Evaluation of the anti-inflammatory activities of 70% ethanol leaves extract and solvent fractions of *Zehneria scabra* (L.f.) Sond (Cucurbitaceae) in rodents

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A thesis submitted to the Department of Pharmacology, School of Medicine, College of Health Sciences, Addis Ababa University in partial fulfillment of the requirements of Master of Science in Pharmacology.

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ABSTRACT

Evaluation of the anti-inflammatory activities of 70% ethanol leaves extract and solvent fractions of *Zehneria scabra* (L.f.) Sond (Cucurbitaceae) in rodents

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Zehneria scarba is one of the medicinal plants used in folkloric medicine of Ethiopia for years to treat various inflammatory disorders. Even though the anti-inflammatory activity of the crude hydroalcoholic extract of the leaves of this plant was evaluated against acute model of inflammation, no further work has been done to screen the anti-inflammatory activities of the crude extract and solvent fractions on subacute and chronic models of inflammation. The present study was, therefore, aimed to further validate the anti-inflammatory activity of crude 70% ethanol leaves extract (70EE) against a sub-acute model and further evaluate the solvent fractions (AF, BF and CF) in an acute (carrageenan induced paw edema), sub-acute (formaldehyde induced arthritis) and chronic (cotton pellet induced granuloma) inflammatory models. The 70EE was first prepared by maceration, and the fractions were obtained by sequential partitioning with chloroform and n-butanol from the aqueous suspension of crude 70EE. The test groups, then, received 100, 200 and 400 mg/kg of the crude 70EE or the fractions (AF, BF, and CF) at the same dose levels, whereas positive controls received aspirin (200mg/kg) or dexamethasone (0.5mg/kg) and negative controls received vehicle (2% tween 80 or distilled water, 10 ml/kg). All tested doses of the crude 70EE showed a significant inhibition of formaldehyde induced arthritis at the 10^{th} day of treatment; on which the 400mg/kg dose showed the maximum anti-arthritic effect (%A = 60.5; $p < 0.001$). In the carrageenan induced paw edema all the three fractions showed a statistically significant effect, in fact, with different onset and magnitude. In this model the AF was found to be the most active fraction, and the 400mg/kg dose demonstrated the maximum effect (%A = 76.25; $p < 0.001$) at 5h post induction which is much better than the effect of aspirin at the dose employed. The overall order of efficacy in inhibiting the exudative component of carrageenan induced paw edema was found to be AF > BF > CF. The AF was also found to be the most

active fraction in inhibiting the exudative component of chronic inflammation in the cotton pellet induced granuloma model, where the maximum effect (%A = 43.10, $p < 0.001$) was exhibited by dose of 400mg/kg. The AF was also the most active fraction in inhibiting formaldehyde induced arthritis, in which the BF and CF relatively showed a comparable effect throughout day 4-10. On the contrary, in the cotton pellet induced granuloma model, the CF was found to be the most active fraction in inhibiting the proliferative and granulomatous component of chronic inflammation, and the overall order of effectiveness was found to be CF > AF > BF. Besides, 400mg/kg of CF demonstrated the maximum inhibition of granuloma formation (%A = 55.52; $P < 0.001$). The phytochemical analysis revealed differential distribution of secondary metabolites into the three fractions which either singly or in concert appeared to be responsible for the observed effects. The data obtained from the present study collectively indicate that the extract and fractions of leaves of *Z.scabra* possessed a significant anti-inflammatory activity, upholding the folkloric use of the plant.

Key Words: Anti-inflammatory activity, *Zehneria Scarba*, caregeenan induced paw edema, formaldehyde induced arthritis, cotton pellet granuloma

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ABBREVIATIONS AND ACRONYMS

5-LOX	5-lipoxygenase
70EE	70% v/v ethanol extract
AA	Arachidonic Acid
AAU	Addis Ababa University
AF	Aqueous fraction
AP-1	Activating Protein-1
BF	Butanol fraction
CF	Chloroform fraction
cGCR	Cytoplasmic Glucocorticoid Receptor
COX	Cyclooxygenase/ prostaglandin G/H synthase
CysLTs	Cysteinyl Leucotriens
ECs	Endothelial Cells
EPHARM	Ethiopian Pharmaceuticals Manufacturing
FDA	Food and Drug Administration
GC	Glucocorticoid
GI	Gatro-Intestinal
ICAM1	Intercellular Adhesion Molecule-1
IL	Interleukin
LTs	Leucotriens
MCP	Monocyte Chemotactic Protein
NF-κB	Nuclear Factor -*κappa*B
nGRE	Negative Glucocorticoid Receptor Response Element

NLRs	NOD-Like Receptors
NOD	Nucleotide-binding Oligomerization Domain
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
P.O.	Per Os (by mouth)
PAMPs	Pathogen-Associated Molecular Patterns
PGs	Prostaglandins
PLA2	Phospholipase-A2
PRRs	Pattern Recognition Receptors
PUFAs	Poly unsaturated fatty acids
TLRs	Toll-like receptors
TNF-α	Tumor Necrosis Factor Alpha
TXs	Thromboxanes
VCAM1	Vascular Cell-Adhesion Molecule-1
VLA4	Very Late Antigen- 4
Z. scabra	*Zehneria scabra*

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1. INTRODUCTION

1.1 Overview of Inflammation

The word inflammation comes from the Latin *inflammare* (to set on fire) (Scott *et al*., 2004). Inflammation is one of the non-specific physiological defensive responses that begin after cellular injury, which may be caused by microbes, physical agents (burns, radiation), chemicals (toxins, caustics), necrotic tissue and/or immunological reactions (Villarreal *et al*., 2001).

On the basis of its plainly visible manifestations, the ancients characterized inflammation by five cardinal signs, namely redness (rubor), swelling (tumour), heat (calor), pain (dolor) and loss of function (functio laesa) (Patrekar *et al*., 2014). The first four of these signs were named by Celsus in ancient Rome (30 - 38 B.C.) and the last by Rudolph Virchow's (1821-1902) in his Cellular Pathology (1858) (Weissmann, 2010).

Hence, in its genesis inflammation was defined by a mix of clinical signs and symptoms and not by its particular pathophysiology. Such definition of inflammation by its cardinal signs is obsolete and unsuitable for guiding adequate therapeutic strategies (Stankov, 2012), as in most cases, the cellular processes that underlie inflammation happen at a subclinical level and don't offer ascent to any warmth, redness, swelling, or agony. Furthermore, areas of swelling, pain, and tenderness may have a wide variety of non-inflammatory causes (Scott *et al*., 2004).

Today it is perceived that inflammation is much more complex than might first appear from the simple description by its cardinal signs and symptoms, and it is more completely described by its pathological definition as a complex reaction to injurious agents, that consists of vascular responses, migration and activation of leukocytes, and systemic reactions (Spicer *et al*., 2011).

1.2 Types of inflammation

Inflammation is a normal defense mechanism that is designed to limit invasions and damage after injury, a process which has been essential for the survival of Homo sapiens in the absence of medication such as antibiotics (Boer, *et al*., 2012). It initiates pathogen killing as well as tissue repair processes and helps to restore homeostasis at infected or damaged sites (Calder, 2010). Despite the fact that such self-limiting acute inflammation is a beneficial response, deregulated and chronic inflammation is a phenomenon that may lead to a host of debilitating chronic pathologies (Munari and Cervera, 2015).

