flavonols and flavan-3-ols). Based on LC-MS, molecules belonging to the flavonol group were found to be abundant with concentrations ranging to 0.16 – 1.47 µg/g. The flavonoid compounds exhibited binding energy -6.9 to -8.6 kcal/mol while phenolic acids, -3.8 to -8.3 kcal/mol. Quercetin (-8.6 kcal/mol) scored the most favorable anti-inflammatory flavonoid compound and chlorogenic acid (-8.3 kcal/mol) for phenolic acids. These results validated the anti-inflammatory capacity of the aqueous extract of the leaves by acting as cyclooxygenases 2 inhibitors. Extensive research should be focused on isolation and purification of compounds from crude extracts and testing for their anti-inflammatory activity at both *in vivo* and *in vitro* basis.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the study

Inflammation is a complex, natural protective response towards different stimuli characterized by the dilation and permeation of the blood vessels with a surge in leukocytes in the tissues (Fujiwara & Kobayashi, 2005). Coordination of the immune cells and biological mediators such as cytokines and chemokine drive the response and have a pivotal role in the chronic inflammatory disease pathology, including but not limited to arthritis, multiple sclerosis, which account for more than 50% of all deaths (Libby, 2007).

Non-Steroidal Anti-Inflammatory drugs (NSAIDs) are used in mediating inflammation by reduction of swelling, redness, fever or headache. They target cyclooxygenases which breakdown arachidonic acid into prostaglandin G2 (PGG2) which is further reduced into PGH2. PGH2 acts as the substrate for the production of other bioactive prostaglandins and thromboxane whose biological functions include: inflammation, platelet aggregation, smooth muscle contraction among others (Pelley, 2012). However, prolonged use of NSAIDs has been associated with heart attacks (Kregiel *et al*., 2018).

Natural compounds of plant origin can also have pharmacological responses similar to NSAIDs by inhibiting cyclo-oxygenases (COX) (Maroon *et al*., 2010). In particular, stinging nettle plants contain compounds such as caffeic malic acids and polysaccharides with anti-inflammatory activities (Hajhashemi & Klooshani, 2013). Plant-based bioactive compounds have been applied in integrative medicine, whose scope focuses on conventional/non-conventional methods and utilizes indicators such as environment, food and health, which collectively form the wheel of health (Hillinger *et al*., 2017). Furthermore, it is essential to note that dietary components influence inflammation through gut microbiome interaction or scavenging free radicals and the reactive oxygen species (ROS) that would otherwise promote oxidative stress (Arulselvan *et al*., 2016).

The stinging nettle has been documented to treat many ailments due to its anti-inflammatory characteristic and can even be used alongside NSAIDs (Di Lorenzo *et al*., 2013). Traditional

medicine utilized urtication (external stinging) or rube faction in treating arthritis, rheumatism and muscular paralysis (Upton, 2013). This technique was applied in testing the efficacy of urtication in alleviating the base of thumb pain using a double-blind, completely randomized test and a dead nettle as the placebo. The analgesic effect is thought to be mediated by serotonin which activates the nociceptive neurons that affect pain perception by an acupuncture-like effect (Randall *et al*., 1999). The East and Central African stinging nettle has been utilized to treat rheumatism, malaria, venereal and urethral diseases. A study by Omwenga and colleagues (2015) highlighted its use in treating skin and stomach infections in Western Kenya (Omwenga *et al*., 2015). Therefore, the purpose of this is to determine the natural bioactive compounds in the stinging nettle that suppress inflammation.

1.2 Problem statement

Use of NSAIDs has been linked to gastro-intestinal and cardio-vascular complications (Gómez-Acebo *et al*., 2018; Vane & Botting, 1998). Specifically, NSAIDs that are COX-1 inhibitors are associated with gastro-intestinal and renal complications while COX-2 inhibitors are associated with ischemic heart failure and myocardial infarctions (Attiq *et al*., 2018).

Moreover, Dona and colleagues have reported allergic reactions to NSAIDs due to specific immunoreactions or imbalance of the arachidonic pathway during inhibition. These reactions are classified into five categories as: NSAIDs-induced urticaria/angioedema (NIUA), single NSAID-induced urticaria/angioedema or anaphylaxis (SNIUAA), single NSAID-induced delayed reactions (SNIDRs), NSAIDs-exacerbated respiratory disease (NERD) and NSAIDs-exacerbated cutaneous disease (NECD) (Dona *et al*., 2016).

Secondary metabolites from plants have the potential of eliciting responses similar to anti-inflammatory drugs. Thus, they can be utilized to avert the mentioned negative consequences. For instance, it was found that the aqueous and lipophilic extracts of the stinging nettle have inhibitory properties against NF-κB. The NF-κB is a primary influencer of inflammation and the immune system, controlling transcription of genes encoding for cyclooxygenase, pro-inflammatory cytokines such as interleukin (and its variants) and tumor necrosis factor (Patil *et al*., 2019). Riehemann *et al*. (1999) demonstrated that the water-soluble extract suppressed the

NF-κB activated by tumor necrosis factor (TNF) and lipopolysaccharide (LPS)) while Johnson *et al*. (2013) reported that the lipophilic extract was more potent (Johnson *et al*., 2013; Riehemann *et al*., 1999).

1.3 Justification

Identifying the active anti-inflammatory compounds provides a deeper outlook on the nettle's pharmacological and immune-modulatory properties, which can be used in drug and nutraceutical development. For instance, stinging nettle cream formulated in an oil-water emulsion was found to alleviate pain in osteoarthritis patients (Rayburn *et al*., 2009). PhytalgicTM is a nutraceutical product for osteoarthritic patients, consisting of fish oils, vitamin E, *Urtica dioca* and zinc (Jacquet *et al*., 2009). Its clinical trial performance was found to be better than intra-articular corticosteroids (Christensen & Bliddal, 2010). Urtidin® F.C drug comprises of dried root extracts and is used as an adjuvant therapy for irritable bladder and voiding problems for patients with stages I and II of Benign Prostatic Hyperplasia.

Moreover, the identified potent compounds will provide a glimpse on which preparation strategies work best to provide the desired effect. So far, the stinging nettle extract is prepared as soup, infusion, decoction or tincture using alcohol (Upton, 2013).

1.4 Research questions

i. What is the stinging nettle species growing in Kenya?
ii. What is the phytochemical composition of the stinging nettle?
iii. Which are the specific anti-inflammatory compounds present in the stinging nettle extract?

1.5 Objectives

1.5.1 General objective

To determine the phytochemical composition and anti-inflammatory potential of the stinging nettle leaf crude extract using a Carrageenan induced paw edema model of inflammation.

1.5.2 Specific objectives

i. To determine the phytochemical composition of the stinging nettle leaf crude extracts.

ii. To evaluate the anti-inflammatory efficacy of different crude extracts of the stinging nettle using a Carrageenan induced paw edema model of inflammation.

iii. To identify the specific active compounds responsible for anti-inflammation using *in silico*, computational tools.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Inflammation

Inflammation is generally characterized as swelling, pain, heat and loss of tissue function. Its aetiology is summarized as either infectious or non-infectious. Infectious agents are bacterial or viral, while non-infectious can be biologically, chemically or physically mediated. Whichever the case, these factors trigger the recruitment of leukocytes that eventually release cytokine, generating an acute phase, and if not resolved, it develops into chronic inflammation (Chen *et al*., 2018).

The infiltrated polymorpho-nuclear leukocytes/neutrophils are directed and attached to the capillary walls through selectins and integrins. Their mechanism by phagocytosis is to remove pathogens and other material. As time progresses, the damaged tissue heal and the neutrophils undergo apoptosis as monocytes flood. These monocytes differentiate into macrophages that take part in removing dead neutrophils. After that, the clearance of macrophages is achieved through apoptosis or the lymphatic system (Freire & Van Dyke, 2013).

The resolution phase capitalizes on lipid mediators whose intent is to restore tissue integrity and homeostasis. Lipoxin A4 is synthesized by oxidation of arachidonic acid using lipoxygenases. It works by activating the G-coupled proteins to prevent neutrophils translocation to endothelial cells, promote apoptosis of leukocytes and allow the influx of macrophages to the inflamed sites (Bannenberg & Serhan, 2010). Resolvins, protectins and maresins are specialized pro-resolving lipid mediators obtained from the oxidation of ω-3 polyunsaturated fatty acids (PUFA). They have a role in anti-inflammatory and resolution properties. Resolvins are produced from docosahexaenoic acid (DHA) by 15-lipoxygenase (LOX), then 5-lipoxygenase in neutrophils or action by aspirin acetylated cyclooxygenase-2 (COX-2) enzymes while protectins result from 15-LOX action on DHA. During phagocytosis of apoptotic cells, macrophages release 14-hydroxy-DHA (14-HDHA) which is further catabolized into maresins1 by 12-lipoxygenase (Serhan *et al*., 2015).

2.2 Inflammatory pathways

Every inflammatory pathway consists of the following subunits: an inducer which could be a biological, chemical or physical substance that initiates the process, and sensors that are linked to mediators that present the inflammation signals such as pain, heat to the effectors, i.e. the tissues or cells (Hannoodee & Nasuruddin, 2020)

2.2.1 Arachidonic acid dependent pathways

Phospholipases A2 (PLA2) are enzymes that break down membrane phospholipases into fatty acids that can be incorporated into the cyclo-oxygenases (COX) or lipo-oxygenases pathway (LOX) (Lehr, 2001). It has the following subtypes: secreted PLA2 found in mammalian tissues, cytosolic PLA2 and calcium-independent PLA2. The cytosolic PLA2 requires calcium to mediate inflammatory processes, while calcium-independent has trans-cyclase and lysophospholipase activity needed in regulation (Burke & Dennis, 2009).

Phospholipases' action on the plasma membrane yields a 20 carbon unsaturated fatty acid, known as arachidonic acid. The action of cyclo-oxygenases/prostaglandins synthase and peroxidase generates prostaglandins PGH2 and subsequently its isomers: prostaglandin D2 (PGD2), prostaglandins E2 (PGE2), prostaglandin F2α (PGF2α), prostacyclin (PGI2), and thromboxane A2 (TXA2). Its G coupled receptors exist in different isoforms by activating adenyl cyclase for cyclic AMP or phosphatidylinositol for calcium mobilization (Ricciotti & FitzGerald, 2011).

The COX gene has three isoforms generated by alternative splicing. They all share the catalytic features. The COX-1 and COX-2 forms exhibit a 61% similarity with the same molecular weight and length, with the upper section of the active site being highly conserved. The COX-3 form results from intron retention 1, a conserved region, and is sensitive to analgesics and antipyretics with low inflammatory activity. COX-1 is a dominant source for prostaglandins with housekeeping functions in gastric epithelial cyto-protection and homeostasis; COX-2, induced by inflammatory stimuli, hormones and growth factors, is responsible for inflammation and proliferative diseases such as cancer. The prostaglandins attach to G coupled receptors to exert an inflammatory effect (Chandrasekharan *et al*., 2002; Whittle, 2000).

Intake of COX-2 inhibitors has been linked with renal failure and early termination of pregnancy since the COX-2 enzyme is expressed in gonads and kidneys. Also, this enzyme is utilized in the protection of cells in the gut. Therefore, individuals with conditions related to gastric should avoid using such drugs. In comparison to COX-1 inhibitors, they have weaker gastrointestinal side effects and ulceration development. Cardiovascular side effects can occur since inhibition of COX-2 yields low prostacyclin, which is needed for arteries protection (Zarghi & Arfaei, 2011).

5-Lipo-oxygenases (LOX) are enzymes actively present in myeloid cells. ATP, Calcium ions, phosphatidylcholine and hydro-peroxides stimulate the enzyme causing its translocation to the nuclear membrane. It interacts with a five-lipoxygenase-activating protein (FLAP), which presents the substrate, arachidonic acid, broken down to 5-hydroperoxy eicosatetraenoic acid (5-HPETE). Thereafter, 5-HPETE is dehydrated to form leukotriene A4 (LTA4), which acts as a source for the generation of other isoforms. For example, LTA4 hydroxylation produces leukotriene B4 (LTB4), conjugation with glutathione forms leukotriene C4 (LTC4) which can be cleaved at certain side chains to generate leukotriene D4 (LTD4) and leukotriene E4 (LTE4) (Hedi & Norbert, 2004). Generally, leukotrienes play a crucial role in the pathology of allergies and asthma (Joshi & Praticò, 2015).

2.2.2 Non-dependent arachidonic pathways

The NF-κB (Nuclear Factor kappa beta) is a common target for therapies by regulating inflammation (Baeuerle & Baichwal, 1997). It is a transcription factor that regulates genes that control inflammation and immunity and the expression of cytokines and proteins involved in leukocyte migration (Sabir *et al*., 2019). NF-κB protein family is made up of Rel proteins (A (p65), B, C), p50/p105/NF-κB1 and p52/p100/NF-κB2); some of these protein forms couple to form biologically active homo and hetero- dimers. P65 and P50 types are bound to inhibitor IkBα in the cytoplasm, and hence they require the IkB kinase (IKK) enzyme for activation (Verma *et al*., 2019). Factors such as pathogenic infections (viral, bacterial, parasitic or fungal), the presence of damaged associated molecular patterns (proteins from damaged cells, DNA, RNA) and cytokines are recognized by Toll-like Receptors, which activate the mitogen-activated protein kinase (MAPK) and IkB kinase (IKK) enzymes (Karunaweera et al., 2015).

MAPK family comprises serine/ threonine kinases: p38 MAPK, c-Jun-N-terminal kinases and extracellular signal-regulated kinases. Once stimulated, they drive the transcription of genes and activate a myriad of transcription factors that have a role in cell signalling, growth and survival (including NF-κB) (Coskun *et al*., 2011).

The IKKβ enzyme is the primary regulator of its activation (Roman-Blas & Jimenez, 2006). A previous study conducted using a negative adreno-virus IKKβ construct showed a reduction in swelling and provided evidence of using NF-κB pathway as a suitable target for arthritis treatment (Tas *et al*., 2006). It consists of three domains: IKKα, IKKβ and (regulatory subunit) IKKϒ or NuclearFactor kappa-β essential modulator (NEMO). Between the IKKα and IKKβ, there is a 50% structural homology, and functionally, they have catalytic properties. Their activation involves phosphorylation at 177 and 181 serine residues for IKKβ while 176 and 180 serine residues for IKK α. NEMO interacts with these subunits at their carboxyl level. Despite its inability to perform catalysis, its modification via ubiquitination results in the recruitment of complexes that can phosphorylate the IKKs (Israël, 2010).

Once the IKK is phosphorylated, it proceeds to activate the NF-κB through canonical or non-canonical means, depending on their reliance on NEMO. Stimulation of the canonical pathway includes a wide array of stimuli such as pathogens, stress, and cytokines; this triggers the IKK to phosphorylate the IKB, which eventually gets degraded by cellular proteasomes. NEMO mediates the IKK used in this pathway. Unlike the canonical, this pathway responds to selective stimuli such as CD40 and Receptor activator of NFκB ligand (RANKL). Instead of IKB degradation, the synergism between NF-κB inducing kinase (NF-KIK) and IKKα to phosphorylate NF-κB2 precursor (P100) is utilized without NEMO mediation. Despite their differences, cross-talk between them accounts for their inflammation and immunity functions (Liu *et al*., 2012; Shih *et al*., 2011b).

The peroxisome proliferator-activated receptors (PPARs) superfamily exists in three isoforms PPARα, PPARβ and PPARϒ. Activation of these transcription factors occurs in the presence of small lipophilic ligands; once activated, they translocate to the nucleus and perform gene regulation influencing glucose and lipid metabolism. Consequently, they have a role in metabolic disorders. In addition, PPAR α and PPAR ϒ have a role in inhibiting inflammation and pain.

PPAR-ϒ accommodates a wide variety of ligands (agonists) from diet, metabolites and synthetic drugs, while PPAR uses fenofibrate (Grygiel-Górniak, 2014; Tyagi *et al*., 2011).

In the presence of oxygen and Nicotinamide Adenine Dinucleotide Phosphate (NADPH), this enzyme can convert arginine to citrulline with nitric oxide (NO) release. The neuronal NOS (nNOS) and constitutive endothelial NOS (eNOS) require calcium ions and calmodulin to perform nitric oxide production, while the inducible NOS (or iNOS) form requires calmodulin-binding only. Depending on NO concentration, they can either favour or repress inflammation. High NO levels are destructive and linked to auto-immune inflammatory conditions (Sharma *et al*., 2007).

NSAID activated gene-1 (NAG-1) gene belongs to the transforming growth factor beta (TGF-β) family and has been implicated in the progression of various cancer forms. Its expression can be regulated by COX inhibitors, dietary compounds and PPARϒ ligands. Moreover, COX inhibitor-induced NAG-1 expression in COX deficient cells. Consequently, it is hypothesized that the anti-tumorigenic effect of NSAIDs is mediated by NAG-1. This can be applied in early cancer treatment (Wang *et al*., 2013).

2.3 Inflammatory mediators

The key inflammatory molecules targeted by anti-inflammatory drugs and phytochemical compounds are: lipid derived molecules, transcription factors, pro-inflammatory cytokines, vaso-active mediators, proteolytic enzymes, reactive oxygen species, and complement system.