1.2.1 Acute inflammation

Local injuries of all sorts (e.g., infection or tissue damage) provoke an immediate, acute response which is basically the same whatever the agent is and hence in essence, a stereotyped and nonspecific response (Cruvinel *et al*., 2010). Acute inflammation is such a nonspecific and short lived response which occurs as long as the injurious stimulus is present and ceases once the stimulus has been removed, broken down, or walled off by scarring (fibrosis) (Vetriselvan *et al*., 2013).

The initiation of acute inflammation involves expression of two families of pathogen-associated molecular pattern (PAMP) receptors of ECs including membrane bound Toll-like receptors (TLRs) and cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These Pattern Recognition Receptors (PRRs) are able to sense components of exogenous microbes, toxins, cytoplasmic pathogen associated molecular patterns (PAMPs), as well as harmful endogenous DAMPS (Mai *et al*., 2013; Cantu *et al*., 2013; Santoni *et al*., 2015).

The activation of TLRs and NLRs by their respective ligands then activates downstream signaling pathways which finally converge on activation of pro-inflammatory transcription factors, such as nuclear factor-*kappa*B (NF*κ*B), activator protein-1 (AP-1) or interferon regulatory factors (IRFs) (Oviedo-Boyso *et al*., 2014). Activation of these pro-inflammatory transcription factors leads to expression of a number of genes that are

involved in the acute inflammatory reaction such as the production of growth factors, adhesion molecules, inflammatory enzymes, and pro-inflammatory cytokines and chemokines including TNF-α, IL-1β, IL-6, IL-8, IL-12 which altogether orchestrate the inflammatory response (Karatzis, 2005; Mogensen, 2009). On the early phase of inflammation these mediators act primarily on the microcirculation, with two main effects: a) vasodilation and exudation of fluid (the vascular response), and b) recruitment and extravasation of white blood cells (the cellular response) (Verma *et al*., 2012).

i. Vascular response and mediators of acute inflammation

The process of acute inflammation is initiated by the blood vessels local to the injured tissue (Vetriselvan *et al*., 2013). Vasodilation of precapillary arterioles increases blood flow to the injured tissue, a condition known as hyperemia, while vasoconstriction of postcapillary venules increases capillary bed hydrostatic pressure, further potentiating exudation of plasma proteins and leukocytes in edema formation (Rubin and Strayer, 2014). These vascular responses are mediated by the release of specific vasoactive mediators such as nitric oxide, histamine, and eicosanoids (Larsen and Holt, 2000).

Eicosanoids (PGs, TXs, and LTs) released by cells recruited to or present at the site of tissue injury, are key mediators of inflammation (Massoumi and Sjölander, 2007; Calder, 2010). They contribute to the development of cardinal signs of acute inflammation due to their effect on the microvasculature to cause vasodilation/constriction. They are generated from 20 carbon PUFAs by the metabolic processes summarized in Fig.1 (Ricciotti and FitzGerald, 2011). Activation of phospholipases (e.g. cytosolic PLA2) by noxious stimulis such as bacterial lipopolysaccharides and pro-inflammatory cytokines releases free AA from the cell membrane phospholipid pools, the initial rate-limiting step in eicosanoids production (Zeldin, 2001; Rocha and Carvalho, 2005).

Arachidonic acid will be then acted upon by PGG/H synthases, colloquially known as COXs, that catalyze the oxidation to PGG2 and a two-electrone reduction of PGG2 to PGH2 (Parente and Perretti, 2003). The unstable PGH2, then serves as a substrate for tissue-specific isomerases (prostaglandin synthase enzymes) in different cells, which are

responsible for the production of the five principal bioactive prostanoids PGE2, PGF2α, PGD2, PGI2 (prostacyclin), and TXA2 (thromboxane) (Hata and Breyer, 2004).

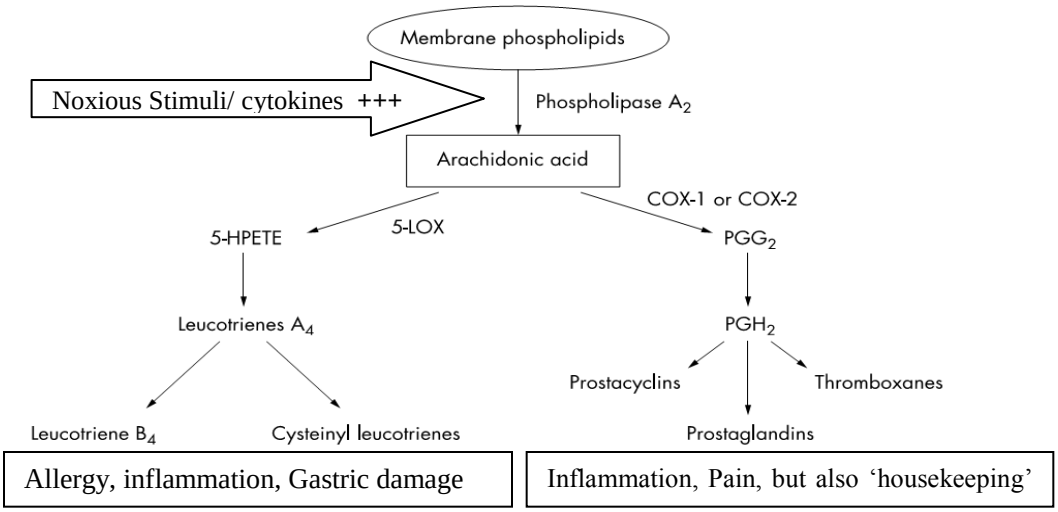

Fig. 1: Products and enzymes of AA metabolism (Pelletier *et al*., 2003).

There are two COX isoforms: COX-1 and COX-2. The predominant constitutive nature of COX-1, together with the observation that expression of COX-2 can be upregulated by inflammatory stimuli like cytokines and bacterial endotoxins has led to the hypothesis of the existence of the 'good' versus 'bad' PGs (Parente and perretti, 2003; Fattahi and Mirshafiey, 2012). According to this concept, COX-1 generates good PGs for physiological 'housekeeping' functions, including platelet dependent homeostasis, gastric mucosal integrity, and regulation of renal blood flow, whereas COX-2 forms the 'bad' PGs responsible for signs and symptoms inflammation (Kocks *et al*., 2004).

However, this simplified paradigm of constitutive COX-1 and inducible COX-2 has many exceptions. Accumulating pieces of evidence suggest that COX-1 and COX-2 have overlapping actions and that both isoforms are involved in homoeostasis processes, just as both are modulators of inflammatory reactions (Martel-Pelletier and Pelletier, 2003). More recent data indicate that COX-2 is constitutively expressed in some healthy organs such as kidney, gastrointestinal tract, brain, and thymus, and appears to have physiological roles such as being involved in electrolyte retention and hence influencing blood pressure (Kirkby *et al*., 2016; Davies and Jamali, 2004).

In addition, COX-2 exhibits a protective role in asthma exhibiting anti-inflammatory and anti-proliferative function in the airways (Belvisi *et al*., 1998). Studies have also demonstrated that COX-2 plays an important role in gastrointestinal mucosal defense in certain circumstances, such as when the mucosa is inflamed or ulcerated, as evidenced by impaired gastric ulcer healing with long-term administration of COX-2 specific antagonist (Schmassmann *et al*., 1998; Zimmermann *et al*., 1998).

Conversely, the role of COX-1 is not only physiological. It has been noted that, the anti-inflammatory effects of selective COX-2 inhibitors was not seen if the dose is not increased above levels which also inhibit COX-1 activity, suggesting that this latter isoform has a significant role in the synthesis of proinflammatory PGs (Li *et al*., 2006). Impaired inflammatory responses have also been reported in both COX-1 and COX-2 deficient mice, although they diverge in time course and intensity. Human data also indicate that COX-1 derived products drive the initial phase of an acute inflammation, with COX-2 upregulation occurring within several hours (Smyth *et al*., 2009).

Thus, the earliest prostlanoid response to deleterious environmental stimuli is dependent on COX-1, and as the inflammatory process progresses, COX-2 becomes a major source of prostanoids (Tilley *et al*., 2001). These studies evidenced the role of both COX-1 and COX-2 in the inflammatory response, their contribution being different depending on the type of insult, the time after insult, and the tissue examined (Aid *et al*., 2008).

Among the eicosanoids, PGE2 and PGI2 (prostacyclin), are the predominant pro-inflammatory prostanoids (Seguineau *et al*., 2011). Given their potent vasodilatory properties, PGE2 and PGI2 increases tissue blood flow which results in the characteristic erythema of inflammation. PGE2 and PGI2, through activation of the EP2 and IP receptors respectively, also increase vascular permeability and leukocyte infiltration and hence the appearance of edema (Pelletier *et al*., 2003; Smyth *et al*., 2009).

Leucotrienes, which are the second main family of AA derivatives, are synthesized from the activity of 5-LOX in concert with 5-lipoxygenase-activating protein (FLAP), which facilitates the interaction between 5-LOX and its substrate AA (Mao *et al*., 2008; Savari

et al., 2014). The biologically active 5-LOX pathway metabolites include cysteinyl-LTs (LTC4, LTD4, LTE4), and LTB4 (Montuschi, 2010). LTB4 is a potent chemotactic agent for leucocytes and induces chemokinesis and adhesion of these cells to the vascular endothelium and enhances vascular permeability (Afonso *et al*., 2012; Werz *et al*., 2002). The CysLTs mediate significant inflammatory changes, such as endothelial cell contraction, eosinophil recruitment, reduction in leukocyte rolling velocity and increase leukocyte adhesion (Pelletier *et al*., 2003; Gennaro *et al*., 2004). They also induce survival of eosinophils, mast cells, and basophils and hence contribute to maintaining the inflammatory reaction (Ogawa and Calhoun, 2006; Jiang *et al*., 2009).

Generally speaking, products of the two enzymes, COX and 5-LOX, are important mediators of inflammation. High levels of LTs and up-regulated expressions of COX-2 and its main products are characteristic hallmarks of inflammation (Massoumi and Sjölander, 2007).

ii. Cellular response of acute inflammation

The monolayer of endothelial cells (ECs) at the inner side of all vessel types, i.e. at the interface between blood and tissue, gate the traffic of molecules and cells across the vessel wall and play an active role in hemostasis, inflammatory reactions, and immunity (Wallez and Huber, 2008). Due to their location, ECs are one of the first cell types to detect foreign pathogens and endogenous metabolite-related danger signals in the blood stream, and hence function as danger signal sensors (Mai *et al*., 2013). In this role, the ECs express cell surface-molecules that orchestrate the trafficking of leucocytes from intravascular space to extravascular sites of inflammation (Limaye and Vadas, 2007; Danese *et al*., 2007).

Leukocyte extravasation from the blood into inflamed tissues follows a multistep cascade that involves the sequential action of molecular signals and adhesion molecules (Weber *et al*., 2007). Following exposure to inflammatory signals such as IL-1, TNF-α, and bacterial lipopolysaccharides, ECs will be activated and produce chemoattractants (e.g., IL-8 and MCP-1) that attract and activate different leukocyte populations. These chemo-

attractant act in concert with de novo or augmented expression of endothelial adhesion molecules at sites of inflammation, to cause capture and extravasation of circulating leukocytes (fig. 2) (Mantovani *et al*., 1992; Nystedt *et al*., 1996).

The L, E and P forms of selectins, on the activated endothelium surface, in combination with the integrins (β_2, β_3 and α_4) have been found to be involved in leukocyte capture. These molecules, along with the participation of chemotactic IL-8 and MCP-1 are involved in starting leucocytes tethering and rolling along the endothelium at a very slow speed, i.e. the 'braking' of leucocytes (Karatzis, 2005; Garrood *et al*., 2005). Following their recruitment, the interactions between leukocyte β2-integrins and their ligand intercellular adhesion molecule 1 (ICAM1), and between the integrin very late antigen 4 (VLA4) and its ligand vascular cell-adhesion molecule 1 (VCAM1) mediate the firm adhesion of the leukocytes (Weber *et al*., 2007; Schnoor *et al*., 2015).

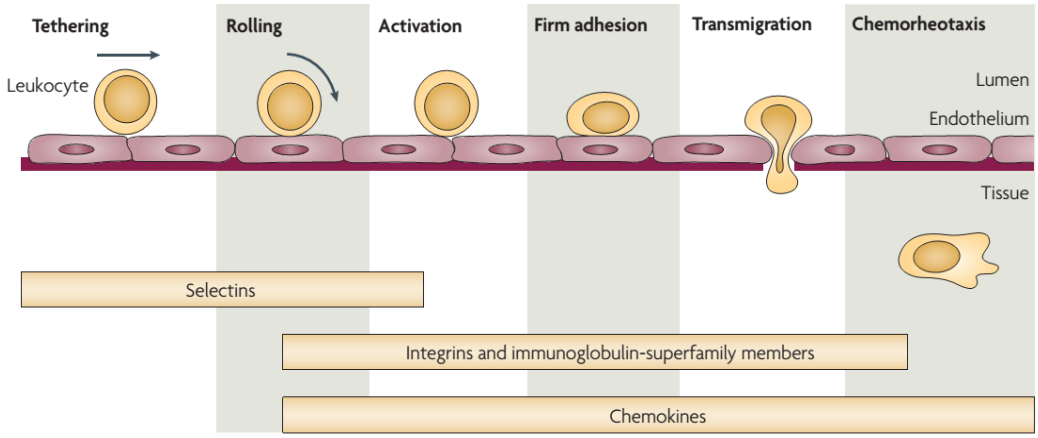

Fig. 2: Physiologic interaction of leukocytes with the endothelium (Weber *et al*., 2007).

Leukocytes adherent to the endothelium then make contact with free flowing leukocytes through the L-selectin molecule, resulting in amplification of leukocyte recruitment to sites of inflammation (Nourshargh and Alon, 2014). The leucocytes then flatten and transmigrate along the endothelium, a process known as diapedesis. Once present in tissues, leukocytes then migrate to the site of injury and initiate the process of eliminating the offending agents and tissue repair will commence (Vetriselvan *et al*., 2013; Rubin and Strayer, 2014).

iii. Resolution of Acute inflammation

The inflammatory response is a spatially and temporally orchestrated event in which cells and mediators collaborate to eliminate the damaging stimuli and allow maintenance of homeostasis (Sousa *et al*., 2013; Alessandri *et al*., 2013). Therefore, where an inflammatory response does occur, it is normally well regulated in order that it does not cause excessive damage to the host, is self-limiting and resolves rapidly (Calder, 2010).