2.3.1 Lipid derived molecules

They originate from the arachidonic pathway as described earlier. Cyclooxygenases form prostaglandins and thromboxane; lipoxygenase form leukotrienes and lipoxins; cytochrome P450 enzymes forming epoxyeicosatrienoic acids (EETs) (Hanna & Hafez, 2018).

2.3.2 Transcription factors

The main transcription factor, NF-κB, has been discussed earlier in section 2.2.2, non- dependent arachidonic pathway.

2.3.3 Pro-inflammatory cytokines

Cytokines are proteins of small molecular weights, non-structural in nature produced by every cell. Anti-inflammatory cytokines is a subclass that suppresses inflammation. They work alongside inhibitors and receptors of cytokines to perform their physiological role. Examples of these cytokines are antagonists of interleukin (IL)-1 receptor and antibodies neutralising tumor necrosis factors. On the flip side, pro inflammatory cytokines promote inflammation cytokines. They include tumor necrosis factors (TNF), interferon, interleukins (IL-1) and colony stimulatory factors. They are responsible for immune and inflammatory processes regulation (Dinarello, 2000; Zhang & An, 2007).

Tumor necrosis factor (TNF) (synonymous to TNFα or cachectin) is primarily produced by activated macrophages and T lymphocytes. Other sources are neutrophils, mast cells, smooth and cardiac muscles, natural killer and endothelial cells, fibroblasts and osteoclasts. TNFs that are membrane-associated and soluble exist as a trimer. The TNF converting enzyme (TACE) or A Disintegrin and Metalloprotease 17 (ADAM 17) is involved in releasing the TNF from the cell surface; synthesize cell membrane associated proteins such as TNF receptors. These TNF receptors are in the form of: TNFR1 and TNFR2. The extracellular section of the TNF receptor has the ligand binding domain and is rich in cysteine while the intracellular portion does not have a distinct enzymatic and signaling pathway activity (Bradley, 2008).

TNF is capable of activating NF-κB and arachidonic pathway (5-HPETE, leukotrienes and prostaglandins). Its presence stimulates downstream production of cytokines and chemokines (Sedger & McDermott, 2014).

Interleukin 1 exists structurally in two forms: alpha and beta (Warren, 1990). IL-1 and TNF inflammatory activities can be countered by neutralization with antibodies, soluble receptors and their antagonist, and protease inhibitors (Dinarello, 2000).

The IL1β is produced by monocytes, macrophage, endothelial cells and fibroblasts in the event of injury, infection or invasion. In neuronal and glial cells, IL1β is linked to prostaglandin E2 production. IL-6 plays a crucial role in nerve injury as it regulates neuropeptide expression and activation of astrocytes and microglial (Opal & DePalo, 2000).

2.3.4 Vasoactive mediators

During a tissue injury, histamine gets secreted from mast cells eliciting contraction of endothelial cells, entry of fluid, vascular permeation and edema formation. It is the primary agent responsible for immediate vascular changes during inflammation. Its receptors, H1R, H2R, H3R, and H4R, belong to the G coupled receptor family. According to their functions, H1 performs vasodilation, bronchoconstriction, migration of cells and nociception; H2-receptor modification of secretion of gastric acid, production of mucal airways, and permeation of blood vessels; H3-receptor influences the development of inflammatory diseases of the nervous system; H4-receptor is involved in inflammation and allergy process (Thangam *et al*., 2018).

They modulate the functions of monocytes, T and B cells, macrophages, eosinophils, neutrophils, and dendritic cells as part of their immune regulatory functions. Histamine also serves as a neuro-transmitter and neuromodulator to growing cells thus affecting brain development (Carthy & Ellender, 2021).

Serotonin (5-hydroxytryptamine) is a neuro-transmitter synthesized from the hydroxylation of L-tryptophan in the enterochromaffin cells found in the intestines (Wu *et al*., 2019). Its peripheral effects include heart functions, homeostasis, organ development, and intestinal motility whereas among the central effects are related to sleep, depression, aggression and psychosis. Nevertheless, both effects have an impact on immune responses. The peripheral effects are usually platelet derived; platelets ensure the release of serotonin in an inflammatory reaction. Subsequent activation of lymphocytes and monocytes is done by serotonin which in turn releases cytokines (Herr *et al*., 2017).

Bradkynins are peptide inflammatory mediators produced from kallikreins. Kallikreins are broken down to release kallidin which gets transformed into bradkyinin. Action of bradykinin leads to endothelial cell separation, vasodilation and augmented vascular permeability (Golias *et al*., 2007; Pirahanchi & Sharma, 2022).

2.3.5 Proteolytic enzymes

During the process of inflammation, human leukocyte elastase gets secreted from polymorphonuclear leukocytes. It hydrolyzes elastin, a protein found in blood vessels, lungs, collagen and immuno-globulins and permits entry of pro-inflammatory mediators (Patil *et al*., 2019).

2.3.6 Reactive oxygen species (ROS)

ROS are metabolites of oxygen which are partially reduced and have strong oxidizing properties. They are released during inflammation and oxidative stresses to the cell. At low and physiological concentrations they assist in cellular defense mechanism by intracellular signaling pathway. Its activity can be affected by the presence of free radical scavengers and peroxides. In comparison, at high concentration they are lethal since they oxidize proteins, lipids and damage DNA (Mittal *et al*., 2014; Patil *et al*., 2019).

2.3.7 Complement system

The complement system is made up of a group of plasma proteins that mark pathogens and develop a cascade of inflammatory reactions to fight the pathogens. Enzymatic reactions become activated before the development of inflammatory reactions. There are three mechanisms in which complement system performs its fight against infection. At the beginning, there is generation of complement proteins that opsonize the pathogens by activation of C3 followed by recruitment of more phagocytes through cleaving with C3b which acts as an opsonin and finally creation of pores in the bacterial membrane by an anaphylatoxin (C3a). C3 activation can activate the lytic enzyme to damage the plasma membrane of cells and bacteria while C5a attracts macrophages and neutrophils (Gani, 2022; Janeway *et al*., 2001).

2.4 *In vivo* models of inflammation

2.4.1 Carrageenan model of inflammation

The carrageenan induced paw edema is used in assessing natural and synthetic compounds for their anti-inflammatory activity (Mansouri *et al*., 2015). Carrageenan is made up of sulfated sugars which activate the phospolipases A2 and eventually the complement system and inflammatory mediators such as cytokines (Patil *et al*., 2019). The induced edema is characterized by arteriole dilation, permeation of post-capillary venules and flooding of

inflammatory cells and fluids (Duwiejua *et al*., 2002). Neutrophil migration relies on the resident cells which release chemotactic mediators (Okoli & Akah, 2004).

Advantages: it is widely used for acute inflammation, reproducible and is used to assess compounds that are capable of inhibiting cyclooxygenase. Therefore, it is used in screening anti-inflammatory drugs (Sarkhel, 2015). Disadvantages: A week prior to the experiment, the animals need to be adapt to their new environment. The researcher requires training in order to operate plethysmometer which records the paw volumes and preparation of the carrageenan solution (Whiteley & Dalrymple, 2001).

2.4.2 Histamine and serotonin/5-hydroxytryptamine induced paw edema

Injection of histamine results in edema through the flow of plasma protein and lymph into the extracellular spaces. This would cause an increase in the flow of lymph and protein content, resulting in edema. Exposure of serotonin to rats increases vascular permeability and leakage of blood and fluid into tissues (Ben et al., 2016; Cole et al., 1995).

Advantages: The histamine and 5-hydroxytryptamine models are effective in assessing the anti-inflammatory effect. They are used as secondary models for drugs or substances that show effect during the first phase of carrageenan induced inflammation. Also, they can be used to assess drugs whose mechanism of action is related to histamine and serotonin. Disadvantages: The histamine and 5-hydroxytryptamine models produce inflammation that is minimal and transient. They are unsuitable for drugs that do not act by histamine or serotonin (Cole *et al*., 1995; Nakamura & Shimizu, 1974).

2.4.3 The Bradykinin-induced paw edema

This model utilizes the arachidonic acid pathway by involving the prostaglandins in the formation of bradykinin.

Advantages: This model can be used in acute inflammation. Results obtained can be used to correlate those of the carrageenan paw model. Besides that, antiprostaglandins drugs can be

screened using this model. Disadvantage: it produces mild and transient edema (Patil *et al*., 2019).

2.4.4 Dextran-induced paw edema

Inflammation caused by dextran has the following properties: increased vascular permeability, kinins activation, and histamine and serotonin release (Coura *et al*., 2015). Neutrophils accumulation is through mast cell degranulation (Okoli & Akah, 2004). Eventually, histamine and serotonin are liberated from the mast cells (Babu *et al*., 2009).

Advantage: Since this model takes into account the action of histamine and serotonin, it can be used to appraise drugs such as anti-histaminic or anti-serotonic drugs for their anti-inflammatory potential. Thus, it can be used to validate the findings from the investigations of carrageenan induced model of paw inflammation. Disadvantage: This model cannot be utilised for non- anti-serotonin or anti-histamine drugs (Patil *et al*., 2019).

2.4.5 Lipopolysaccharide (LPS)-induced paw edema

The lipopolysaccharide induced model represents gram negative bacteria induced inflammation. It induces the NF-κB-dependent pathway leading to the production of cytokines: TNF-α, IL-1β (Okoli & Akah, 2004).

Advantage: It is suitable for testing anti-inflammatory drugs that act on modulating cytokines and analgesics (Patil *et al*., 2019). Disadvantage: Intraperitoneal introduction of LPS in inducing neuroinflammation has low specificity in creating brain changes (Skrzypczak-Wiercioch & Salat, 2022).

2.4.6 Arachidonic acid-induced ear edema

The arachidonic acid-induced mouse ear edema produces metabolites such as prostaglandin and leukotriene by the action of cyclo-oxygenases and lipoxygenases. Inflammation caused by topical application of arachidonic acid results are characterized by the following: accumulation

of neutrophils, edema and intense erythema. The anti-inflammatory compounds identified using this model are associated with the antihistaminic and antioxidant properties (Young *et al*., 1983).

Advantages: the arachidonic acid induced model of ear edema is suitable for acute inflammation and in identifying anti-inflammatory compounds through the eicosanoid pathway. Disadvantage: At the end of experiment animals are sacrificed (Patil *et al*., 2019).

2.4.7 TPA-induced ear edema

The 12-O-tetradecanoylphorbol-13-acetate (TPA) - induced ear edema is used to test cutaneous anti-inflammatory activity by observing cellular hyper-proliferation. It induces expression of pro-inflammatory cytokines in keratinocytes (Boller *et al*., 2010). The ear inflammation can be used to determine anti-inflammatory characteristics of synthetic and botanical ingredients in drugs (Tamura *et al*., 2009).

The pro-inflammatory activity is through the stimulation of protein kinase followed by activation of mitogen activated protein kinase (MAPK) and phospholipase A2 which initiates the arachidonic pathway. Therefore, arachidonic pathway inhibitors of COX, LOX and phospholipases can be used to reduce TPA induced inflammation (Young *et al*., 1983).

Advantage: it is suitable for testing both steroidal and non-steroidal anti-inflammatory drugs, lipoxygenase inhibitors and cyclooxygenase inhibitors. Disadvantage: the mechanism of TPA induced ear oedema is not fully understood (Boller *et al*., 2010; Inoue *et al*., 1989).

2.4.8 Oxazolone-induced ear edema

Oxazolone is an allergen used in initiating allergic contact dermatitis in both induction and elicitation phases. Its inflammatory effect is through the increase in arachidonic acid metabolites and promotion of nitric oxide synthase expression in langerhan cells and keratinocytes. Inhibitors of cytokine expression and corticosteroids are capable of deterring hypersensitivity caused by oxazolon. Furthermore, oxazolone causes interferon-γ production by CD8+ Tc1 cells which activates inflammatory cells, keratinocyte proliferation and epidermal thickening thus develop dermatitis (Bas *et al*., 2007).

Advantages: oxazolone induced ear edema model is good for delayed type hypersensitivity. Disadvantages: it cannot screen drugs that act by non-immune mechanisms in their anti-inflammatory mechanism (Patil *et al*., 2019).

2.4.9 Acetic acid or Compound 48/80-induced vascular permeability

The compound 48/80 has the ability of releasing histamine from the mast cells which results in increased vascular permeability (Hossen *et al*., 2006; Segawa *et al*., 2007).

In acetic acid-induced vascular permeability test, there is an increase in mediators which are responsible for vasodilation and vascular permeability (Chen *et al*., 2018).

Advantages: This model is appropriate for assessing acute anti-inflammatory effect. Moreover, it is used to screen drugs that are anti-histaminic or linked with mast cell stabilization. Disadvantages: the use of acetic acid leads to severe irritation and this violates animal welfare and could raise ethical issues. Also, at the end of experiment animals are sacrificed (Patil *et al*., 2019).

2.4.10 Leukocyte migration test / Pleurisy model

Pleurisy model depicts exudative inflammation in living organisms. It uses phlogistic agents such as antigen, carrageenan, dextran, and compound 48/80. Pleurisy induced by carrageenan in rats is measured by the biochemical parameters in the exudate, fluid extravasation and leukocyte migration (Hou *et al*., 2022).

Advantages: the pleurisy model is used for acute anti-inflammatory screening. Moreover, it can assess the inflammatory indicators such as biochemical parameters in the exudate, the leukocyte migration etc (Patil *et al*., 2019). Disadvantages: this method may inflict pain or develop infections to the animals if the phlogostic agent is not sterile. After the experiment, the laboratory animals are sacrificed (Rachmawati *et al*., 2016).

2.4.11 Sub-acute inflammation

The granuloma pouch model involves introduction of an irritant substance into the air pouch at the subcutaneous level. This act causes inflammation by the following characteristics: tissue proliferation, infiltration of macrophages and leukocytes. Such tissues may end up being carcinogenic or mutagenic (Patil *et al*., 2019).

Advantages: this is a model of sub-acute inflammation brought by having a direct contact between the compounds being tested and target tissues. Disadvantages: the procedure needs anesthesia since subcutaneous injection of the air pouch creates pain. Also, at the end of experiment animals are sacrificed (Patel *et al*., 2012)

2.4.12 Chronic Inflammation

2.4.12.1 Cotton pellet induced granuloma

The cotton pellet induced granuloma method evaluates the pathology of chronic inflammation: monocyte and neutrophil infiltration ; proliferation of fibroblasts, angiogenesis and exudation (Babu *et al*., 2009). This model assesses the efficacy of a drug against the proliferative characteristic phase of inflammation (Amresh *et al*., 2007). The amount of granulomatous tissue formed correlates to the dry weight of the cotton pellet while transudate tallies with the weight of moistened cotton pellets. The dry weight of granuloma is inhibited by steroidal drugs (Gupta *et al*., 2005; Meshram *et al*., 2015).

Advantages: the cotton pellet induced is for chronic inflammation. Proliferative changes occurring during chronic inflammation can be obtained through biochemical analysis of granuloma. Therefore, additional biomarkers can be assessed through this method. Disadvantages: there is need for surgery to implant and removal of cotton pellets and granuloma. Sepsis can occur due to the implantations and this could distort the observations (Gupta *et al*., 2005; Panthong *et al*., 2003).

2.4.12.2 Formaldehyde-induced model

Formaldehyde-induced model can be used to assess both anti-arthritic and anti-inflammatory properties. This model embodies the proliferation phase of inflammation produced by

cyclooxygenase mediators. Formaldehyde induced edema of ipsilateral paws has a biphasic response (Ben *et al*., 2016). Inflammation process is biphasic: the first component is by a neurogenic substance, P and bradykinin then the involvement of histamine, serotonin, prostaglandins and bradykinin (Amresh *et al*., 2007).

On the other hand, the central nervous system drugs suppress both phases while NSAIDs, corticosteroids act through the peripheral nervous system hence they inhibit the second phase. Advantages: This model closely resembles the human arthritis. Moreover it can predict the influence of anti-inflammatory drugs on the central or peripheral components. Disadvantages: Use of formaldehyde irritates and generates severe pain to experimental animals (Lalrinzuali *et al*., 2016; Segawa *et al*., 2007).

2.4.12.3 Complete Freund Adjuvant- Induced arthritis model

The complete freund's adjuvant (CFA)-Induced arthritis model involves synovial hyperplasia (brought by proliferation of leukocyte, increase in cytokines and release of reactive oxygen species), cartilage and bone destruction (Mbiantcha *et al*., 2017). Determination of the probable mechanisms in CFA-induced paw inflammation can be achieved through hematological, histo-pathological, radiological and biochemical evaluations, visual system of scoring arthritis, content of nitrite and anti-oxidant investigations (Patil *et al*., 2019).