The resolution phase can be defined histologically as the interval from maximum neutrophilic infiltration to the point when they are lost from the tissue (González-Périz and Clària, 2010; Kamaly *et al*., 2013). Hence, during the resolution phase, neutrophils undergo apoptosis, a highly regulated cell death mechanism that prevents the release of histotoxic cellular contents (Barnig and Levy, 2015). During apoptosis, alterations in neutrophil cell surface markers (e.g. external expression of Phosphatidylserine) and morphological changes (e.g. nuclear condensation, loss of phospholipids asymmetry, plasma membrane blebbing, and decomposition of the cytoskeleton) results in increased recognition by professional phagocytes (macrophages), which mediate effective clearance of dying cells by a process termed efferocytosis (Chaurio *et al*., 2009; Maskrey *et al*., 2011). Once phagocytosis is complete, macrophages in turn are cleared from the inflamed tissue either by egression to the lymphatic system or die locally by apoptosis (Lawrence and Gilroy, 2007; Freire and Dyke, 2013).

To restore homeostasis, avoidance of provocative stimulus can lead to such passive decrease in pro-inflammatory effectors. In the traditional view, it was widely accepted that catabolism of pro-inflammatory mediators was the 'turn off' signal of inflammation and sufficient for the inflammatory response to switch off passively and just "fizzled out" (Serhan *et al*., 2007; Carlo and Levy, 2008; Dwivedi *et al*., 2012).

In fact, resolution involves halting of recruitment and influx of inflammatory neutrophil and apoptosis of granulocytes followed by nonphlogistic clearance by macrophages (Croasdell *et al*., 2015). This process induces a phenotypic switch in macrophages from a pro-inflammatory M1 to anti-inflammatory M2 phenotypes (Fig. 3). Anti-inflammatory

M2 macrophages, then, alter their metabolism of lipids from pro-inflammatory PGs and LTs to pro-resolving autacoids such as lipoxins, resolvins, protectins, maresins, and IL-10 (Sousa *et al*., 2013; Headland and Norling, 2015). Hence, the termination of acute inflammation involves active biosynthetic processes producing novel anti-inflammatory lipid mediators whose specific role is summarized in Table 1 (Serhan *et al*., 2008).

In sum, late in the inflammatory reaction, potent molecules that signal via specific receptors to block inflammatory responses are synthesized and released. This demonstrates that resolution of inflammation is an active process, and not merely what happens when pro-inflammatory stimuli cease (Nathan and Ding, 2010).

Table1: Lipid mediators of resolution phase of inflammation (Freire and Dyke, 2013).

Mediators	**Cellular functions**
Lipoxin A4	Decreases adherence of leukocytes, Reduces vascular leakage, Inhibits neutrophil recruitment, Attenuates expression of NF-κB gene, Promotes lymphatic removal of phagocytes
Resolvin E1	Inhibits neutrophil infiltration, Modulates cytokine synthesis, Activates lymphatic removal of phagocytes, Reduces lymphocyte recruitment, Attenuate expression of the NF-κB gene
Protectin D1 /AT-PD1	Decreases inflammatory actions of COX-2, Inhibits neutrophil infiltration, Modulates chemokine/ cytokine synthesis, Regulates T-cell migration, Regulates macrophage function, Inhibits pain signals, Suppresses eosinophil chemotaxis and adhesion
Maresin -1	Reduces neutrophil numbers in exudates, Enhances macrophage phagocytic functions, Decreases transendothelial polymorphonuclear cell migration
interleukin (IL)-10	inhibit transcription of LPS-induced genes such as cell surface receptors, chemokines & cytokines , regulates apoptosis in B cells

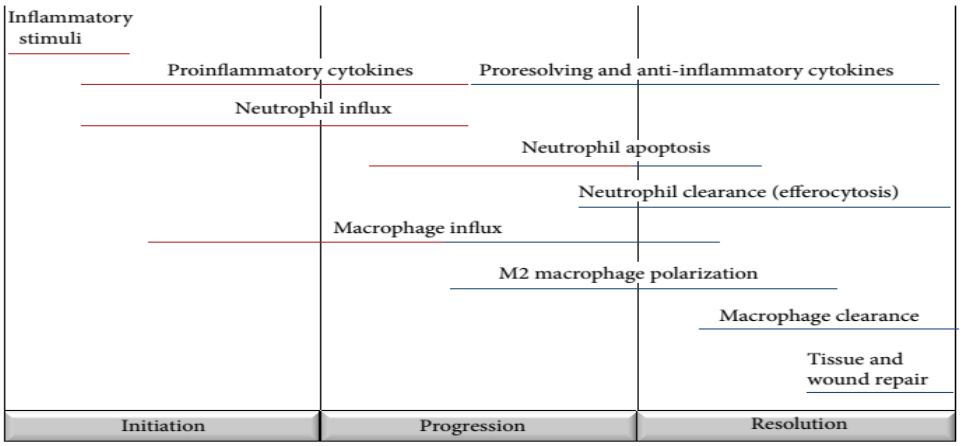

Fig. 3: Cells and phases of acute inflammation (Croasdell *et al*., 2015).

1.2.2 Chronic inflammation

Although it is often defined simply in terms of time course, with lesions of over 6 weeks' duration traditionally being regarded as chronic, chronic inflammation is most appropriately defined in terms of the process, in which continuing active inflammation and attempted tissue healing by repair occur concurrently, rather than consecutively (Wakefield and Kumar, 2001; Bajpai *et al*., 2014). This is because chronic inflammation may develop from unresolved symptomatic acute inflammation or may evolve insidiously over a period of months without apparent acute onset of clinical manifestations (Schottenfeld and Dimmer, 2006; Tandon *et al*., 2014).

Persistent presence of inflammatory stimuli or deregulation of mechanisms of the resolution phase (Maskrey *et al*., 2011), combined with failed attempts at therapy, can induce a long-term, deregulated inflammatory response that affects several organ systems and sets in motion a vicious cycle of inflammation → damage → inflammation. This feed forward loop can lead to persistent, deregulated inflammation that promotes organ dysfunction and perhaps death (Wu and Wu, 2007; Vodovotz *et al*., 2010).

Thus, although restricted inflammation is beneficial, excessive or persistent inflammation which runs unchecked can lead to a host of chronic inflammatory pathologies, such as remodeling of the artery wall in atherosclerosis (Razavian *et al*., 2011), remodeling of the

airways in asthma and COPD (Chung, 2005; Shifren *et al*., 2012), development of insulin resistance in type 2 diabetes mellitus (Shoelson *et al*., 2006; De Luca and Olefsky, 2008), rheumatoid arthritis (McInnes and Schett, 2011), crohn's disease and ulcerative colitis, (Nathan and Ding, 2010) and such neurological disorders as stroke, parkinson's disease, alzheimer's disease, and Multiple sclerosis (Dammann and Leviton, 2013).

iv. Cells and characteristics of chronic inflammation

Chronic inflammation is not characterized by the classic cardinal signs of acute inflammation. Moreover, unlike acute inflammation in which neutrophils predominate, chronic inflammation is well characterized histopathologically by predominant infiltration of mononuclear immune cells (monocytes, macrophages, and lymphocytes), with angiogenesis and fibrosis (Schottenfeld and Dimmer, 2006; Vetriselvan *et al*., 2013).

The actual mechanisms that control the transition from neutrophil to mononuclear cells recruitment during the switch from acute to chronic inflammation are poorly understood. But, it is possible that the IL-6/soluble IL-6 receptor α (sIL-6Rα) complex plays an important role (Gabay, 2006). A complex of IL-6 and sIL-6Rα can regulate a switch in chemokine secretion by ECs to IL-8 and MCP-1, so that mononuclear immune cells are directed to sites of inflammation (Kaplanski *et al*., 2003; Rubin and Strayer, 2014).

These predominant cells of chronic inflammation then form multiple focal aggregates known as granulomas (Zumla and James, 1996). Fibroblasts, being recruited by macrophages, will build collagen in larger quantities leading to the formation of a fibrous capsule and granuloma rich with multinucleated giant cells generated by macrophage fusion (Lucke *et al*., 2015). Hence, the entire inflammatory lesion will be bounded by a zone of granulation tissue which is typical histological feature of chronic inflammation (Matsushima *et al*., 2011). Furthermore, chronic inflammation tends to be systemic. During the prolonged exposure to the risk factor(s), inflammatory mediators from the primary site of inflammation travels via blood vessel to various distant sites of the body, attacks cells at distant organs and tissues creating new inflammation sites and new inflammatory diseases (Wu and Wu, 2007).

1.3 Management of inflammation

Non-steroidal anti-inflammatory drugs (NSAIDs), and Corticosteroids are the major classes of drugs extensively used for the management of inflammatory conditions. These conventional anti-inflammatory drugs have been developed for their capacity to inhibit or antagonize the production or action of pro-inflammatory mediators (Ayroldi *et al*., 2012; Sousa *et al*., 2013). In recent years particular efforts have been made to design biological agents targeting inflammatory cytokines such as anti-TNF-α and anti- IL-1 therapy (Murdaca *et al*., 2009).

1.3.1 Non-Steroidal Anti-inflammatory Drugs

NSAIDs have over the years been the therapeutic agents of first choice for the treatment of inflammatory conditions. They act by inhibiting the enzyme COX, and hence block the production of pro-inflammatory prostanoids (PGs and TXs) (Ajmone-Cat *et al*., 2010; Crofford, 2013). Structurally, most NSAIDs are organic acids with low pK values that lend themselves to their accumulation at sites of inflammation, areas that often exhibit lower pH than uninvolved sites (Crofford, 2013; Shah and Piyush, 2015).

NSAIDS can be classified as single- or dual-action drugs. NSAIDS such as aspirin, naproxen, flurbiprofen, and ibuprofen are COX inhibitors only and are single-action drugs. Other NSAIDs such as diclofenac and ketoprofen are dual-action NSAIDs blocking both the COX and LOX pathways of AA metabolism. The latter NSAIDs may therefore have a more powerful anti-inflammatory effect than the single-action NSAIDs (Connolly *et al*., 2003).

Most conventional NSAIDs are nonselective in their COX inhibition; exerting their anti-inflammatory effects primarily through the inhibition of COX-2, but having adverse effects (such as GI mucosal damage and nephrotoxicity) primarily due to inhibition of COX-1. Currently, a range of new and specific COX-2 inhibitors, such as celecoxib and rofecoxib, were developed with the hope of reducing possible GI side effects. However, growing awareness of the cardiovascular adverse effects of selective COX-2 inhibitors

has raised the question of safety, especially for patients with established cardiovascular disease (Russell, 2001; Al-Saeed, 2011; Stanos, 2013).

COX-2 specific inhibitors could potentially increase the thrombotic risk, because they block the production of PGI2 (potent vasodilatory and anti-aggregatory) through COX-2 inhibition, but, as they do not inhibit COX-1, the only isoform expressed in platelets, they do not block the formation of TXA2 (vasoconstrictive and pro-aggregatory) from platelets. This tip of balance allows TXA2 to function unopposed, leading to increased risk for cardiovascular adverse events (Pelletier *et al*., 2003; Ong *et al*., 2007). Generally speaking, NSAIDs has recently come under scrutiny because of recent focus in the various adverse effects that can occur specially with either long term or high dose administration (Al-Saeed, 2011).

1.3.2 Corticosteroids

Corticosteroids are the most important drugs in treatment of a variety of inflammatory diseases, and represents the first successful exploitation of an endogenous anti-inflammatory mediator, cortisol (Serhan *et al*., 2007; Sousa *et al*., 2013). Although there are several mechanisms by which GCs reduce inflammation, their predominant effect is to switch off multiple inflammatory genes encoding for cytokines, chemokines, adhesion molecules such as ICAM-1, inflammatory enzymes such as COX-2, receptors and proteins (Barnes, 2006; Dinarello, 2010).

These actions are caused by passage of GCs through the plasma membrane, high-affinity binding to inactive cytoplasmic steroid receptors cGCRα, formation of the activated GC/cGCRα complex and translocation of the complex into the nucleus (Spies *et al*., 2011). Once translocated, activated GC/cGCRα complex bind to either a negative GR response element (nGRE) in the promoter of inflammatory genes inhibiting gene transcription or, more commonly, by physically interacting with transcription factors, such as NF-κB or AP-1, and impairing their ability to activate gene expression (Fig.4) (Adcock and Lane, 2003; Ayroldi *et al*., 2012; Ramamoorthy and Cidlowski, 2013).

At higher concentrations, GC/cGCRα homodimers also interact with DNA recognition sites to activate transcription of anti-inflammatory genes. Corticosteroids, for example, induce synthesis of Annexin 1 (an inhibitor of PLA2 thus block the initial cleavage of AA from the cell membranes), IL-10, and the inhibitor of NF-κB and thus resulting in a more complete anti-inflammatory effect than most commonly used single-action NSAIDs (Connolly *et al*., 2003; Barnes, 2006; Iudicibus *et al*., 2011).

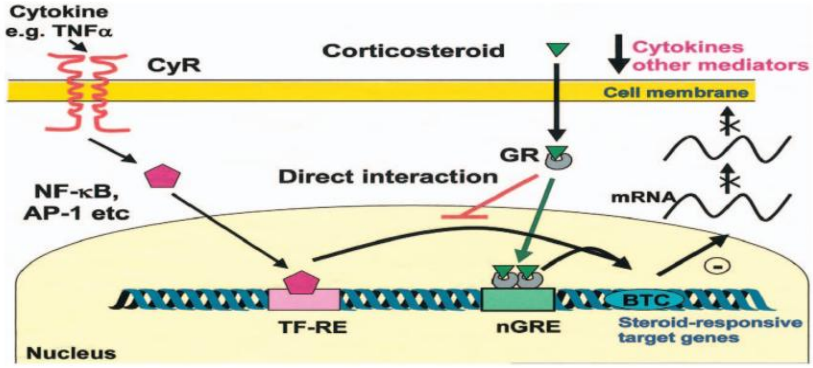

Fig.4: How Corticosteroids switch off pro-inflammatory genes (Adcock and Lane, 2003).

1.3.3 Newer anti-inflammatory agents

Recent advances in biotechnology have allowed for the commercial development of agents that specifically target inflammatory cytokines. In the last 10 years, FDA approved eight biological agents (etanercept, infliximab, anakinra, adalimumab, certolizumab, golimumab, abatacept, and rituximab) which are used worldwide for the treatment of inflammatory conditions like rheumatoid arthritis, psoriatic arthriris, crohn's colitis, and ankylosing spondilitis (Moreland, 2009; Jangid *et al*., 2015).