Advantages: The complete Freund's adjuvant (CFA)-Induced arthritis model is robust with the capacity of assessing immune-inflammatory and arthritic conditions at acute and chronic states. Disadvantages: Animals are euthanized at the end of the protocol. The protocol is time consuming and stressful to the animal. Also, the experiment requires use of specialized instruments such as plethysmometer to measure changes in the paw volume and Von-Frey apparatus measures pain threshold. The preparation and induction of CFA should be carefully done as it affects the arthritic response (Kshirsagar *et al*., 2014; Mbiantcha *et al*., 2017)

2.5 Inflammatory diseases

2.5.1 Rheumatoid arthritis

Rheumatoid arthritis is a chronic condition characterized by the inflammation of joints of hands and feet due to infiltration of CD4+T cells, B cells and macrophages and destruction of bones

and cartilage. It has been linked to the Human Leukocyte Antigen-Death Receptor (HLA-DR) genes with a common amino acid motif (QKRAA) in the HLA-DRB1 region which resides in the major histocompatibility complex and plays a role in the presentation of the antigen (Firestein, 2003).

Autoantibodies produce rheumatoid factor, and anti-citrullinated proteins antibodies act as the sero-markers. Environmental factors coupled with genetic factors increase the risk of disease development. Smoking, pathogens such as *E.coli* and their products, periodontal disease and the gut microbiome environment have been associated with this disease (McInnes & Schett, 2011).

Patients tend to use NSAIDs, Disease-Modifying Anti-Rheumatic Drugs (DMARDs) and glucocorticoids to target pain, inflammation and disease modification. For DMARDs, their synthetic forms have either their mode of a mechanism known (targeted synthetic DMARDs) or not known (conventional synthetic DMARDs), such as methotrexate. Others are biological DMARDs in the form of TNF inhibitors, anti-B cell, T-cell and IL 6R. However, the degree of adverse effects produced from natural and targeted forms is more significant than conventional ones (Smolen *et al*., 2016).

2.5.2 Inflammatory Bowel Syndrome (IBS)

IBS involves inflammation of the gut existing as ulcerative colitis and Crohn disease. Clinical symptoms are abdominal pain, rectal bleeding and potential weight loss. Through genome-wide association studies (GWAS), the identified loci are related to either innate or adaptive immune pathways. For instance, the nodulation (NOD) gene encodes for a pattern recognition receptor and its presence signifies a greater risk of developing an aggressive IBS. The coexistence of the commensal microbiome and the immune system achieves equilibrium; distortion of the equilibrium generates dysbiosis and triggers the development of this condition. Treatment of IBS depends on its severity: aminosalicylates for mild conditions while biologics (anti-TNF) for severe conditions (Shapiro *et al*., 2016).

2.5.3 Multiple sclerosis

Multiple sclerosis is caused by chronic inflammation that infiltrates T-cell lymphocytes alongside TNF alpha and IL-6. It leads to demyelination and neural degeneration. At the onset,

the immune system gets attacked, leading to internal immune reactions in the central nervous system (CNS) as the disease progresses. The genetic factor is the HLA-DRB1*15 gene with/or alleles that are strongly linked to it. Over 150 genetic polymorphisms have been identified and found to reside near genes that have an immune function. Genetic interactions with ecological factors such as smoking constitute disease development. Vitamin D and obesity have been positively correlated as risk factors (Correale *et al*., 2017; Dobson & Giovannoni, 2019).

2.5.4 Psoriasis

This is an auto-immune inflammatory disease, primarily affecting the keratinocytes of skin and nails. The psoriasis-susceptibility loci (PSORS1) and its polymorphisms, Human leukocyte antigen Cw6 and major histocompatibility complex (MHC) are among the positively identified loci with a role in psoriasis either targeting the skin or immunity. Treatment therapies used are derivatives of vitamin D and gluco-corticosteroid (Boehncke & Schön, 2015).

2.6 Anti-inflammatory drugs

2.6.1 Glucocorticoids

Glucocorticoids are often used in the suppression of chronic inflammation. Drugs such as hydrocortisone (natural glucocorticoid), prednisone, methylprednisolone, and dexamethasone (synthetic glucocorticoid) belong to this class (Thornton, 2010). Glucocorticoids are known to decrease the activation of NF-κB (Patil *et al*., 2019). Chronic use of glucocorticoids increases the likelihood of developing hypertension, osteoporosis and hyperglycemia (Yasir *et al*., 2021).

2.6.1.1 Mechanism of action: genomic

Direct interaction with the DNA: the glucocorticoid receptor is located in the cytoplasm; in its inactive state, it is complexed with chaperones. Otherwise when activated, the receptor dissociates from the chaperones and the newly formed glucocorticoid-glucocorticoid receptor is released to the nucleus. There, it binds to the glucocorticoid response element, DNA sequence found at the promoter region of gluco-corticoid genes. This results in either gene activation or gene repression. The gene activation process is attributed to the co-activator proteins which influence transcription activities. Indirect interaction with the DNA: instead of glucocorticoid

receptors binding with the glucocorticoid response element, it binds with transcriptional elements (co-activators and co-repressors) directly. This mechanism is the most significant for inflammatory diseases (Grzanka *et al*., 2011).

2.6.1.2 Mechanism of action: non-genomic

The glucocorticoid acts on the glucocorticoid receptor in the cell membrane. This activates secondary messengers such as cyclic adenosine monophosphate, cylic guanine monophosphate, calcium ions, inositol triphosphate and diacylglycerol. Successively, kinase activity such as protein kinase A and C, mitogen activated protein kinases (MAPK), tyrosine kinase and lipid kinase are induced and affect the G and ion channels. For dexamethasone, a glucocorticoid drug, binding to its receptor results in chaperone, Hsp90, dissociation and release of kinase Src tyrosine which inhibits the release of arachidonic acid. The non-receptor mechanism of glucocorticoid is poorly deciphered. However, it is believed to have a correlation with the membrane ion channel regulation of sodium, potassium, chloride and calcium ions (Grzanka *et al*., 2011).

2.6.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

They serve not only as anti-inflammatory but also as antipyretic and analgesic drugs. They are made up of weak acids with lipid soluble activity. Bacchi *et al*., (2012) classified NSAIDs based on their chemical structure, inhibition activity and half-life.

2.6.2.1 Classification based on chemical structure

Salicylic acid: This is a simple phenol of benzoic acid derivative (Aldred *et al*., 2009). Its derivatives are acetylsalicylic acid or aspirin (synthetic form), salicin (naturally occurring), sodium salicylate, sulfasalazine, salicylamide, methyl salicylate, olsalazine, fendosal and diflunisal (Paumgartten *et al*., 2022). Acetylsalicylate binds to both COX isoforms (1 and 2) covalently and irreversibly and causes acetylation of serine 530. Moreover, at high doses, aspirin and sulfasalazine correlates negatively NF-κB activation (Patil *et al*., 2019).

Acetaminophen is a weak inhibitor of cyclooxygenase.

Arylpropionic acid derivatives (ibuprofen, ketoprofen, flurbiprofen, and naproxen) are usually better tolerated than other NSAIDs such as aspirin and indomethacin.

Hetero-aryl acetic acid derivatives include diclofenac, tolmetin, ketorolac and zomepirac. The sodium form of diclofenac is one of the potent NSAIDs known clinically.

Oxicam family (meloxicam, tenoxicam, piroxicam) belongs to the enolic acid group: piroxicam is used for the treatment of rheumatoid and osteo- arthritis. A summary of the NSAIDs is presented in (Table 1).

Table 1: Classification of NSAIDs based on structure alongside their characteristics

Class structure	**Drug**	**Properties**
Salicylic acid[a]	Acetylsalicylic acid, sodium salicyclate	Inhibit COX-1, COX-2
Indole acetic acid[a]	Etodolac	Inhibit COX-1, COX-2
Hetero-aryl acetic acid[a]	Diclofenac	Inhibit COX-1, COX-2
Aryl propionic acid[a]	Ibuprofen Naproxen	Inhibit COX-1, COX-2
Enolic acids[a]	Meloxicam	Inhibit COX-1, COX-2 (Preferential to COX 2)
Diarylheterocycles[a]	Celecoxib	Inhibit COX-1, COX-2 (Preferential to COX 2) Strong COX-2, weak COX-1 inhibition
	Rofecoxib	
Para-aminophenol[b]	Acetaminophen/paracetamol	Inhibit COX-3

Key: a: (Bacchi *et al*., 2012); b:(Vane & Botting, 1998)

2.6.2.2 Inhibition activity and selectivity

NSAIDs inhibition activity can be grouped according to Bacchi *et al*., (2012):

i) NSAIDs that poorly select and inhibit both COX-1 and COX-2 include ibuprofen, diclofenac, aspirin, piroxicam, naproxen.

ii) Inhibition by NSAIDs to COX-1 and COX-2 but has a preferential selection to COX-2; for example celecoxib, meloxicam, nimesulide, etodolac

iii) NSAIDs that are strong inhibitors to COX-2 but weak to COX-1: rofecoxib

iv) NSAIDs that are weak inhibitors of both COX-1 and COX-2 such as sodium salicylate

Inhibition of both COX iso-zymes (COX-1 and COX2) causes damage to the lower gastrointestinal tract (Maseda & Ricciotti, 2020).

2.6.2.3 Half-life in serum

NSAIDs can be classified as short acting (lasts less than six hours) and long acting (more than six hours). Short acting NSAIDs are used for acute pain and long lasting for chronic pain (Bacchi *et al*., 2012; Bindu *et al*., 2020).

2.6.2.4 Mechanism of action

NSAIDs mode of action is by inhibition of cyclooxygenase (COX) enzyme. Cyclooxygenase is needed for the synthesis of prostaglandins thromboxane (needed for platelet adhesion) and prostaglandins prostacyclins (responsible for anti-nociception, vasodilation) from arachidonic acid (Ghlichloo & Gerriets, 2021). Selective COX-2 inhibitors selectively block the COX-2 isoform which is responsible for prostaglandins prostacyclin (PGI2) production. These drugs are unlike the traditional anti-inflammatory drugs which block COX-1 and COX-2. Blocking of COX-1 has been linked to stomach ulceration and bleeding.

2.6.3 Monoclonal antibodies

Monoclonal antibodies are capable of inhibiting inflammation by blocking pro-inflammatory mediators such as TNFs, interleukin (IL) isoforms of 1, 6 and 8. They are antigen specific, can target virtually any cell, efficient and safe. Monoclonal antibody drugs such as anti-IL-1 and anti-IL-6 receptors, anti-tumor necrosis factor, anti-α4 integrin subunit, and anti-CD20 agents have been used in the treatment of chronic inflammation (Kotsovilis & Andreakos, 2014).

Examples of such drugs are adalimumab, infliximab and remicade are used in treatment of rheumatoid arthritis while adalimumab, infliximab, golimumab, and certolizumab pegol for inflammatory bowel disease (Andreakos *et al*., 2002; Di Paolo & Luci, 2021).

2.7 Selected natural products that have anti-inflammatory properties

Polyphenols (lignans, stilbenes, phenolic compounds, and flavonoids) from plant sources possess anti-inflammatory properties and have been studied using different pathways.

2.7.1 Flavonoids

Ruiz and Haller (2006) studied the molecular mechanisms by which plant-derived flavonoids interact with signaling pathways using non-carcinoma mouse cell lines. 3- Hydroxy flavone, apigenin, and luteolin repress the expression of Interferon-inducible Protein -10 (IP-10), which was induced by Tumor Necrosis Factor (TNF). Since the IP promoter is interlinked to other transcription factors such as Nuclear Factor Kappa Beta (NF-kB), IP repression affects IKKβ, Akt, and NF-kB binding to pro-inflammatory genes (Ruiz & Haller, 2006). Lipopolysaccharide stimulated macrophage cells pretreated with luteolin, genistein, and quercetin stimulated cells retarded the production of cytokines such as TNF-α and IL-6 with quercetin and luteolin performance being highly effective.

Flavonoid hesperetin and eriodictyol inhibit only TNF alpha release (Xagorari *et al*., 2001). Epigallocatechin gallate antitumor activity was reported by inhibiting enzymes p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated protein kinase (ERK) (Kundu *et al*., 2003). Anti-inflammatory screening of 22 compounds by targeting COX, iNOS revealed that quercetin, resveratrol, and curcumin as the most potent compounds (Gerhäuser *et al*., 2003).

Prenylated flavonoids such as papyriflavonol and sophoroflavonol are COX and LOX inhibitors. This dual inhibition is beneficial since COX inhibition results in LOX-mediated inflammation. Kaempferol and quercetin are also LOX inhibitors (Chi *et al*., 2001).

A summary of selected flavonoids with anti-inflammatory properties is presented in (Figure 1).

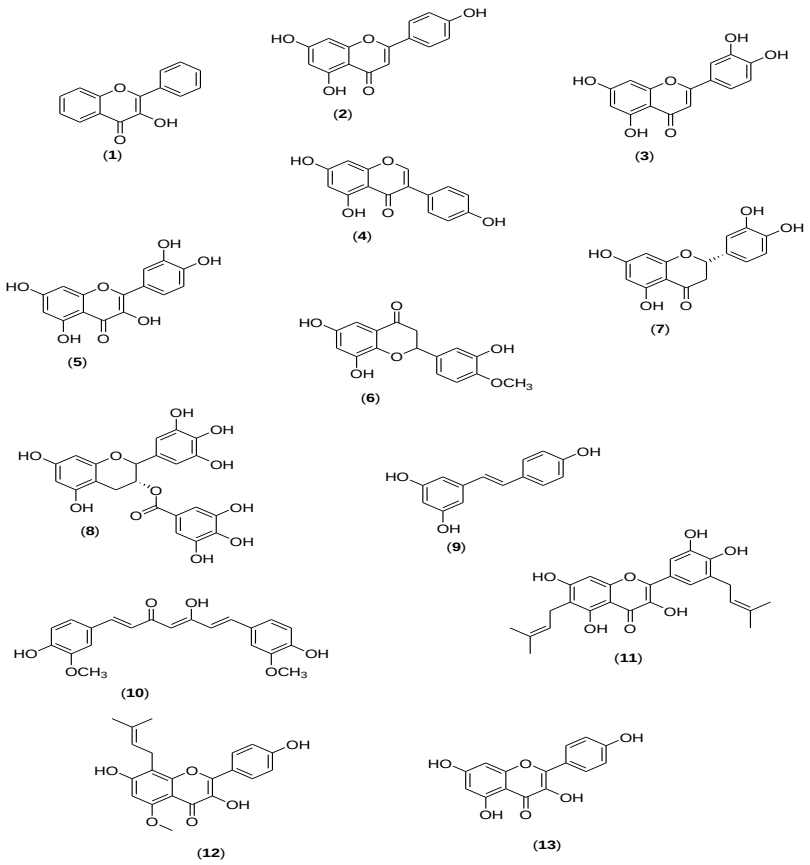

Figure 1: A structural summary of selected flavonoids with anti-inflammatory properties (1: 3-hydroxyflavone, 2: apigenin, 3: luteolin, 4: genistein, 5: quercetin, 6: hespertin, 7: eriodictyol, 8: epigallocatechin gallate, 9: resveratrol, 10: curcumin, 11: papyriflavonol, 12: sophoroflavanol, 13: kaempferol).

2.7.2 Stillbenes

Resveratrol is a polyphenol present in red wine and grapes. In 12- O –tetra decanoylphorbol-13-acetate (TPA) induced cells, it was able to inhibit COX-2 expression by preventing the activation of IKK, ERK, and MAP enzymes (Kundu *et al*., 2006).

2.7.3 Phenolics

Curcumin, also known as 1, 6-Heptadiene-3, 5-dione, 1, 7-bis (4-hydroxy-3-methoxyphenyl)-, (E, E) - or diferuloylmethane), is the primary polyphenol in turmeric. It has been used in treating pain, inflammatory and metabolic conditions (Karunaweera *et al*., 2015).

Its activity can be through direct or indirect molecular mechanisms. Direct mechanisms constitutes direct interaction of curcumin (10) with enzymes (COX, LOX, glucose synthase kinase, phosphorylase-3 kinase, xanthine oxidase, N-amino peptidase, DNA polymerase, pp60 src tyrosine kinase, ubiquitin isopeptidase, thioredoxin reductase and topoisomerase II), protein (amyloid protein, glutathione, human a1-acid glycoprotein, albumin, P glycoprotein, tubulin, and toll-like receptor) and divalent metals (iron, manganese, zinc) (Aggarwal & Sung, 2009).

Secondary or indirect mechanisms involve up regulation and down regulation. Up regulation involves activation of the following transcription factors: peroxisome proliferator-activated receptor g (PPAR-g) that suppresses cyclin D1 expression and epidermal growth factor; p53 which mediates cell cycle dependent kinase inhibitor p21 and Nrf2 which induces Glutathione S-transferases and nicotinamide adenine dinucleotide phosphate (NADPH). In contrast, down regulation involves: transcription factors (NF-kB, activator protein (AP-1), specificity protein (SP)-1, Signal transducer and activators of transcription protein 3 (STAT)-3, and b-catenin), growth factor receptors, protein kinases, chemokine and its receptors and inflammatory biomarkers (IL-1, IL-6, TNF, 5-LOX, prostate specific antigen (PSA), COX-2, and C-reactive protein (CRP) (Aggarwal & Sung, 2009).

Trans-cinnamaldehyde and 2-methoxycinnamaldehyde from *Cinnamomum cassia* inhibit NF-kB by preventing DNA binding and, inevitably, the transcription of the pro-inflammatory genes (Reddy *et al*., 2004).

2.7.4 Capsaicin

A quinone compound found in hot peppers has been extensively studied concerning its effect on NF-kB activation. In a dose-dependent mechanism, capsaicin blocks inhibition of NF-kB activation brought by TNF or phorbol ester. Similarly, in LPS stimulated macrophages cells, capsaicin prevented COX-2 activity and expression of iNOS (Kim *et al*., 2003; Singh *et al*., 1996).

2.8 Stinging nettle

2.8.1 Botany of the stinging nettle

The stinging nettle belongs to the Urticaceae family. It consists of 12 genera such as Urtica, Urera, Obetia, Nanocnide and Zhengyia and over 200 species (Zy *et al*., 2013). Local names for stinging nettle are mpupu (Swahili), thabai/hatha (Kikuyu), Isambakhulu (Luhya), Siwot (Kipsigis), Ayela (Luo) and endamejoi (Maasai) (Wasike, 2013).

It is known for its skin allergenic reaction upon touch. The non-stinging hairs are found on the serrated leaves and stem while stinging ones with touch-sensitive tips are found at the apex (Grauso *et al*., 2020).

2.8.2 Pharmacological properties of Urtica species

The therapeutic uses of stinging nettle can be found through its macerated, infused or boiled portions in the treatment of hepatic diseases and stomach aches (Kokwaro, 1993). Seeds of *Urtica dioica* manifested hepato-protection when examined against aflatoxin-induced liver injury of rats (Yener *et al*., 2009).

Using the disc diffusion method, ethyl acetate extracts of the stinging nettle inhibited the activity of *Aeromonas hydrophila, Bacillus cereus, Escherichia coli, Salmonella typhi* and *Staphylococcus aureus* (Ghaima *et al*., 2013). On the other hand, their Minimum Inhibitory Concentrations were found to be comparable to gentamycin which was used as the positive control. The leaves portion has been widely studied for its anti-inflammatory properties. The hexane extracts exhibited anti-inflammation by measuring paw oedema of rats against

indomethacin, a known anti-inflammatory drug (Dar et al., 2013). Another study by the Food Research International used in-vitro inhibition of nitric oxide and they found that *Urtica urens* had a higher activity compared to *Urtica dioica* and *Urtica membranacea* (Carvalho *et al*., 2017).

The stinging nettle roots contain lectins and polysaccharides as the active ingredients responsible for anti-prostatic property (Wagner *et al*., 1994). This was further confirmed by Hyrb and colleagues; using the aqueous root extract, it inhibited the binding of 125I-Sex Hormone Binding Globulin to its receptor in a dose dependent trend (Hryb *et al*., 1995).

Nettle water extracts showed anti-ulcer and analgesic activities using acetic acid induced stretching and ethanol induced ulcerogenesis (Gülçin *et al*., 2004). The hypoglycemic property of the extract is due to its ability to promote the secretion of insulin by the islets of Langerhans. An *in vivo* study of normal and diabetic rats induced with streptozotocin treated intra-peritoneally with the nettle extract revealed a decrease in glucose after half an hour while an increase in insulin after one and a half hour (Farzami *et al*., 2003). The hypoglycemic mechanism of the extract was elucidated as preventing decrease in islet cells size and stimulates regeneration of beta cells (Gohari *et al*., 2018).

A rat model of fructose-induced insulin resistance validated the capacity of stinging nettle to have an anti-hyperlipidemic effect by decreasing the low density lipoprotein (LDL) / high density lipoprotein (HDL) ratio and increasing very low density lipoprotein (VLDL) (Ahangarpour *et al*., 2012).

The anti-asthmatic effect of the leaves of *U. dioica* aqueous extract has been evaluated by Zemmouri *et al*.,(2017). Male adult Wistar rats were divided into negative control group, positive control (ovalbumin induced) group, experimental group that received the extract orally with the experimental protocol and another experimental group which received only the extract orally. Through histopathological and hematological analysis, the extract ($p < 0.01$) was capable of suppressing the propagation of the inflammatory cells thus protecting against airway inflammation.

Among the cosmetic applications of nettle is its anti-aging activity. Its extracts have been found to inhibit collagenase and elastase possibly by the action of ursolic acid and quercetin which are strong anti-oxidants (Bourgeois *et al*., 2016).

2.8.3 Chemical composition of *Urtica* species

2.8.3.1 Proximate composition

Table 2: Proximate composition of *Urtica dioica* in percentage according to Adhikari *et al*., (2015)

Chemical group	%content
Protein	33.7
Moisture	7
Ash	16.21
Fat	3.5
Fiber	9.08
Carbohydrates	37.39
Tannins	0.93
Total Phenolic (GAE/g)	128.75
Carotenoids (μg/g)	3496.67

An extensive analysis of these food groups using nuclear magnetic resonance for polar compounds (amino acids, organic acids, sugars) and gas chromatography mass spectroscopy for non-polar compounds (fatty acids) revealed the following (Table 3) (Grauso *et al*., 2019).

Table 3: Chemical groups with their respective constituents for Urtica dioica

Chemical group	Constituents
Amino acids	Alanine, 4-aminobutyrate, glutamic acid, isoleucine, leucine, phenylalanine, proline, tyrosine, valine.
Organic acids	malic acid, acetic acid, citric acid, succinic acid, formic acid
Sugars	inositol, glucose, rhamnose, sucrose
Steroids (found in the roots only)	sitosterol (3-β-sitosterol, sitosterol β D glucoside, sitostetol-3-O-β-D-glucoside, 7-β hydroxysitosterol, 7 α hydroxysitosterol
Fatty acids	lauric acid , dodecanedioic acid , myristic acid ,myristoleic acid, palmitic acid, palmitoleic acid, heptadecanoic acid, stearic acid, cis -9-oleic acid ,cis-9-12 linoleic acid, arachidonic acid, α-linolenic acid, gondoic acid, erucic acid ,heneicosanoic acid, cis 11,14 eicosadenoic acid, 9 hydroxy-10,12 octadecadienoic acid
Carotenoids	neoxanthin, violaxanthin, lutein, isoprenoid and lycopene

2.8.3.2 Previously identified phytochemical compounds from *Urtica* species

Metabolite profiling of *Urtica dioica* by the use of nuclear magnetic resonance unveiled caffeoyl derivatives such as 3-caffeoylquinic acid or chlorogenic acid, 5-caffeoylquinic acid or neo-chlorogenic acid, monocaffeoyl tartaric acid or caftaric acid and dicaffeoyl tartaric acid or cichoric acid; flavonoids such as apigenin-7-glucoside, luteolin-7-glucoside, luteolin-7-rutinoside, quercetin-7-rutinoside, choline and trigonelline (Grauso *et al*., 2019).

High performance liquid chromatography combined with mass spectroscopy mass spectroscopy (HPLC-MS-MS) analysis of the leaves, stems, flowers and inflorescence informed on a wide array of the phenolic compounds present in *Urtica dioca*. For instance: For instance: p-hydroxybenzoic, gentisic, protocatechuic, vanillic, quinic, p-coumaric, caffeic, ferulic, and 5-O-caffeoylquinic acids, esculetin, scopoletin, secoisolariciresinol, chrysoeriol, kaempferol, isorhamnetin, catechin, kaempferol 3-O-glucoside, quercetin 3-O-glucoside, quercetin, rutin and amentoflavon (Orčić *et al*., 2014).

Zeković and coworkers used atypical methods of extraction (subcritical water extraction, ultrasound and microwave assisted techniques) to obtain the stinging nettle bioactive compounds. The microwave assisted extract had the highest relative abundance in phenolic compounds: bioactive compounds. The microwave assisted extract had the highest relative abundance in phenolic compounds: p-Hydroxybenzoic acid, cinnamic acid, protocatechuic acid, gentisic acid, p-Coumaric acid, gallic acid, ferulic acid, syringic acid, sinapic acid, quinic acid, umbelliferone, esculetin, scopoletin, apigenin, luteolin, chrysoeriol, kaempferol, quercetin, isorhamnetin, myricetin, genistein, naringenin, catechin, kaempferol-3-O-glycoside, luteolin-7-O-glycoside, quercetin-3-O-rhamnoside and quercetin-3-O-rutinoside, rutin. The subcritical water extract was devoid of umbeliferone, chrysoeriol, isorhamnetin, luteolin 7-O glycoside and quercetin 3-O rhamnoside while for ultrasound assisted extract: protocatechuic acid, gentisic acid, umbeliferone and genistein. For all the three extracts, rutin and sinapic acid were the highest flavonoid and phenolic acid respectively (Zeković *et al*., 2017).

An Ultra Performance Liquid Chromatography Mass Spectroscopy, UPLC-MS/MS, analysis of nettle leaves and stalks identified classes of benzoic, cinnamic and other phenolic acids, flavonols, flavan-3-ols, flavones, isoflavones, flavanones and coumarins. Flavonols and their glycosides were found to be the abundant class of flavonoid polyphenols (Repajić *et al*., 2021).
The roots are low in phenolic compounds characterized by the presence of secoisolariciresinol, coumarins and scopoletin / lignans (Francišković *et al*., 2017).

A similar study utilized ultra performance liquid chromatography-ultra violet-mass spectroscopy UPLC-UV-MS to analyse *U. dioica*, *U. platyphylla and U. flabellate* extracts. Phenolic acids such as (Hydroxycinnamic: 3-O-Caffeoylquinic acid, 5-O-Caffeoylquinic acid ,O-Caffeoyl-

threonine, 4-O-Caffeoylquinic acid , 3-(4-Hydroxycinnamoyl) quinic acid, caffeoyl malic acid, O-feruloyl quinic acid, p-coumaroyl malate, feruloyl malate, dicaffeoyl quinic acid , and caffeoyl feruloyl tartaric acid ; lignans which include (neoolivil-4-O-glucopyranoside); fatty acyl glycoside (3-Hexenyl-primeveroside and hexenyl-primeveroside); flavonoids such as (Isorhamnetin hexoside, quercetin dihexoside, rutin, isoquercetrin , kaempferol 3-O-rutinoside, isorhamnetin 3-O-rutinoside, kaempferol 3-O-glucoside); terpenes including (Pinene-9-ol-O-glucoside, pinene-ol-O-glucoside, megastigmane hexoside and fatty acids, (Dodecenedioic acid, hydroxy octadecanedioic acid, dihydroxy octadecatrienoic acid, corchorifatty acid D, japonic acid, dihydroxyoctadecadienoic acid, 9-Hydroxyoctadecatrienoic acid, 9-Hydroxy-10,12-octadecadienoic acid and linolenic acid) (Farag *et al*., 2013).

Chlorogenic acid is highest in cultivated nettle leaves while 2- O-caffeoylmalic acid in wild nettle leaves. Flavonoids are the most abundant in nettle stalks and in particular anthocyanins are only found in the stalks and not the leaves (Pinelli *et al*., 2008).

Table 4: List of selected compounds from *Urtica species*

Group	Compound	References
Hydroxy-cinnamic acid	Caffeic acid	(Kregiel *et al*., 2018)
	Ferulic acid	(Orčić *et al*., 2014)
	p-coumaric acid	(Francišković *et al*., 2017)
	2-O-caffeoylmalic	(Pinelli *et al*., 2008)
	O-caffeoyl-threonine	(Farag *et al*., 2013)
Chlorogenic acid	5-O-Caffeoylquinic acid	(Kregiel *et al*., 2018)
	4-O-Caffeoylquinic acid	(Orčić *et al*., 2014)
	3-O-Caffeoylquinic acid	(Francišković *et al*., 2017)
	Dicaffeoylquinic acid	(Farag *et al*., 2013)
Anthocyanins	Peonidin 3-O-rutinoside	(Pinelli *et al*., 2008)
	Rosinidin 3-O-rutinoside	
Flavonol	Kaempferol-3-O-Glucoside	(Kregiel *et al*., 2018)
	Quercetin 3-O-Glucoside	(Orčić *et al*., 2014)

	Quercetin 3-O-rutinoside	(Francišković *et al*., 2017)
	Isorhamnetin 3-O-rutinoside	(Pinelli *et al*., 2008)
	Isoquercetin	(Farag *et al*., 2013)
Isoflavonoid	Gentistic acid	(Kregiel *et al*., 2018)
		(Orčić *et al*., 2014)
Quinic acid (its derivatives)	Quinic acid	(Kregiel *et al*., 2018)
	3-(4-Hydroxycinnamoyl	(Orčić *et al*., 2014)
	quinic acid)	(Francišković *et al*., 2017)
	O-Feruloyl quinic acid	(Farag *et al*., 2013)
Indol (alkaloids)	Histamine	(Cummings & Olsen, 2011)
Protoalkaloids	Adrenaline	(Randall *et al*., 1999)
	Nor- adrenaline	
Malic acid and its derivatives	p-coumaroyl malate	(Farag *et al*., 2013)
	Feruloyl malate	
Hydroxybenzoic	p-hydroxybenzoic acid	(Kregiel *et al*., 2018)
	Protocatechuic acid	(Orčić *et al*., 2014)
		(Francišković *et al*., 2017)
Bicyclic monoterpenoids	2-Pinene-9-ol-O-glucoside	(Farag *et al*., 2013)
Lineolic acid	Corchorifatty acid D	(Farag *et al*., 2013)
7-hydroxycoumarins	Scopoletin	(Kregiel *et al*., 2018)
		(Orčić *et al*., 2014)
		(Francišković *et al*., 2017)
6, 7-dihydroxycoumarins	Esculetin	(Kregiel *et al*., 2018)
		(Orčić *et al*., 2014)
		(Francišković *et al*., 2017)
Di-benzylbutanediol lignans	Secoisolariciresinol	(Kregiel *et al*., 2018)
		(Orčić *et al*., 2014)
		(Francišković *et al*., 2017)
3'-o-methylated flavonoids	Chrysoeriol	(Kregiel *et al*., 2018)
Serotonins	5-hydroxytryptamine	(Cummings & Olsen, 2011)

		(Randall *et al*., 1999)
Acylcholine	Acetylcholine	(Cummings & Olsen, 2011)
		(Randall *et al*., 1999)

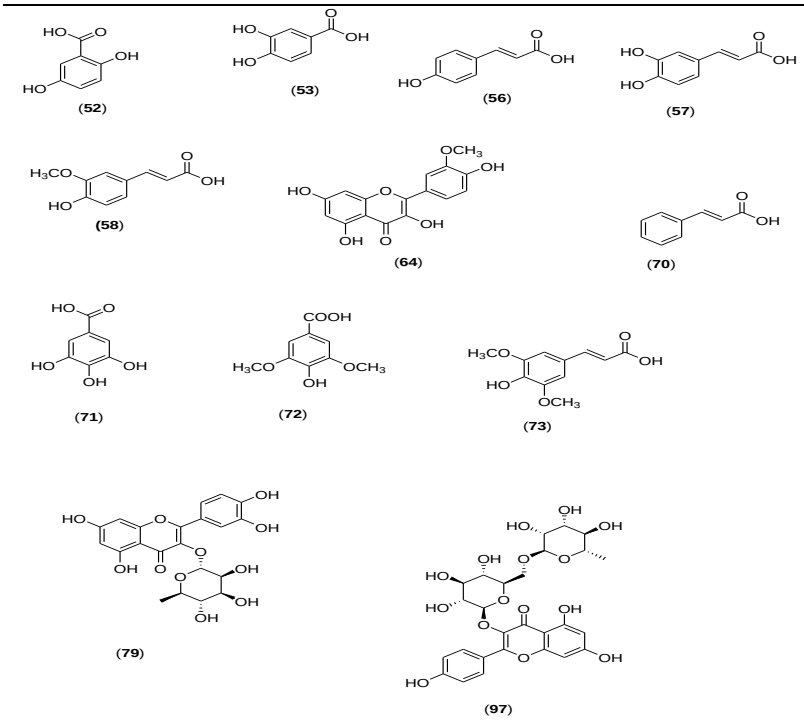

Figure 2: A summary of previous phytochemical compounds characterized from Urtica species (52: gentisic; 53: protocatechuic, 56: p-coumaric, 57: caffeic acid, 58: ferulic, 64: isorhamnetin, 70: cinnamic acid, 71: gallic acid, 72: syringic acid, 73: sinapic acid, 79: quercetin-3-O-rhamnoside, 97: kaempferol 3-O rutinoside).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Phytochemical composition of stinging nettle leaf crude extract in aqueous and organic phases

3.1.1 Plant sample collection

The samples were collected from indigenous stinging nettle plants growing in Limuru, Central Kenya (1.1069° S, 36.6431° E) kept in kraft paper bags and transported to the Center for biotechnology and bioinformatics (CEBIB). The leaves were air dried in a room and ground into a fine powder using a Hammer mill (Muharatta mechanical grinder) (Kama-Kama *et al*., 2016).

3.1.2 Molecular identification of the plant material

From the sample leaves genomic DNA was extracted using Cetyltrimethylammonium bromide (CTAB) protocol and checked on 1% agarose gel (Wu *et al*., 2013). Polymerase chain reaction (PCR) amplification was done in a total volume of 25 µl (2.5 µl 10 PCR buffer, 2.5 µl magnesium chloride (25 mM), 2.0 µl dNTP mixture (2.5 mM), 0.75 µl each 206 primer (10 µM), 0.125 µl Taq polymerase (5 U/µl), 2 µl template DNA (containing 50 ng genomic DNA), and finally distilled deionized water to toped up to 25 µl). The PCR profile for the primers *rbcL* and internal transcribed spacer (ITS) were used with an initial denaturation step at 94 °C for 1 minute, followed by 30 cycles of 50 seconds at the same temperature, 1 minute at 52 °C for *rbcL* and 55 °C for ITS, 80 seconds at 72 °C and a final extension at 72 °C for 10 minutes. The PCR products were checked on 1% agarose gels, followed by PCR cleanup. The final PCR products were sent for Sanger sequencing to Inqaba Biotec East Africa Ltd.