These biological agents antagonize the effect of pro-inflammatory cytokines such as TNF-α and IL-1 (Donnelly *et al*., 2009). The approach to TNF-α inhibition has followed two different mechanistic paths: i) the selections of monoclonal antibody which binds with high affinity to TNF-α and inhibits the effective binding of TNF-α with its receptors, and ii) the synthesis of a soluble TNF-receptor (TNF-R) fusion protein which directly binds to and blocks TNF-α receptors (Murdaca *et al*., 2009; Jangid *et al*., 2015). Infliximab, Adalimumab, Golimumab and Certolizumab, are examples of monoclonal

antibodies which acts by the first mechanism, whereas, Etanercept is a direct receptor blocker (Keystone and Ware, 2010). Anakinra on the other hand is a recombinant human IL-1 receptor antagonist, first approved by the FDA in 2001 for the treatment of rheumatoid arthritis (Donnelly *et al*., 2009).

1.4 Plant medicines in management of inflammatory diseases in Ethiopia

The use of plants as medicines predates written human history and almost all cultures in the world have a body of expertise concerned with the therapeutic properties of the local flora (Gedif and Hahn, 2003). Ethiopia is characterized by a wide range of ecological, edaphic, and climatic condition that accounts for the wide diversity of its biological resources both in terms of flora and fauna (Andarge *et al*., 2015). There are about 6,500 species of higher plants in Ethiopia, making the country one of the most diverse floristic regions in the world (Birhanu, 2013).

It is well known that traditional medical practices are common in Ethiopia in which about 80% of the population use plant based medicine by indigenous knowledge as their major primary health care system (Mesfin *et al*., 2013). The reliance on medicinal plants is partly owing to the high cost and inaccessibility of modern medicine, and due to cultural acceptability, affordability, effectiveness, and fewer side effects of the system (Mesfin *et al*., 2013; Teklay *et al*., 2013).

In this regard, many plants are being used for treatment of different inflammatory conditions and symptoms in traditional medical practice of Ethiopia. Some of these plants include: *Cucumis fi cifolius* (arthritis), *Kalanchoe lanceolata* (edema, inflammation) (Birhanu, 2013), *Albuca abyssinica* (rheumatism) (Mesfin *et al*., 2013), *Lepidium sativum* ('mich'), *Eucalyptus globules Labill* ('mich') (Mesfin *et al*., 2009), *Conyza sp*. (rheumatism), *Plectranthus caninus* (alergic reaction and wound), *Plectranthus ornatus* (allergic reaction), *Rhynchosia orthobotrya* (allergic reaction), *Salvia nilotica* (allergic reaction) (Andarge *et al*., 2015), *Capparis tomentosa* (‘mich’), *Cynoglossum coeruleum* (‘mich’), *Phragmanthera* sp. (‘mich’), *Premna schimperi* (inflammation of skin), *Tapinanthus* sp. (‘mich’), *Vernonia adoensis* (‘mich’) (Giday *et al*., 2007), Malvaceae

Sida ovate (eczema), Solanaceae *Datura stramonium* (eczema) (Gedif and Hahn, 2003), *Ocimum lamiifolium* ('mich', alergic reaction) (Andarge *et al*., 2015; Giday *et al*., 2007), and *Zehneria scabra* (febrile conditions, headache, Inflammation) (Gedif and Hahn, 2003; Akele, 2012; Birhanu, 2013). Among these, *Zehneria scabra* is the one which is the focus for the present study.

1.5 *Zehneria scabra*

The cucurbitaceae family includes mostly prostrate or climbing herbaceous annuals comprising about 90 genera and 700 species that are further characterized by commonly having 5 angled steams and coiled tendrils (Abdulrahaman *et al*., 2011). *Zehneria scabra* (L.f.) Sond, vernacular name is called Areg-resa (Amharic), Etse-sabieq (Ge'ez), Hafaflo (Tigrigna), Daaymii (Afan Oromo), Kiete (Gedeo), is one of the important medicinal climbers of this family and attracts greater attention because of its high medicinal value in herbal folklore practices (Gedif and Hahn, 2003; Teklay *et al*., 2013; Tekle, 2014; Kefalew *et al.,* 2015).

It is a climbing or trailing herb that can go up to 10 m, with stem inter-node length of 6 - 7cm which become woody with corky-ridged bark as the plant grows old (Tadesse *et al*., 2014; Agogbua *et al*., 2015). The leaves are ovate-suborbicular with cordate base, angular or 3-5 lobed (Fig. 5); flowers greenish-white; fruits Oval, finely reticulate, red when ripe and seeds ovate-oblong (Murthy *et al*., 2013; Agogbua *et al*., 2015).

It inhabits forest and on forest margins, riverine fringes and exotic plantations across 900 - 2100 m above sea level. It is native to South America, the common name "sandia de raton" describes the fruit as mouse-sized watermelons, and widespread in tropical Africa, Ethiopia, South Africa, Arabia, India, Java and the Philippines (Abew *et al*. 2014).

In Ethiopia, *Z. scabra* is one of the traditional medicinal plants commonly used for the treatment of a number of illnesses. By Amhara ethinic group of Bahir Dar zuria district for example, the leave juice is used to treat fever and headache (Ragunathan and Abay, 2009). Peoples of Kilte Awulaelo district of Tigray use the leaves for treatment of paralysis, michi, and external wounds, while the root is used for abdominal pain and

ascariasis (Teklay *et al*., 2013). The flowers of the plant have also been used for topical treatment of alopecia, wound and eczema being mixed with other herbals (Messele *et al.*, 2004). In addition, the root has also been used for anemia by people of Guangua Sub-district of the Agew-Awi zone of the Amhara Regional State (Giday *et al*., 2007).

Furthermore, the plant has enormous ethnobotanical value, as used by other tribes. For example, in Tamil people of southern India and Sri Lanka, it is used to treat fever and stomach pain in the form of root and leaves extracts (Anand and Jeyachandran, 2004). People of Marakwet Community in Kenya use the leaves crushed and administered for common colds and whole plant boiled with other herbs for Cancer (Kipkore *et al*. 2014).

On top of this, a recent study confirms the ethanol and ethyl acetate shoot extract of *Z.scabra* to have antimicrobial activity against the most common bacterial pathogens, i.e. *Staphylococcus aureus* and *E.coli* (Anand *et al*., 2012). The ethanolic tuber extract was also proved to have potent antifungal properties against five types of fungi including *A. fumigatus, A. niger, A. flavus, M. indicus* and *C. albicans* (Arulappan *et al.*, 2015). Furthermore, the 80% methanolic leaves extract have been proven to have activities including, antidiarrheal and antisecretory (Tadesse *et al*., 2014), antimalarial (Tesfaye and Alamneh, 2014), and anti-nociceptive and anti-inflammatory activity (Akele, 2012).

Fig. 5: *Zehneria scabra*

1.6 Rationale for the Study

Inflammation is central to many of the diseases that affect both developed and emerging nations. Virtually all acute and chronic diseases are either driven or modulated by inflammation (Vodovotz *et al*., 2010). Despite this fact, the complex interplay between beneficial and harmful arms of the inflammatory response underlies the lack of safe and fully effective therapies for many of the pathologies (Vodovotz *et al*., 2009). On top of this, the majority of existing drugs suffer from diverse adverse events, especially at higher doses and longer duration of therapy (Spies *et al*., 2011).