The forward and reverse sequences' read (.abi) files were each converted to a text file and then concatenated. From the combined file, a contig sequence was generated using the Cap Contig Assembly program in BioEdit® (Hall, 1999). This contig sequence was then subjected to a homology search using BLASTN under the default parameters (Altschul *et al*., 1990). Sequences of the related homologs were subjected subjected to multiple sequence alignment using T-Coffee under default parameters (Notredame *et al*., 2000). Phylogeny was reconstructed using MrBayes (Ronquist & Huelsenbeck, 2003) by converting the alignment file to a nexus file followed by Bayesian analysis with four independent runs of 10,000 generations. The phylogeny tree was viewed using FigTree (Rambaut, 2010).

3.1.3 Extraction of crude extract

3.1.3.1 Aqueous extraction

Powdered sample weighing 50 g was soaked in 1000 ml of distilled water, boiled for 15 minutes, cooled, and filtered using a Whatmann filter paper No.1 (Whatman International Limited, Madstone England) (Kama-Kama *et al*., 2016; Telo *et al*., 2017). The filtrate was concentrated by Freezer-1 incubator (Analis,Suarlee, Belgium) and then stored at 4 °C for further analysis.

The percentage yield of the aqueous extract was calculated as: (Wcrude extract / Wsample) * 100%

3.1.3.2 Organic extraction

Powdered plant material, 100 g, was used to carry out extraction by cold percolation a mixture of methanol: dichloromethane (1:1). The extraction procedure was done in a three-stage phase whereby the first and second phases were each done after four hours, and the last step was done overnight. The final filtrate was concentrated using a rotary evaporator vacuum pump (Heidolph WB2000, Germany) and stored at 4 °C for further analysis while the insoluble part was discarded (Ebrahimzadeh *et al*., 2015; Kama-Kama *et al*., 2017).

The percentage yield of the organic extract was calculated as: (Wcrude extract / Wsample) * 100%

3.1.4 Phytochemical composition of crude extracts

3.1.4.1 Total phenolic content using Folin-Ciocalteu (FC) reagent

The plant extract sample (1 mg) was prepared in 1ml of methanol (1 mg/ml). A sample aliquot of 1 ml was introduced into a 10 ml volumetric flask and was mixed with 2.5 ml of Folin-Ciocalteu (FC) reagent (1:10 diluted with distilled water) followed by the addition of 2 ml of 7.5% sodium carbonate. The assay mixture was topped up to the mark using distilled water. The mix was incubated at 45 °C for 15 minutes, and absorbance was measured at 765 nm by a double beam UV-Vis spectrophotometer Hitachi 2900. The total phenolic content (mg/ml) was calculated using gallic acid as standard (Sangeeta & Vrunda, 2016).

Total phenolic content (TPC) was expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg GAE / g)

TPC = C (V/M)

C = concentration of gallic acid (mg/ml), Dilution factor = V/M

V = volume of extract, M = mass of extract

3.1.4.2 Total flavonoid content using aluminum chloride method

The plant extract sample (1 mg) was prepared in 1ml of methanol (1 mg/ml). A sample aliquot of 1 ml was introduced into a 10 ml volumetric flask and was mixed with 4 ml of distilled water, then accompanied by 0.3 ml of 5% sodium nitrite. The assay mixture was allowed to stand for 5 minutes then 0.3 ml of 1% aluminum chloride was added. The resulting mixture then stood for another 5 minutes, followed by the addition of 2 ml, 1 M sodium hydroxide, and topped up to the mark using distilled water. Absorbance was measured at 510 nm by a double beam UV-Vis spectrophotometer Hitachi 2900. The total phenolic content (mg/ml) was calculated using quercetin as standard (Sangeeta & Vrunda, 2016).

Total flavonoid content (TFC) was expressed as mg quercetin per gram of sample in dry weight (quercetin mg/g)

TFC =C (V/M)

C = concentration of quercetin (mg/ml), Dilution factor=V/M

V = volume of extract, M = mass of extract

3.1.4.3 Raman spectroscopy

Spectral measurement by STR Raman microscope from Technos Instruments constituted the following conditions: 785 nm with × 10 objective lens, 1% power, 600 BLZ grating, 98 - 1800 cm^{-1} range, 1050 cm^{-1} center wavelength, 10 sec exposure time, and five accumulations.

The vibrational spectral range constitutes from 600 to 1800 cm^{-1} for chlorogenic acid and its derivatives measurement (Eravuchira *et al*., 2012). Phenolic acids (hydroxybenzoic acid and its derivatives, hydroxycinnamic acid, and its derivatives) and flavonoids have the spectral range between 50 - 3599 cm^{-1} (Pompeu *et al*., 2018)..

Data collected from Raman spectroscopy was analyzed using the ChemoSpec package in R software (Hanson, 2016). Data analysis involved the following actions: pre-processing and feature extraction.

3.2 Ethical approval

Ethical approval was obtained from Biosafety, Animal use, and Ethics committee of the Faculty of Veterinary Medicine, Department of Veterinary Anatomy and Physiology, reference letter FVM BAUEC/2021/314.

3.3 Anti-inflammatory activity using the Carrageenan model of inflammation

A total of twenty, female and male albino rats weighing 180 - 250 g were randomly divided into 4 experimental groups with each having a sample size of 5. Group A and B received the aqueous and methanol: dichloromethane extracts each at 750 mg/kg body weight. Group C, the reference/positive control, received both the carrageenan and indomethacin, an NSAID, at a dosage rate of 10 mg/kg. Finally, group D received carrageenan treatment and saline water at 0.9% (w/v) (carrageenan/negative control).

The protocol for acute inflammation was adopted from the Carrageenan model. Group A and B were each treated orally with 750 mg/kg. This treatment was introduced 30 minutes before injection of carrageenan 1% pure at 100 µl into the sub-plantar region of the right hind paw of every rat (Winter *et al*., 1962). Changes in volume were measured using a plethysmograph at 0, 0.5, 1, 1.5, 2, 2.5 and 3 hours after carrageenan administration. An increase in paw volume was calculated as the difference between the "0" time and at respective hours.

Paw volumes is the difference in paw volume before and after administration of the extracts or controls. From the paw volume measurements, the percentage inhibition of edema was calculated as the difference between the initial and final paw volumes of the control group and test group recorded each at the same time (Pérez G, 1996).

$$\% \text{ inhibition of edema} = (A - B)/A \times 100$$

A - Paw volume of the control (negative) group

B - Paw volume of the test group

3.4 Active anti-inflammatory compounds determination from the potent extract using computational tools

The identified potent extract from the anti-inflammatory biological test was subjected to spectral analysis.

3.4.1 Reverse Phase High Pressure Liquid Chromatography Mass Spectrometry (LC-MS)

Air-dried powder sample of 100 g was extracted in high pressure liquid chromatography (HPLC) grade methanol while being sonicated for two days and filtered. The filtrate was then evaporated using Buchi Rotary Evaporator in a water bath with vacuum of 600 Psi to achieve 3.5 g of the extract. The 1 g of the extract was weighed with Sartorius analytical balance and centrifuged at 15,000 rpm for 2 minutes (J2-21 MIE, JA-20 rotor, Beckman, UK) and using a glass pipette and transferred into small clean labeled vials. Centrifugation was undertaken to remove all particulates from the extracts, thus preventing the possibility of column blockages. From the extract, the stock solution was prepared of 1 mg/ml, and 1 ml of the stock solution was placed into an HPLC vial (pyrex) and 20 µl was injected into liquid chromatography mass spectroscopy (LC-MS) electrospray ionization (ESI) vial (Agilent Technologies, 2013).

The LC-MS-ESI operating conditions were as follows: a quaternary LC pump (Model 1200) coupled to Agilent MSD 6120-Single quadruple MS with electrospray source (Palo Alto, CA) was used. The system was controlled using ChemStation software (Hewlett-Packard). Reversed-phase liquid chromatography was performed on an Agilent technologies 1200 infinite series, Zorbax SB C18 column, 2.1 x 50 mm, 1.8 µm (Phenomenex, Torrance, CA) using the following gradient program 0 minute, 5% B; 0-5 minutes, 5-50% B; 5-10 minutes, 50-80% B; 10-15 minutes, 80-100% B; 15-25 minutes 100% B; 25-30 minutes 5% B; 30-35 minutes 5% B (Water: Acetonitrile. The key working parameters were: flow rate at 1 ml/minute, injection volume at 20.0 µL, dwelling time was 50 ms, capillary voltage, 3.0 kV, cone voltage, 70 V, extract voltage, 5 V; RF voltage, 0.5 V; source temperature, 110 ºC; nitrogen gas temperature for desolvation, 380 ºC; and nitrogen gas flow for desolvation, 400 l/h. Using rutin as the standard (1-100 ng/µl) the linear calibration curve (peak area vs. concentration) obtained was [y=6008.9x – 5250.3 (R2 = 0.9987)] and it served as a basis for external quantification (Siji, 2016). The single point external standards analyte response is assumed to be linear over concentration. Therefore, all peaks for the analyte were subjected to the same method of quantitation. LC-MS in full scan mode to generate a linear calibration curve (peak area vs. concentration) with the following

equation; [y=6008.9x – 5250.3 (R2 = 0.9987)], which served as a basis for external quantification (Siji, 2016).

3.4.2 Screening of the ligands against the known inhibitors

3.4.2.1 Pairwise comparison between the active compounds and indomethacin
Pairwise comparisons were done between the known inhibitors (indomethacin) and ligands on the Google Collab note books using rd-kit modules (Appendix III) (Landrum, 2010).

3.4.2.2 Molecular docking

3.4.2.2.1 Protein receptor preparation

The following actions were applied using BIOVIA Discovery Studio 2021 Client: removing ligands: protoporphyrin IX containing Fe (HEM), 2-acetamido-2-deoxy-beta-D-glucopyranose (NAG) and indomethacin (IMN) then adding polar hydrogen atoms. Next, the grid box configuration was determined by obtaining the x, y and z configuration values of indomethacin (Dassault Systemes, 2021). The resulting protein file was converted to pdbqt by OpenBabel (O'Boyle *et al*., 2011). The pdbqt file was loaded to Auto Dock Tools 4.2.6 for the following operations: Deletion of water, addition of Kollman charges, computation of Gasteiger charges, and a pdbqt file was generated (Morris *et al*., 2009).

3.4.2.2.2 Ligand preparation

The ligands were retrieved from Pub Chem as Canonical SMILES. Preparation of the ligand was done using Chimera's minimize structure command: Addition of hydrogen and charges using Gasteiger method. The prepared ligand was subsequently configured by Auto Dock Tools 4.2.6 to form a pdbqt file (Morris *et al*., 2009).

3.4.2.3.3 Docking process

The final configuration parameters for docking using Autodock Vina (version 1.1.2) were : center_x = 14.949600, center_y = 52.163200 and center_z = 68.722120; size_x = 20, size_y = 20 and size_z = 20; exhaustiveness = 8 (Trott & Olson, 2010). Values with the lowest binding energy and below 2 Å in root mean square deviation (RMSD) were selected for each ligand (Diallo *et al*., 2021).

3.5 Data analysis

The differences between treatments for paw edema were analyzed using a one-way analysis of variance (ANOVA). Significant results were considered at a p-value < 0.05 followed by post hoc Tukey test.

CHAPTER FOUR

4.0 RESULTS

4.1 Molecular identification of the plant material

4.1.1 Contig assembly and BLASTN (Basic Local Alignment Search Tool)

To unravel the identity of the resultant consensus sequences, a blast search against the national center for biotechnology information (NCBI) database sequences was done for *rbcL* and ITS markers. Identification of the plant samples was based on percentage similarity and respective E-values. According to the results alignment for the *rbcL* marker, *Urtica sp.* registered the highest identity of 99.13% with 0.0 E-values and 95% query coverage (Table 5).

Table 5: BLASTN results consisting of total score, querycover (%), E-value, percentage identity (%) and accession length for *rbcL* markers

Species	Total score	Query cover (%)	E value	Percentage identity (%)	Accession length	Accession
Urtica sp. Lixinhui	1317	95	0	98.54	741	KF138264.1
Urtica angustifolia	1317	95	0	98.54	741	KF138256.1
Urtica macrorrhiza	1314	96	0	96.28	146747	NC_062304.1 MT465759.1
Urtica angustifolia	1314	96	0	98.28	146679	MZ145046.1
Urtica dioica	1301	94	0	98.52	1414	MG946931.1
Urtica fissa	1290	95	0	97.87	741	KF138260.1
Urtica triangularis	1290	95	0	97.87	741	KF138266.1
Urtica	1284	95	0	97.74	741	KF138257.1

ardens						
Urtica hyperborean	1279	95	0	97.60	741	KF138262.1
Urtica dioica	1275	93	0	98.23	725	MH358163.1
Urtica linearifolia	1275	91	0	98.75	1071	KT626794.1
Urtica dioica sub	1243	88	0	99.13	689	MH358164.1

Results for the ITS marker identified *Urtica sp.* as the plant species with percentage similarity, query cover and E value of 93.48%, 92% and 0, respectively (Table 6).

Table 6: BLASTN results consisting of total score, querycover (%), E-value, percentage identity (%) and accession length for ITS markers

Species	**Total score**	**Query cover (%)**	**E value**	**Percentage identity (%)**	**Accession length**	**Accession**
Urtica sp. Lixinhui	1989	92	0	93.48	700	KF137941.1
Urtica atrovirens	1928	91	0	92.75	681	MH357956.1
Urtica massaica	1909	84	0	94.70	605	KX271389.1
Urtica massaica	1896	84	0	94.54	604	KX271388.1
Urtica massaica	1869	82	0	94.58	592	KM586424.1

Urtica massaica	1858	82	0	94.41	592	KM586438.1
Urtica mairei	1837	91		91.72	685	EU747118.1
Urtica atrichocaulis	1830	92	0	91.25	702	KF137935.1
Urtica simensis	1826	84	0	93.54	605	KX271387.1
Urtica bianorii	1824	84	0	93.38	605	KF558917.1
Urtica dioica sub	1810	84	0	93.21	605	KF558900.1

From the BLASTN results, the identified species from both markers were subjected to phylogenetic analysis. The generated dendrogram has a well–resolved phylogeny, robust bootstrap values and revealed three strongly supported clades and an out-group. The largest clade (highlighted in yellow) consists of sub clades with the following: (*Urtica sp. Lixinhui, U. mairei, U. atrichocaulis* and *U. atrovirens*); (*U.massaica* and *U.simensis*); *U. bianorii*; *U. dioica sub*. The second clade (highlighted in maroon) predominantly consists of *U. dioica* and the last clade in green has *U. angustifolia, U. fissa, U. ardens, U. triangularis* and *U. hyperborean* species (Figure 3).

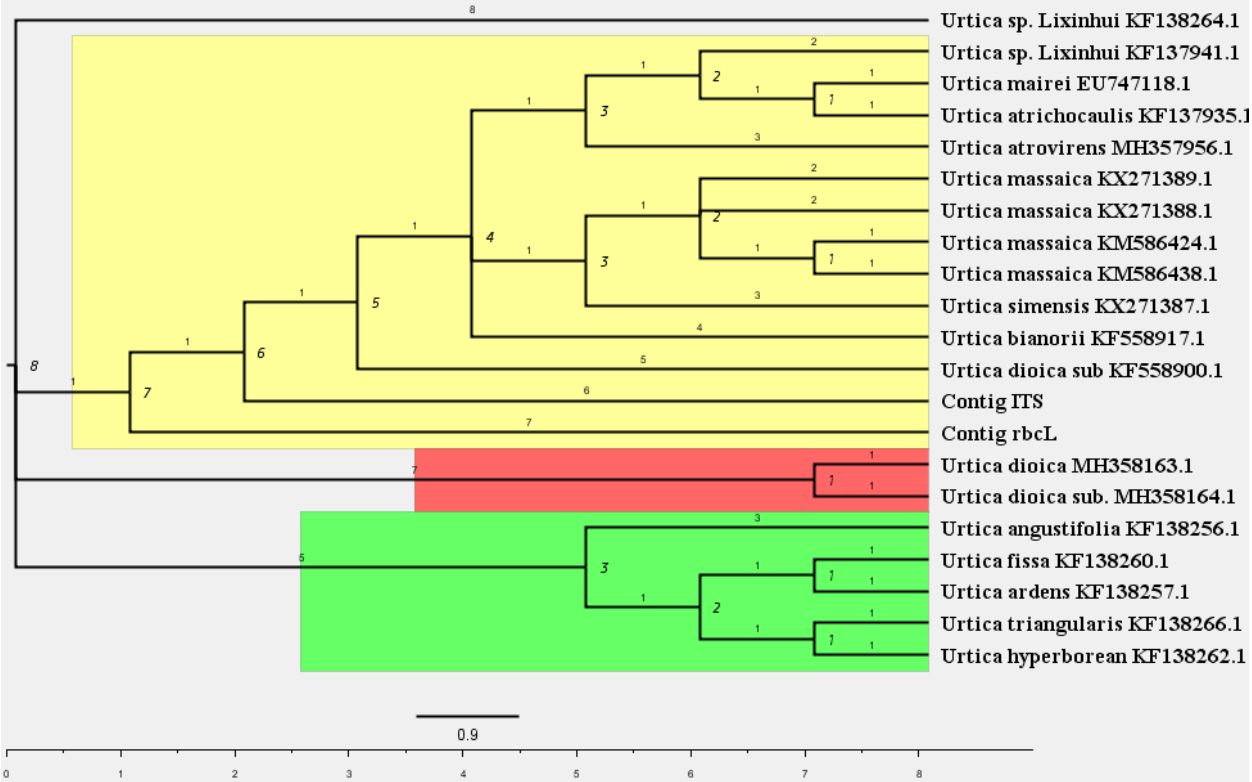

Figure 3: Phylogenetic tree displaying the evolutionary relationships of the Urtica species. The numbers (in italics) are the bootstrap values, branch labels as the branch lengths and the bottom is the scale axis.