Non-steroidal antiinflammatory drugs (NSAIDs), for example, are associated with development of gastric or duodenal ulceration, nephrotoxicity, bronchospasm and exacerbation of symptoms of asthma, an increase in blood pressure, and increased incidence of myocardial infarction and stroke (Ong *et al*., 2007; Stanos, 2013). Corticosteroids on the other hand, are associated with manifold side-effects such as diabetes mellitus/glucose intolerance, hypertension, obesity, osteoporosis, immune suppression, glaucoma, and growth retardation in children (Spies *et al*., 2011; Rhen, and Cidlowski, 2005). In view of this, WHO advocates the inclusion of herbal medicines of proven safety and efficacy in the healthcare programs because of the great potential they hold in combating various diseases (WHO, 2002).

Z.scabra is one of these plants, whose 80% methanol leave extract has been proved to have anti-inflammatory and analgesic activity. Obviously, such a plant exhibiting anti-inflammatory, analgesic (Akele, 2012), antifungal (Arulappan *et al*., 2015) and antibacterial activity (Anand *et al*., 2012) would improve patient compliance and has economic importance. However, there is a dearth of scientific evidences to further substantiate the therapeutic potential of the plant in different inflammatory models.

Hence, this study focuses on an *in-vivo* anti-inflammatory activity of 70EE and solvent fractions of *Z.scabra* leaves using acute (caregeenan induced paw edema), sub-acute (formaldehyde induced arthritis) and chronic (cotton pellet induced granuloma) models of inflammation with a view to validate its acclaimed use by the traditional practitioners.

Moreover, it also tries to identify the most active fraction to give a clue for further investigation in search for the specific agent(s) responsible for the anti-inflammatory effect of the plant. Besides, the finding of this study might provide a clue about the possible mechanisms of anti-inflammatory action of the plant and it might serve as baseline information for scientific community to further investigate the plant by initiating advanced studies on molecular mechanisms with identification of the specific agent(s) responsible for the anti-inflammatory effect of the most active fraction.

2. OBJECTIVES

3.1 General objective

- To evaluate anti-inflammatory activity of 70% ethanol extract (70EE) and solvent fractions of leaves of *Zehneria scabra* (L.f.) Sond (Cucurbitaceae) in rodents.

2.2 Specific objectives

- To assess acute toxicity of 70EE and solvent fractions of *Zehneria scabra* leaves in mice
- To evaluate the effect of crude 70EE of *Zehneria scabra* leaves on formaldehyde-induced arthritis in mice
- To evaluate the effect of chloroform, n-butanol and aqueous fractions of *Zehneria scabra* leaves on carrageenan-induced paw edema in mice
- To evaluate the effect of chloroform, n-butanol and aqueous fractions of *Zehneria scabra* leaves on formaldehyde-induced arthritis in mice
- To evaluate the effect of chloroform, n-butanol and aqueous fractions of *Zehneria scabra* leaves using cotton pellet induced granuloma in rats
- To determine the phytochemical constituents of the 70EE and solvent fractions of *Zehneria scabra* leaves

3. MATERIALS AND METHODS

3.1 Drugs and chemicals

Aspirin active (EPHARM, Ethiopia), Dexamethasone (Medico Labs, Lot E6A00, Syria), Carrageenan (Sigma Aldrich, Germany), thiopental sodium (NEON Laboratories, India); ethanol (Lot 30320601EX) and formaldehyde (Research-Lab Fine Chem Industries-India); n-butanol (Lot 10061), chloroform (Lot 10077), and glacial acetic acid (Lot MR0478) (BDH chemical LTD poole, England); acetic anhydride (Lot A13/45/67/A) and Mayer's reagent (May & Baker LTD Dagenham, England); Dragendroff's reagent and sulfuric acid (Lot 30326) (Fisher Scientific, UK); ammonia, hydrochloric acid, and ferric chloride (Lot 10111) (BDH Laboratory Supplies Poole, England); normal saline (Addis Pharmaceutical Factory, Ethiopia), distilled water (Biochemistry Laboratory of AAU, Ethiopia), tween 80 (UNI-CHEM Chemical Reagents, India), were used in the study and all were of analytical grade.

3.2 Materials and Instruments

Rotary evaporator (Heidolph, Germany), lyophilizer (OPERON, OPR-FDU-5012, Korea), digital plethysmometer (Ugo Basile-Cat no 7140, Italy) electronic balance (KERN-ALJ 220-4, Germany), mini orbital shaker (SSM1-STUART), Tissue Drying Oven (Medite - Medizin technik, Germany), separatory funnel, flasks, Cotton pellets, syringes with needles, feeding tube, blunt forceps, scissors, suturing set.

3.3 Plant material collection and authentication

The fresh leaves of *Zehneria scabra* were collected in Lideta sub city, Addis Ababa, Ethiopia, in December 2014. Identification and authentication of the plant specimen was done at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University and a voucher specimen was deposited with voucher number RB 001/2014 for future reference. The leaves were washed gently by rinsing with running water to remove dust particles, air dried under shade and then size reduced into coarse powder with mortar and pestle.

3.4 Experimental animals

Healthy male (for anti-inflammatory test) and female (for acute toxicity test) Swiss albino mice weighing 20-30 g, and male albino Wistar rats weighing 180-220 g obtained from the animal house of the Ethiopian Public Health Institute and Department of Pharmacology, School of medicine, Addis Ababa University were used. They were kept in plastic cages at room temperature on a 12 h light-dark cycle with free access to pellet food and water *ad libitum*. The animals were acclimatized to laboratory condition for one week prior to commencement of the experiments. All experiments were carried out during the light period of the day (9:00 a.m. - 5:00 p.m.) and in accordance with the guideline for the care and use of laboratory animals (Institute for Laboratory Animal Research, 1996; OECD, 2008). The study was conducted after approval by Addis Ababa University, College of Health Sciences Institution Review Board (IRB).

3.5 Preparation of crude extracts and solvent fractions of *Z.scabra* leaves

The air dried and powdered leaves of *Z.scabra* were first defatted by macerating with petroleum ether for 24 hours at room temperature and with occasional shaking, followed by filtration. The solvent (petroleum ether) was removed from the residue by exposing to open air and the defatted coarse powder was divided into two equal halves of 665gm each for the extraction process. The first half (665gm of the leave) was macerated in a flask containing 70% ethanol (1:5 w/v) for 72 hrs and the other half was macerated with distilled water with the same ration and for the same period of time. The maceration was undertaken with occasional shaking using mini orbital shaker being tuned to 120 rpm for 72 hrs at room temperature. Then, the extract was filtered first using muslin cloth and then using Whatman filter paper (No 1) and the marc was re-macerated for a second and third time by adding another fresh solvent.

The filtrates from the three batches of the 70EE were combined and concentrated in a rotary evaporator with temperature of $40C^0$. The concentrate was then placed in a deep freezer operating at negative $20C^0$ till it forms an ice, and then, the remaining solvent (water) was removed using lyophilizer. After water removal, a black powder residue

weighing 88 gm was obtained, giving rise to a percentage yield of 13.23%. The filtrates from the three batches of the aqueous extract were also combined, placed in a deep freezer at -20C^0 to form an ice and lyophilized. The water extract provides a yield of 39.34 gm of black powder (5.92% w/w). The powders were kept in tightly stoppered bottles and stored in a in a deep freezer at -20C^0 till commencement of the pilot study.