4.2 Extraction of crude extracts

The percentage yield for the stinging nettle aqueous extract, at 14%, was significantly higher than the organic extract (methanol: dichloromethane), at 9.6%.

4.3 Phytochemical composition of the crude extracts

The aqueous extract contained 3.75 ± 0.43 mg GAE/g dry sample while methanol: dichloromethane extract had 6.26 ± 0.276 mg GAE/g dry samples. The total flavonoid concentration of the methanol: dichloromethane (1.76 ± 0.315 mg quercetin/g dry sample) was nearly thrice that of water (0.3872 mg quercetin/g dry sample) (Table 7).

Table 7: Total phenolic and total flavonoid in stinging nettle aqueous and methanol: dichloromethane crude extracts

Extract	Total phenolic content (mg GAE/g)	Total flavonoid content (mg quercetin/g)
Aqueous	3.75 ± 0.43	0.3872
Methanol: dichloromethane	6.26 ± 0.276	1.76 ± 0.315

4.4 Raman Spectroscopy

4.4.1 Preprocessing

First, the acquired spectra were corrected for background fluorescence. Subtraction for background fluorescence can be achieved through instrumental (shifted excitation and time grating) or computational approaches (polynomial fitting, Fourier transformation and spectral shifting) (Zhao *et al*., 2007). For this study the Vancouver Raman Algorithm was used; its basis of operation is signal filtering and polynomial fitting (León-Bejarano *et al*., 2019).

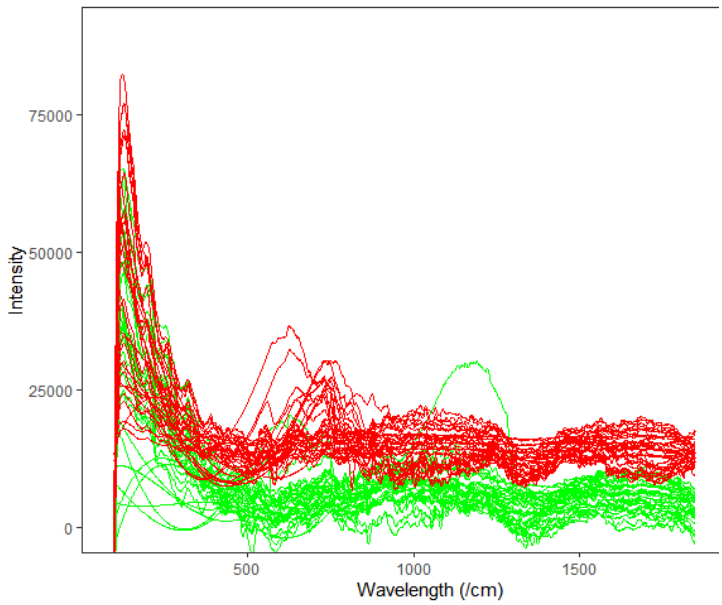

Figure 4: Pre-processed Raman spectra for aqueous and methanol: dichloromethane extracts of the stinging nettle. Key: green spectra for methanol: dichloromethane extract and red for aqueous extract.

Baseline correction estimates the unknown background by polynomial baseline fitting, Savitzky-Golay smoothing coupled with first or second order differentiation.

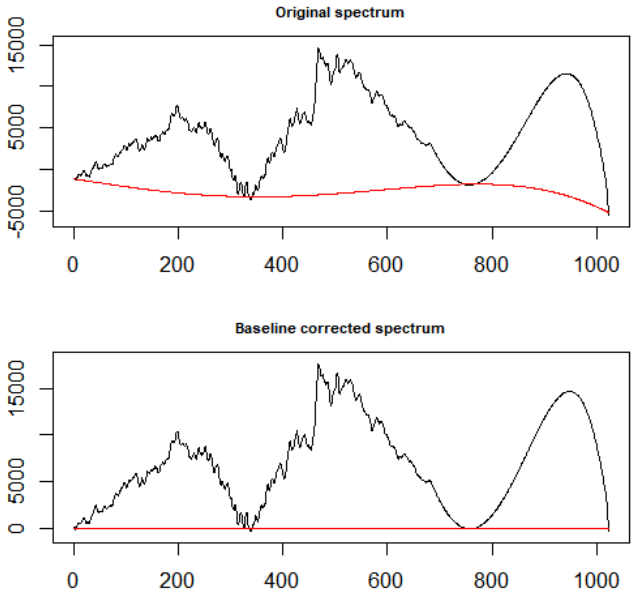

Figure 5: Baseline correction of the pre-processed spectrum of the stinging nettle crude extracts

The purpose of normalization is to adjust for sample and experimental variables in the sample.

Common methods used for this step are vector normalization and min-max normalization.

PCA aims at reducing the spectra data into the significant principal components that influence spectral variance whilst deducting the background noise. It is a key process for both preprocessing and feature extraction (Butler *et al*., 2016).

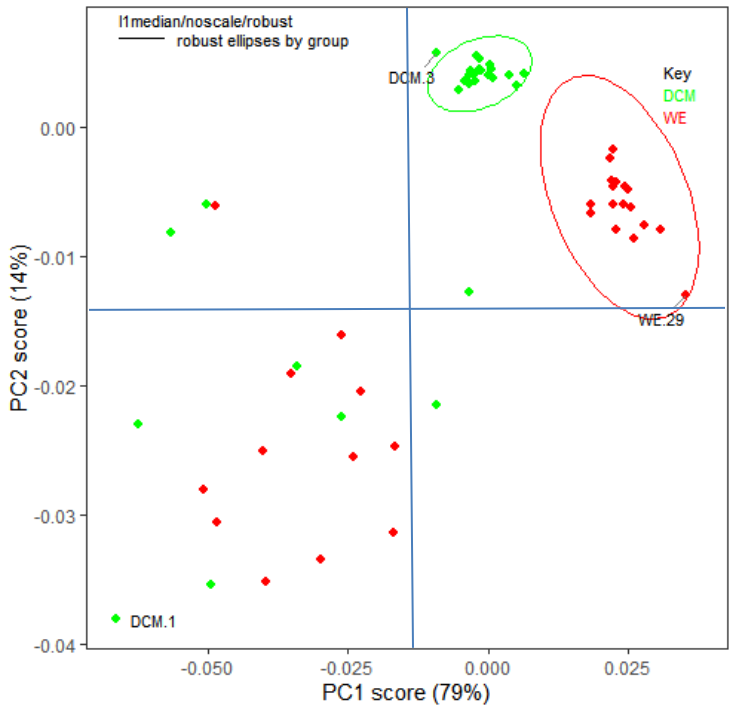

Figure 6: Principal Component Analysis of the stinging nettle crude extracts. Key: aqueous extract (WE in red) and methanol: dichloromethane extract (DCM in green)

4.4.2 Feature extraction of the processed spectra

Feature extraction consists of two strategies: feature construction (creating new feature from a dataset) and feature selection (marking out the existing features from the data). From the noise corrected data, feature selection involved identifying the compounds present. This was manually carried out by identifying peaks using OriginLab. OriginLab employs the following methods: window search, local maximum, first derivative, residual after 1st derivative for hidden peaks, second derivative for hidden peaks and Fourier self deconvolution (Origin, 2003) .

4.4.3 Compound identification

The Raman spectrum for the aqueous extract captured compounds ranging from 100 to 1800 cm^{-1} (Figure 8) in wavelength. The main characteristic bands were associated with caffeoylquinic acid and its derivatives (733 cm^{-1}), epigallocatechin (733 cm^{-1}) and ferulic (1241 cm^{-1}) due to ω(C=O) bonds (Eravuchira *et al*., 2012; Pompeu *et al*., 2018) (Table 8).

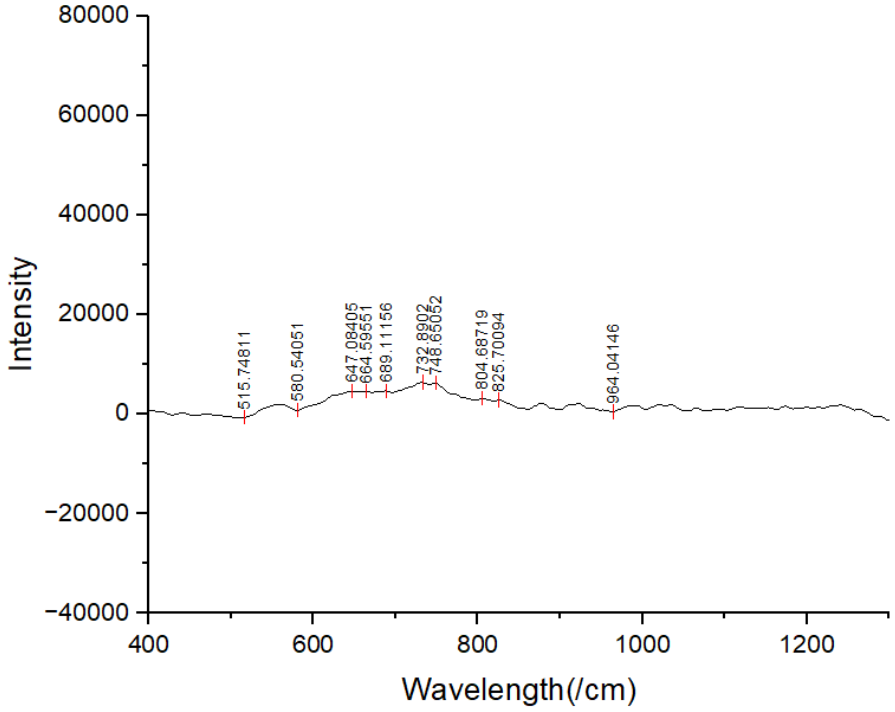

Figure 7: Raman spectra for stinging nettle aqueous extract ranging from -15,000 to 35,000 in intensity and 100 to 1800 cm-1 in wavelength.

Table 8: The vibrational assignment of the Raman band for the stinging nettle aqueous extract and its tentative compounds

Wavelength[a] (cm^{-1})	**Compound**	**Vibrational assignment**
$733_{(w)}$	3-CaffeoylQuinic acid	Cycl. [ω(C=O)]
$733_{(m)}$	5-CaffeoylQuinic acid	Cycl. [ω(C=O)]
$733_{(m)}$	3,4-diCaffeoylQuinic acid	Cycl. [ω(C=O)]
$733_{(m)}$	3,5-diCaffeoylQuinic acid	Cycl. [ω(C=O)]

$733_{(m)}$	4,5-diCaffeoylQuinic acid	Cycl. [ω(C=O)]
$733_{(m)}$	Epi-gallocatechin	-

Key: [a]***w***, weak; [a]***m***, medium. The assignments were obtained from (Eravuchira *et al*., 2012; Pompeu *et al*., 2018).

The Raman spectrum for the methanol: dicholoromethane extract captured compounds ranging from 200 to 1800 cm^{-1} (Figure 9) in wavelength. The main characteristic bands were associated with caffeoylquinic acid and its derivatives (733 cm^{-1}), epigallocatechin (733 cm^{-1}), genistein (1064 cm^{-1}), tannic (1091 cm^{-1}), quercetin (1175 cm^{-1}) and 4-hydroxybenzoic (1313 cm^{-1}) due to ω(C=O) bonds (Eravuchira *et al*., 2012; Pompeu *et al*., 2018) (Table 9).

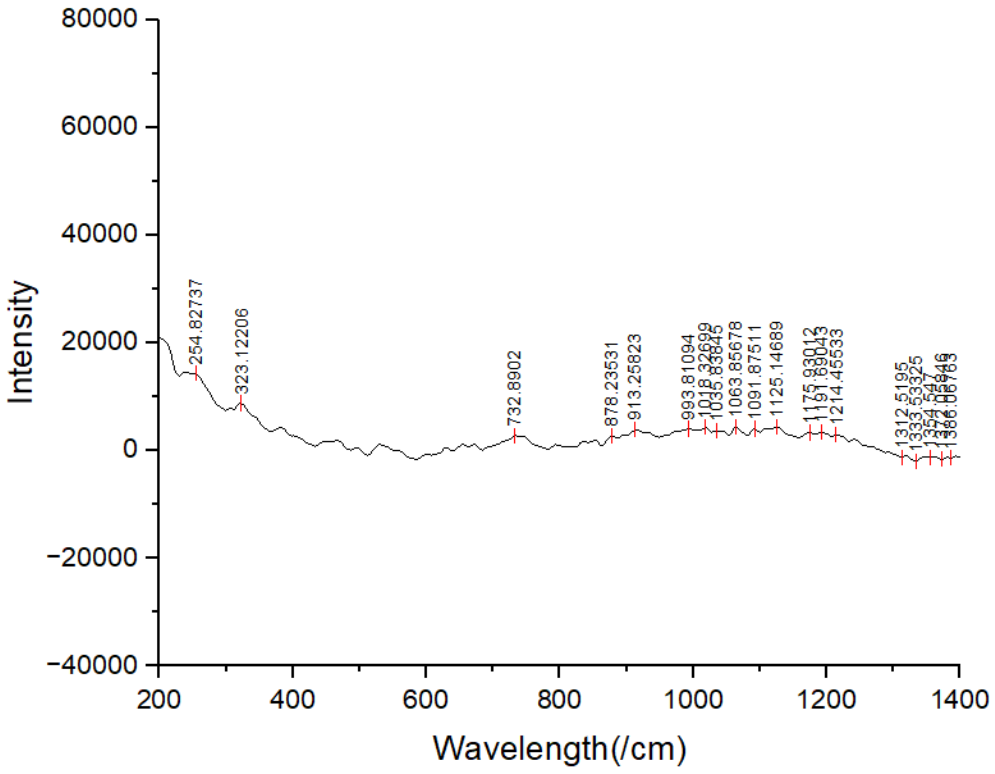

Figure 8: Average Raman spectra for the stinging nettle methanol: dichloromethane extract compounds ranging from -10,000 to 25,000 in intensity and 200 to 1800 cm-1 in wavelength.

Table 9: The vibrational assignment of the Raman band for stinging nettle methanol: dichloromethane extract and its tentative compounds

Wavelength[b] (cm^{-1})	Compound	Vibrational assignment
$733_{(w)}$	3-CaffeoylQuinic acid	Cycl. [ω(C=O)]
$733_{(m)}$	5-CaffeoylQuinic acid	Cycl. [ω(C=O)]
$733_{(m)}$	3, 4-diCaffeoylQuinic acid	Cycl. [ω(C=O)]
$733_{(m)}$	4,5- diCaffeoylQuinic acid	Cycl. [ω(C=O)]
$733_{(vs)}$	Epigallocatechin	-
$1064_{(vw)}$	Genistein	-
$1091_{(vw)}$	Tannic	-
$1175_{(w)}$	Quercetin	-
$1313_{(w)}$	4Hydroxybenzoic	-

Key: [b]***w***, weak; [b]***m***, medium; [b]***vs***, very strong; [b]***vw***: very weak The assignments were obtained from (Eravuchira *et al*., 2012; Pompeu *et al*., 2018).

4.5 Anti-inflammatory activity using the Carrageenan model of inflammation

The anti-inflammatory effect of the aqueous extract was found to be significantly higher than the methanol: dichloromethane extract. Towards the end of the experiment (2.5 hours), the paw volume of the aqueous extract was lower than the positive control (Table 10).

Table 10: Paw volumes for the stinging nettle aqueous, methanol: dichloromethane extracts and control groups

	Paw volumes for different extracts					
Extract	Initial	½ hour	1 hour	1.5 hour	2 hours	2.5 hours
Negative Control	12.04 ±0.483	13.32 ±0.215	13.06 ±0.252	13.96 ±0.354	15.04 ±0.770	15.08 ±0.595
Methanol: dichloromethane extract 750 mg/kg	9.36 ±0.285	11.08 ±0.344	12.96 ±0.462	13.16 ±0.457	14.64 ±0.858	15.44 ±0.655
*Aqueous extract 750 mg/kg	9.2 ±0.433	11.04 ±0.406	11.2 ±0.772	10.84 ±0.553	12.28 ±0.48	12.6 ±0.707
Positive control 10 mg/kg	9.52 ±0.205	11.32 ±0.338	12.12 ±1.23	12.16 ±1.23	12.44 ±0.934	13.08 ±1.055

Table 11: P values from multiple comparisons between various treatments using Tukey HSD test

Treatment A	Treatment B	Mean difference	Significance level
Aqueous extract	Positive extract	-.580	.913
	Negative extract	**-2.557***	**.042**
	Methanol :Dichloromethane	-1.580	.312
Positive extract	Water extract	.580	.913
	Negative extract	-1.977	.151
	Methanol :Dichloromethane	-1.000	.678
Negative extract	Water extract	2.557*	.042
	Positive extract	1.977	.151
	Methanol :Dichloromethane	.977	.694
Methanol :Dichloromethane extract	Water extract	1.580	.312
	Positive extract	1.000	.678
	Negative extract	-.977	.694

Values are expressed in paw volume values were expressed as mean, with ± as the standard deviation for a sample size of 6; *$p<0.05$ significant comparison to the negative control. At $p<0.05$, the stinging nettle aqueous extract was found to be statistically different from the negative control group.

*Significant differences between extracts were analysed using one-way ANOVA ($p<0.05$) followed by post hoc Tukey HSD test. The aqueous extract (750 mg/kg) showed a maximum of 22.35% inhibition in carrageenan induced inflammation after 1.5 hours. On the other end, the methanol: dichloromethane had negative trend in inhibition indicating minimal activity in anti-inflammation (Table 12).

Table 12: Percentage inhibition for the methanol: dichloromethane extract, aqueous extract and positive control by comparing with the negative control group

	Percentage inhibition at time h				
Extract	½ hour	1 hour	1.5 hour	2 hours	2.5 hours
Methanol: dichloromethane extract	16.81	0.766	5.73	2.66	-2.3
Aqueous extract	17.11	14.24	22.35	18.35	16.44
Positive control	15.01	7.19	12.89	17.28	13.26

4.6 Liquid chromatography mass spectroscopy (LCMS) analysis of the potent crude extract

The LC-MS analysis yielded 19 compounds that exhibited positive mass to ion (m/z) mode. Out of the nineteen, 10 were phenolic acids with their following respective peaks: m/z 248.13 for cinnamic acid, m/z 354.13 for p-coumaric acid/ 4 hydroxycinnamic acid, m/z 566.22 for caffeic acid, m/z 487.21 for sinapic acid, m/z 611.20 for ferrulic acid, m/z 348.12 for chlorogenic, m/z 342.17 for gallic acid , m/z 342.17 for syringic acid, at peak at m/z 308.08 for gentisic acid/dihydroxybenzoic acid and m/z 248.13 for protocatechuic acid. Furthermore, 9 were flavonoids with their following respective peaks: a m/z of 252.23 for quercetin, then m/z 350.14 for myricertin, m/z 413.27 for rhamnetin, m/z 224.20 for kaempferol hexoside or kaempferol 3-O hexoxyl hexoside, m/z 409.16 for kaempferol-3-rutinoside, m/z 467.19 for isorhamentin rutinoside or isorhamnetin-3-O-rutinoside and m/z 248.13 indicating the presence of epigallocatechin gallate.

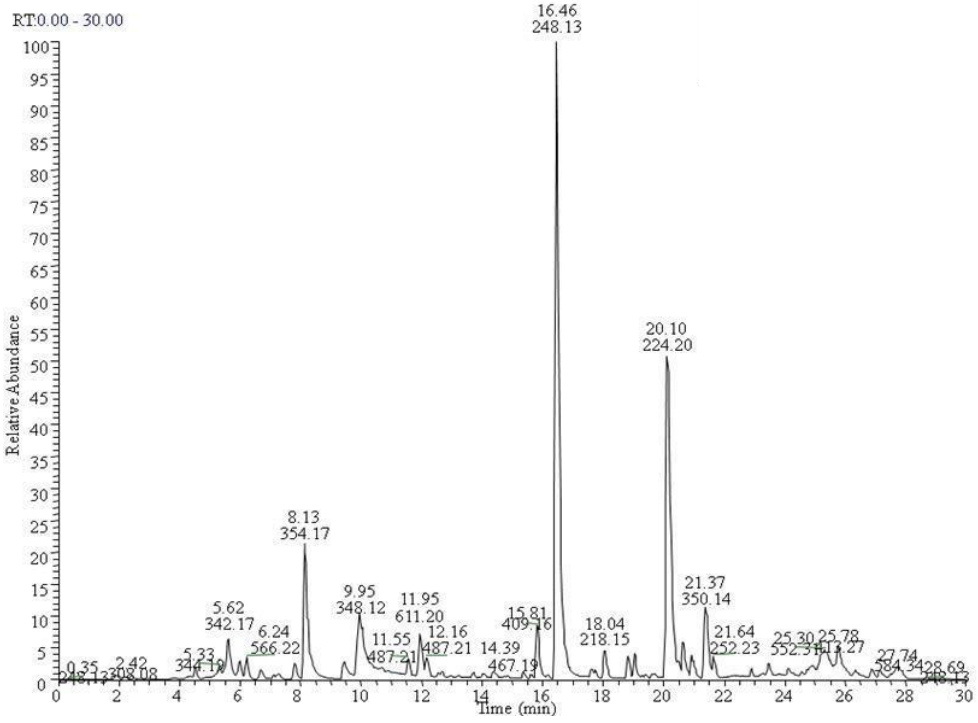

Figure 9: LC-MS spectra of methanol extract of the stinging nettle

Table 13: Identification and quantification of compounds (µg analyte/g extract) from stinging nettle methanolic extract using LC-MS

					Sample 1
	Retention time	**Name**	**Ion Mode**	**Mass to charge ratio (M+H)**	**Cong (µg/g)**
1	0.35	Protocatechuic acid	+ve	248.13	0.0288
2	2.42	Gentisic acid	+ve	308.08	0.0212
3	5.33	Syringic acid	+ve	342.17	0.0198
4	5.62	Gallic acid	+ve	342.17	0.0213
4	6.24	Caffeic acid	+ve	566.22	0.0373
5	8.13	P-Coumaric acid	+ve	354.17	0.1681
6	9.95	Chlorogenic acid	+ve	348.12	0.1654
7	11.55	Cinnamic acid	+ve	487.21	0.0702
8	11.95	Ferrulic acid	+ve	611.20	0.0567
9	12.16	Sinapic acid	+ve	487.21	0.0302
10	14.39	Isorhamentin rutinoside	+ve	467.19	0.2134
11	15.81	Kaempferol-3-rutinoside	+ve	409.16	0.0352
12	18.04	Quercetin rhamnoside	+ve	218.15	1.4785
13	20.10	Kaempferol hexoside	+ve	224.20	1.1314
14	21.37	Myricertin	+ve	350.14	0.0412
15	21.64	Quercetin	+ve	252.23	0.0276
16	25.30	Quercetin-3 glucoside	+ve	552.31	0.0137
17	25.78	Rhamnetin	+ve	413.27	0.0167
18	27.44	Quercetin	+ve	584.34	0.0313
19	28.69	Epigallocatechin gallate	+ve	248.13	0.0297

The calibration curve for quantitative determination of compounds, y=6008.9x – 5250.3, R2 = 0.9987.

4.7 Pairwise comparison between the active compounds and indomethacin

The efficacy of phytochemical compounds was compared against indomethacin, which is a standard NSAID drug. The mean pairwise score was 0.367 ± 0.015. The top compounds that were structurally similar to indomethacin, a non-selective inhibitor for cyclooxygenase 2 were isorhamnetin rutinoside, rhamnetin, kaempferol 3-rutinoside, quercetin rhamnoside, epigallocatechin and syringic acid.

Table 14: Comparing similarity between the stinging nettle polyphenols in the methanol extract and indomethacin

Compound	Score
Protocatechuic acid	0.35
Gentisic acid/dihydroxybenzoic acid	0.327
Syringic acid	0.404
Gallic acid	0.315
Caffeic acid	0.321
P-Coumaric acid/ 4 hydroxycinnamic acid	0.275
Chlorogenic acid	0.367
Cinnamic acid	0.18
Ferrulic acid	0.385
Sinapic acid	0.396
Isorhamentin rutinoside (Isorhamnetin-3-O-rutinoside)	0.452
Kaempferol-3-rutinoside	0.419
Quercetin rhamnoside (Quercetin 3-rhamnoside)	0.414
Kaempferol hexoside (kaempferol 3-O hexoxyl hexoside)	0.391
Myricertin	0.368
Quercetin	0.368
Quercetin-3 glucoside	0.391
Rhamnetin	0.429

Epigallocatechin gallate	0.414

Note: In the table, column 'score' is the pairwise score. It is a quantitative measure of the relatedness between a compound and indomethacin.

4.8 Molecular docking

The average binding energy for docking was -7.12 kcal/mol; molecules with the good binding scores were: flavonoids (quercetin, rhamnetin, quercetin rhamnoside, epigallocatechin gallate) and phenolic acid (chlorogenic acid). Quercetin attained the highest docking score while protocatechuic had the lowest score (Table 15).

Table 15: Molecular docking results of the stinging nettle polyphenols and indomethacin

Ligand	**Binding energy (kcal/mol)**	**Number of modes**
Protocatechuic acid	**-3.8**	**9**
Gentisic acid/dihydroxybenzoic acid	-6.3	9
Syringic acid	-6.1	9
Gallic acid	-6.6	9
Caffeic acid	-7.2	9
P-Coumaric acid/ 4 hydroxycinnamic acid	-7.0	9
Chlorogenic acid	**-8.3**	**9**
Cinnamic acid	-6.8	9
Ferrulic acid	-7.1	9
Sinapic acid	-6.7	9
Isorhamentin rutinoside (Isorhamnetin-3-O-rutinoside)	-6.9	2
Kaempferol-3-rutinoside	-7.4	3
Quercetin rhamnoside (Quercetin 3- rhamnoside)	**-8.1**	**7**

Kaempferol hexoside (kaempferol 3-O hexoxyl hexoside)	-6.9	6
Myricertin	-7.9	9
Quercetin	**-8.6**	**9**
Quercetin-3 glucoside	-7.1	8
Rhamnetin	**-8.5**	**9**
Epigallocatechin gallate	**-8.0**	**9**

Note: in the table, binding energy is the intermolecular force holding the receptor-ligand complex. The lower the binding energy, the higher the docking scores. The number of modes is the number of poses when the ligand orients to the binding site of the receptor during the flexible ligand fixed receptor docking.

Comparing quercetin and protocatechuic with indomethacin docked to COX-2 revealed molecular interactions (Table 16). This was further elaborated by 2D and 3D representations (Figure 12 and Figure 13). Quercetin has similar interactions with indomethacin with hydrogen bonding at Serine 530 and Van der Waal forces at Tyrosine 355, Tyrosine 348 and Phenylalanine 518 amino acid residues. Moreover, it has distinct Pi-sulphur bond by interacting with methionine 522. For protocatechuic, it is devoid of hydrogen, Pi-Sulpur and Pi-Pi bonds but at Phenylalanine 381, it has Pi-alkyl bond similar to indomethacin.

Table 16: Interaction types and amino acids involved in the inhibition of COX-2 by quercetin, indomethacin and protocatechuic

Interaction type	Van der Waal forces	Hydrogen	Pi-sulphur	Pi-Pi	Pi –sigma	Pi-alkyl	CH
Quercetin	Tyrosine 348 Valine 349 Tyrosine 355 Histidine 90 Arginine 513 Glycine 192 Alanine 516 Isoleucine 517 Phenylalanine 518 Leucine 384 Glycine 526 Phenylalanine 381	Serine 530 Tyrosine 385	Methionine 522	Valine 523 Leucine 352	Tryptophan 387	Valine 523	-
Indomethacin	Valine 116 Tyrosine 355 Leucine 352 Tyrosine 348 Glycine 520 Arginine 120 Serine 353 Phenylalanine 518 Leucine 359	Serine 530	-	Tyrosine 385 Tryptophan 387	Valine 349 Alanine 527	Phenyl 381 Methionine 522 Leucine 384, 352 Valine 523	Phenylalanine 518 Tyrosine 348
Protocatechuic	Asparagine 375 Phenylalanine 529 Glycine 533 Serine 530 Glycine 227	-	-	-	Phenylalanine 209	Phenylalnine 381 Leucine 534 Valine 228 Isoleucine 337 Phenylalanine 209	-

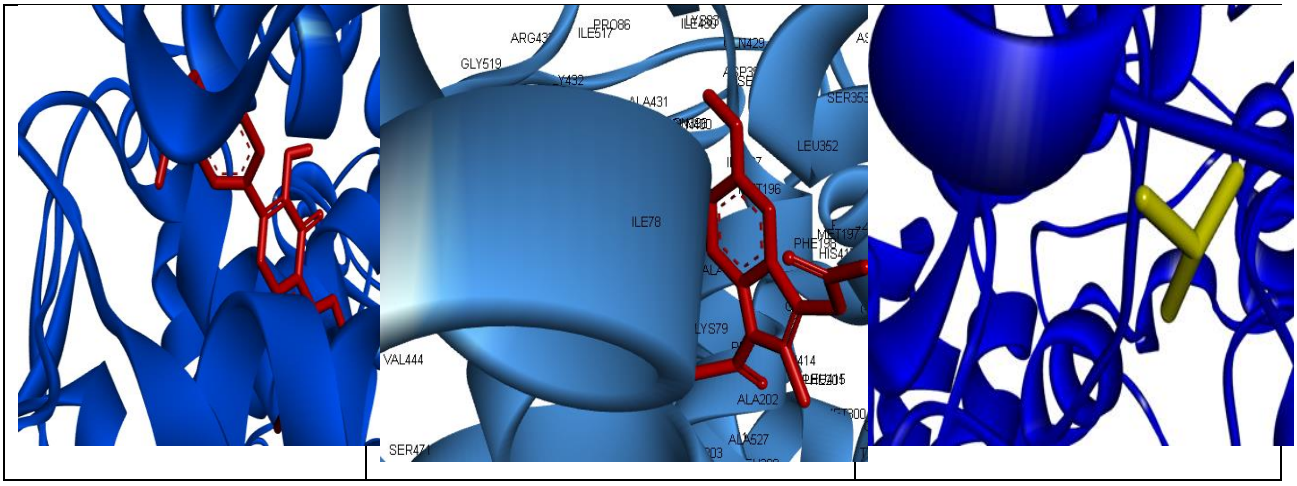

Figure 10: 3D molecular interactions between cyclooxygenase and quercetin (right), indomethacin (center) and protocatechuic (left).

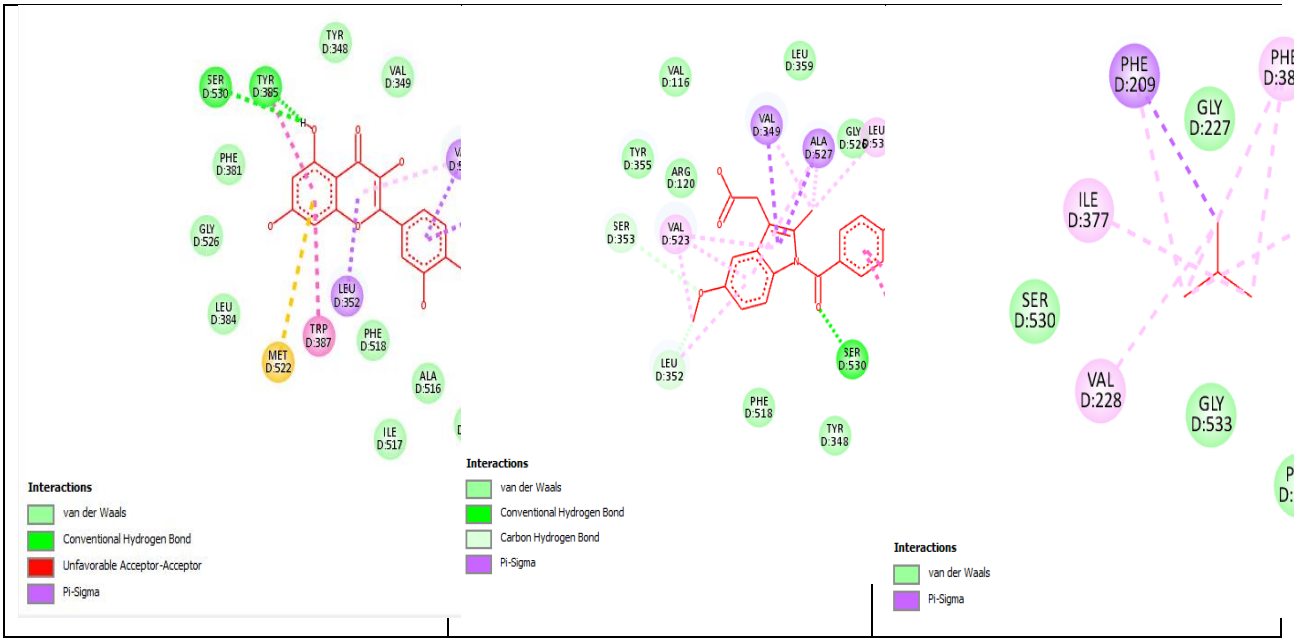

Figure 11: 2D molecular interactions between cyclooxygenase and quercetin (right), indomethacin (center) and protocatechuic (left).

To study the relationship between binding energy and similarity score from the pairwise test, a correlation analysis was performed. From the scatter plot, there was a negative correlation between the similarity score and binding energy i.e. the lower the binding energy the higher the similarity score (Figure 13).

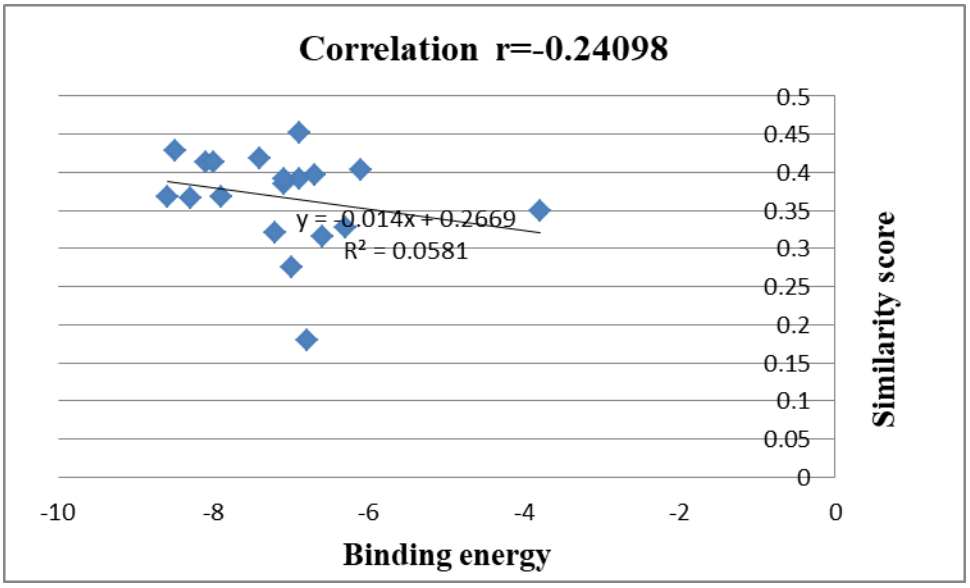

Figure 13: Correlation analysis between similarity score (y axis) and binding energy (x axis) has a negative coefficient, -0.24098, revealing a negative relationship between similarity score and binding energy.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The use of natural products or medicinal plants is considered a key form of complementary medicine (Wachtel-Galor & Benzie, 2011). According to the National Center for Complementary and Alternative Medicine (NCCAM), complementary medicine connotes practices, products and systems that can be integrated with conventional medicine (Langhorst *et al*., 2015). The rational for its use is due to the fact that medicinal plants have pharmacological responses that are comparable to conventional drugs such as anti-inflammatory, analgesic, as well as hemostatic and immune-stimulatory (Vickers & Zollman, 1999). The stinging nettle is an alimurgic considered not only as a weed but also an edible plant (Bandiera *et al*., 2016) with potential pharmacological applications. Therefore, this study aimed at deciphering its pharmacological characteristics as an anti-inflammatory agent.

The stinging nettle plant sample was identified based on DNA bardcoding using *rbcL* and ITS markers. From the BLASTN analysis of both ITS and *rbcL* marker sequences, the sample was identified as *Urtica sp*. This identified species corroborates with the study done by Wu and colleagues on the molecular phylogeny of nettle (Wu *et al*., 2013). Notably, the ITS marker, captured *Urtica massaica* and *Urtica simensis* among other species, in the BLAST analysis. *Urtica massaica* has been documented in Kenya (Njoroge, 2012) while *Urtica simensis* grows indigenously in Ethiopia (Keflie, 2020). According to the Consortium for the Barcode of Life (CBOL) Plant Working Group, *rbcL* is considered a standard marker in DNA plant barcoding due to its universality, comparability and easy amplification (CBOL Plant Working Group, 2009), while ITS is used a supplement barcode (Kang *et al*., 2017).

Phylogenetic tree was constructed to determine the relationship between the different species based on the combination of chloroplast and nuclear datasets. The observations in the largest clade are consistent with a previous study by Grosse-Veldmann and colleagues. Using nuclear ribosomal internal transcribed spacer region (rITS) and three plastid markers, the species *U. massaica Milbr* and *U. simensis* which are found in Africa and *U. atrovirens*, *U. bianorii* and *U. dioica* subsp. Cypria in Mediterranean formed a clade. This clade was found to be sister clade to

U. dioica of Eurasia (Grosse-Veldmann *et al*., 2016). Despite the presence of relatedness between *U. bianorii* and *U. simensis*, their analysis of absolute genome size differs (Rejlová *et al*., 2019).

Prior to preparation of crude extracts, the sample leaves were air dried to prevent the degradation of polyphenols (Tsao, 2010). The mode of preparation affects the bioavailability of the required compounds (Gibson *et al*., 2006). Among the factors that are critical during the preparation stage are the solvent type and system of extraction. With reference to the solvents used in this study, water is the most polar followed by methanol then dicholoromethane. Therefore, water and polar organic solvents capture most of the polyphenols which are hydrophilic in nature such as glycosides, oligomers and aglycones. The extraction systems used in this study were hot water extraction and cold percolation. They require longer extraction times and high amounts of solvent; this can be solved by using modern extraction methods such as microwave assisted extraction, pressurized liquid extraction and super critical fluid extraction (Tsao, 2010; Zhang *et al*., 2018).

Phytochemicals are plant secondary metabolites with a wide scope of biological effects including antimicrobial, anticancer, anti-inflammatory, anti-allergic among others (Neilson *et al*., 2017). In this study, significantly higher phenolic content was observed in methanol: dichloromethane extract. The high level of phenolic content in methanol: dichloromethane extracts might contribute to its therapeutic/pharmacological potential. These results are in agreement with previous studies that confirmed the presence of high phenolics in organic extracts obtained from leaf samples of the genus *Urtica* (Chahardehi *et al*., 2009). Comparison with flavonoid content of methanolic extracts of *Urtica dioica*, at 0.0081- 0.0180 mg quercetin/g, revealed a lower concentration (Begić *et al*., 2020).

The phenolic content was higher than that of flavonoid. Conversely, aqueous extracts of *Urtica dioica* demonstrated equal amounts of phenolics and flavonoids. Variation of flavonoid content could be due to different standards used for measuring flavonoids. For instance, the flavonoid of *Urtica dioica* was found to be 133.916 ± 12.006 using catechin as the standard which is higher than that reported by Fattahi *et al*., (2014). *Urtica simensis* contains 2.18–4.84 mg gallic acid and 1.35–4.46 mg catechin per gram of dry sample (Bayba *et al*., 2020). Other reports revealed 15.75

to 22.67 mg and 6.89 to 9.03 mg of gallic acid and catechin equivalent per g of dried leaves respectively (Seifu *et al*., 2017).

From the Carrageenan model of inflammation aqueous extracts from stinging nettle leaves exhibited anti-inflammatory activity. The inflammation observed in the carrageenan model is associated with the activation of cyclooxygenases and lipoxygenases. The cyclooxygenases are regulated by the transcription factor, NF-κB (Gepdiremen *et al*., 2004; Patil *et al*., 2019). The development of inflammation is in a biphasic fashion. The initial phase commences immediately after the injection and disappears within 50 minutes. Thereafter, the second stage commences at 50 minutes and remains through till the end. It was hypothesized that the initial inflammation is due to neurotransmitters, histamine and serotonin action which perform increased permeability of the blood vessels. As for the second phase, the delayed inflammation is attributed to the effect of prostaglandins on the release of bradykinins which mediate the vascular responses (Gepdiremen *et al*., 2004). Moreover, the second phase has been reported to be more sensitive to anti-inflammatory drugs (Boominathan *et al*., 2004).

The aqueous extract of the stinging nettle and the positive control exhibited the maximum inhibition at the second phase, 22.31% at 1.5 hours and 17.28% at 2 hours, respectively. This suggests that both of them are capable of blocking prostaglandins release in the second phase at their optimum level. Consequently, the water extracts of the stinging nettle performed anti-inflammatory functions by inhibiting cyclooxygenases through inhibition of prostaglandins synthesis which is regulated by the NF-κB. Interestingly, the aqueous extract exhibited a higher inhibition rate than the indomethacin, positive control. This behavior can be either attributed by the additive effect or synergistic effect of the active compounds in the aqueous stinging nettle. Additive effect is the sum potency of the different compounds while synergistic is whereby the exhibited effect is greater than the sum effect of the individual compounds. Synergism leads to enhanced bioavailability and targets various inflammatory pathways (Zhang *et al*., 2019)

A hybrid approach was used in the identification of the poly-phenolic compounds through Raman spectroscopy and LC-MS. Polyphenols or phenolic compounds are a conglomerate of subclasses: phenolic acids (hydroxyl-cinnamic acid and hydroxyl-benzoic acid), flavonoids

(flavone, flavonol, flavan-3-ol, iso-flavone, flavanones, anthocyanids and anthocyanins) and other secondary metabolites (lignans, stillbenes, tannins, xanthones, lignins, chromones and anthroquinones) (Martinez *et al*., 2017). In this study, the Raman spectra captured from 733 to 1241 cm^{-1} was assigned to different polyphenols in both the aqueous and methanol: dichloromethane extracts. In other investigations, hydroxycinnamic acids (part of phenolic compounds) were observed from 1050-1450 cm^{-1} or 1000-1600cm^{-1} in wine samples (Deneva *et al*., 2019). Krysa and collegues attempted to compile the spectra of different flavonoids whereby at 1160-1250 cm^{-1}, the bands were assigned to flavones, isoflavones and flavonols (Krysa *et al*., 2022). This is consistent with the results since at band 1160-1250 cm^{-1}, quercetin, a flavonol, was identified.

Liquid chromatography – mass spectroscopy has the ability to identify the concentration level of each compound in the crude extracts. The results from aqueous extract from this study are harmonious with previous findings for *Urtica dioica* that identified chlorogenic acid, quercetin-3-O rhamnosyl glucoside / rutin and iso-quercetin (Francišković *et al*., 2017; Orčić *et al*., 2014); rutin and chlorogenic acid (Otles & Yalcin, 2012). Another study found that 2-O-caffeoyl malic acid, chlorogenic acid, and rutin are the most abundant (Vajić *et al*., 2015). Phytochemical analysis of hydromethanolic extracts of *Urtica massaica* unmasked the presence of anthocyanins, flavonoids, saponosides and tannins (Nahayo *et al*., 2008).The compounds identified in the anti-inflammatory aqueous extract by LC-MS were subjected to in-silico analysis of clustering, pairwise testing and molecular docking.

The docking results identified flavonoids (quercetin, rhamnetin, quercetin rhamnoside, epigallocatechin gallate) and hydroxycinnamic acid (chlorogenic acid) as the potent anti-inflammatory compounds in the crude extract. In the literature, flavonoids and hydroxyl-cinnamates are water soluble and possess anti-inflammatory properties proven by rodent assays (Paumgartten *et al*., 2022). This is in agreement with the findings from this study.

5.2 Conclusions

i. The aqueous and methanol: dicholoromethane extracts are rich in phenolics and flavonoids.
ii. The aqueous extracts contain anti-inflammatory properties using the Carrageenan induced paw edema model of inflammation
iii. Based on in silico docking studies, the active compounds responsible for anti-inflammtion are flavonoids (quercetin, rhamnetin, and quercetin rhamnoside and epigallocatechin gallate) and phenolic acid (chlorogenic acid).

5.3 Recommendations

The recommendations from this study are;

i. Isolation and purification of compounds from stinging nettle crude extract and screening for their anti-inflammatory activity on not only *in-vivo* but also *in-vitro* basis
ii. The anti inflammation activity of the stinging nettle by targeting other inflammatory pathways such as NF-kB pathway

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APPENDIX

Appendix I

Codes for Mr. Bayes

i. Execute alignment.nexuss (name of .nexus file)
ii. lset nst = 6 rates = invgamma
iii. mcmc ngen = 10,000 samplefreq = 10 (independent runs were done till the standard deviation becomes lower than 0.01)
iv. sump burnin = 250
v. sumt burnin = 250

Appendix II

```
library("ChemoSpec")

#read the csv file and identify the number of columns

data <- read.csv("data_dcm_we.csv")

is.data.frame(data)

ncol(data)

# Reading the csv file as a matrix data file stored in the working directory

rawspec <- matrix2SpectraObject(gr.crit = c("DCM","WE"),

                                gr.cols = c("green","red"),

                                freq.unit = "Wavelength (/cm)",

                                int.unit = "Intensity",

                                descrip = "data_dcm_we",

                                in.file = "data_dcm_we.csv",

                                out.file = "data_dcm_we",

                                chk = TRUE,
```

```
                    sep = ",",
                    dec = "."


# Summarizing the data
sumSpectra(rawspec)
#creating a title
title<-expression(bolditalic(Raman)~bold(Spectra))
# Plotting all the spectra
plotSpectra(rawspec,
        main = title,
        which = c(1:60),
        yrange = c(0,90000),
        xlim =c(0,1850),
        offset = 300,
        showGrid = FALSE,
        lab.pos = 2000)
#abline(h=600,v=30)
#installing baseline
install.packages("baseline")


#baseline correction
```

```
base_raw <- baselineSpectra(rawspec,
                    int = FALSE,
                    method = "modpolyfit", retC = TRUE)


# normalization
spec3<-normSpectra(rawspec)
plotSpectra(spec3,
          main = title,
          which = c(2,5,8),
          yrange = c(0,250),
          xlim =c(249,1300),
          offset = 100,
          lab.pos = 1280)
#PCA analysis
pca<-r_pcaSpectra(spec3, choice = "noscale")
plotScores(spec3, pca,
         main ="Stinging nettle extracts", pcs = c(1,2), ellipse = "rob", tol = 0.01)
abline(h =0,v=0)
# getting the PC scores for each PC from PCA attributes
attributes(pca)
pca_scores <-pca[["x"]]
```

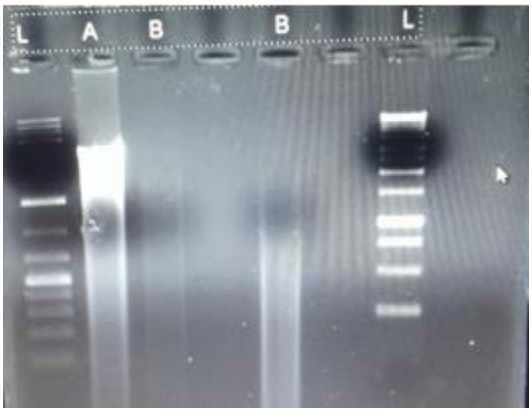
```
pca_scores


#potential outliers
diagnostics<-pcaDiag(spec3,
                     pca,
                     pcs = 2,
                     #quantile = 0,95,
                     plot = c("OD","SD"))


#number of pcs needed to describe data
plotScree(pca,
          style = "alt"
)
# From the plot, we need 5 PCs to reach the 95% confidence interval
# determine which variables affect the scores
plotLoadings(spec3, pca, loads = c(1,2))
```

Appendix III

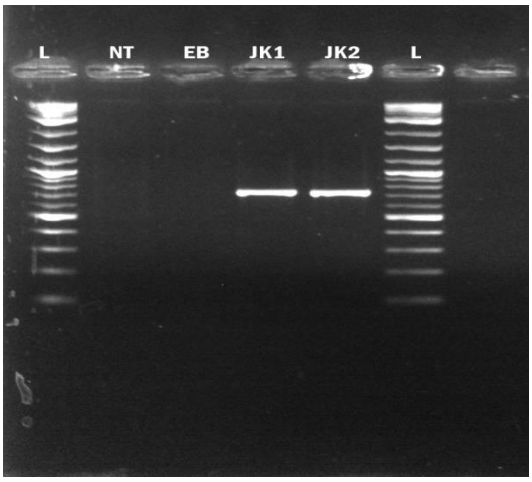
Pair wise comparison analysis:

https://colab.research.google.com/drive/1ASMTabYrY3LRZiaXTfZ1ZEaLB8MEuUp0?usp=sharing

Appendix IV



Gel electrophoriesis analysis for the genomic DNA collected



Gel electrophoriesis analysis for the PCR products

Appendix V

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

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Direct Line. 4448648

REF: FVM BAUEC/2021/314

Ms. Wambui Jacquiline Emmah.
Dept. of Biochemistry
University of Nairobi
20/09/2021
Dear Jacquiline,

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

Phytochemical Screening of Anti-inflammatory compounds of the stinging nettle (*Urtica massaica mildbr*).

Wambui Jacquiline Emmah I56/34968/2019

We refer to your MSc. proposal submitted to our committee for review and your application letter dated 11th September 2021.We have reviewed your application for ethical clearance for the study. The number of rats and the Carrageenan model of inflammation used in evaluating the anti-inflammatory activity of *Urtica massaica mildbr* meets the minimum standard of the Faculty of Veterinary medicine ethical regulation guidelines.

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

Yours sincerely,

Kaluwa

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