Based on the pilot experiment performed, the hydroalcoholic extract (70EE) (which had a better activity) was selected for further fractionation. A total of eighty gram of powdered residue of the 70EE was divided into four equal parts (20gm each) and then employed for the fractionation. At a time, an aliquot of the 70EE (20 g) was suspended in distilled water (100 ml) and then sequentially partitioned with chloroform and *n*-butanol at room temperature using a separatory funnel. Partitioned layers of each solvent were pooled together and concentrated on a rotary evaporator at 40C^0 followed by oven at room temperature for 48 h, yielding 13.4 gm of black gummy residue from the chloroform fraction (16.75% w/w), and 16.73 gm of light brown slightly hygroscopic powder from the *n*-butanol fraction (20.91% w/w). The aqueous residue was lyophilized to give 38.85gm of black dried powder (48.56% w/w) fraction. The fractions were kept in tightly stoppered bottles and stored in a deep freezer at -20C^0 till commencement of the actual experiment. Finally the fractions were reconstituted in distilled water/2% tween 80 at appropriate concentrations for the various experiments to be conducted.

3.6 Acute oral toxicity test

Acute oral toxicity test for 70EE and solvent fractions of the leaves of *Z.scabra* was performed according to the Organization for Economic Cooperation and Development (OECD) guideline 425; "Limit Test at 2000 mg/kg" (2008). Five female albino mice of 6-8 weeks were used for each test. All mice were fasted for 4 h before and 2 h after the administration of the extract/fractions. First, a sighting study was performed to determine the starting dose, in which, a single female mouse was given 2000 mg/kg of the extract/fractions as a single dose by oral gavage. Since no death was observed within 24 h, additional four mice were used for each of the extract and fractions, and administered the same dose of extract/fractions. The animals were observed continuously for 4 h with

30 min interval and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicities such as changes in skin and fur, eyes and mucous membranes, somatomotor activity and behavioural pattern, salivation and diarrhea, weight loss, tremor and convulsions, lethargy and paralysis, food and water intake and mortality.

3.7 Pilot study

Pilot study was done using Carrageenan-induced paw edema acute model of inflammation on 70% ethanol leave extract (70EE), aqueous leave extracts (AE), and petroleum ether extract (PE) of *Z.scabra*. All the three extracts were administered at doses of 100, 200 and 400mg/kg and three animals per group were used in all the dose levels. The result indicated that both the AE and 70EE have anti-inflammatory activity; even though the 70EE had showen a better anti-inflammatory effect (%A) at all employed doses (maximum %A at the 5^{th} h = 37% for 100mg/kg, 45% for 200mg/kg, and 52% for 400mg/kg) than the AE (maximum %A at the 5^{th} h = 19% for 100mg/kg, 28% for 200mg/kg, and 41% for 400mg/kg). The PE extract, on the other hand, failed to demonstrate anti-inflammatory activity at all employed doses. Hence, the 70EE was opted for further study and fractionation.

3.8 Animal grouping and dosing

The animals were randomly assigned into twelve groups of six animals in each group to perform anti-inflammatory activity test in three models. The first two groups served as negative controls and the vehicles for the fractions (distilled water and 2% tween 80 at a dose of 10 ml/kg) were administered. The third group served as positive control and the standard drugs Aspirin (200mg/kg p.o. in the acute and sub-acute models) or Dexamethasone (0.5mg/kg p.o. in chronic model) was administered to this group. The first three test groups (4-6) received three different doses (100, 200, and 400mg/kg) of the aqueous fraction. The next three test groups (7-9) received n-butanol fraction at doses of 100, 200, and 400mg/kg, while the final three test groups (10-12) received the chloroform fraction at the same three dose levels. The same dose levels were also applied during anti-

inflammatory test of the crude extract using formaldehyde-induced arthritis in mice during which a total of five groups, i.e. two control groups (positive and negative) and three test groups of 70EE (100, 200, and 400mg/kg) were used. The dose levels (100, 200 and 400mg/kg) were selected based on results of acute oral toxicity test and pilot study.

3.9 Determination of anti-inflammatory activity

3.9.1 Carrageenan-induced paw edema

The method described by Mequanint *et al.* (2011) was followed in this model to study the effect of solvent fractions of *Z.scabra* leaves on acute inflammation. Mice were fasted for 12 h with free access to water until the experiment starts and grouped and treated by oral gavage as described under section 3.8. Aspirin 200mg/kg p.o. was administered as standard drug in this model. The right hind paw was marked with ink at the level of lateral malleolus so that it could always be immersed to the same extent in the measurement chamber of the plethysmometer.

An hour later, edema was induced by injecting 0.05 ml of 1% w/v carrageenan in normal saline into the right hind paw of each mouse. Increased volume of the right hind paws was taken as a sign of paw oedema. Paw volume was determined, by volume displacement technique using Ugo-basile plethysmometer, just before carrageenan injection (initial volume (v_0)), and 1, 2, 3, 4 and 5 h after carrageenan injection (final volumes (v_f)). The degree of swelling i.e., oedema was evaluated by the delta volume (v_f - v_0) (Sanusi *et al*., 2013).

$$\text{Oedema (I)} = V_f - V_0$$

Where V_0 is the volume of paw before carrageenan injection and V_f is the volume of paw after carrageenan injection at a given time.

In addition, the anti-inflammatory effect of the fractions expressed in percentage (%A) was calculated according to the formula given by Sanusi *et al*. (2013):

$$\% \text{ inhibition } (\%A) = \left(1 - \frac{It}{Ic}\right) x\ 100$$

Where It and Ic are the mean inflammation (Oedema) values reached at a given time in treatment and control groups respectively.

3.9.2 Formaldehye induced arthritis

The method described by Mehta *et al*. (2012) and Cui *et al*. (2014) was used as a subacute model of inflammation. Mice (20-30 g) were fasted for 12 h with free access to water until commencement of the experiment. The right hind paw was marked with ink at the level of lateral malleolus so that it could always be immersed to the same extent in the measurement chamber of the plethysmometer. The control, standard and test groups of mice received distilled water (2% tween 80 in case of chloroform fraction), Aspirin (200mg/kg p.o.) and extract/fractions, respectively, as described in section 3.8. On the first day, the basal paw volume (V_0) of right hind paw of each mouse was measured using Plethysmometer. On day 1 and day 3, mice were injected into the sub-plantar region of the right hind paw with 0.05 ml of 2 % v/v formaldehyde in normal saline. Dosing with vehicles, standard drug (Aspirin) and extract/fractions was started on same day an hour before induction of arthritis, and continued for 10 consecutive days by oral gavage. The mice paw volume was recorded daily by using Plethysmometer after 1 h of drug administration, but at day 1 measurement was taken 3 h after formaldehyde injection. Finally the percentage inhibition of oedema was calculated as described in section 3.9.1.

3.9.3 Cotton pellet induced granuloma method

The method previously described by Afsar *et al*. (2013) was used to assess the transudative and proliferative (granulomatous) components of chronic inflammation. Male albino wistar rats (180-220 g) were fasted for 12 h with free access to water until commencement of the experiment. The control, standard and test groups of rats received distilled water (2% tween 80 in case of chloroform fraction), dexamethasone (0.5 mg/kg p.o.) and fractions, respectively, as described in section 3.8.

Cotton pellets weighing 10 ± 1 mg were sterilized in an autoclave for 30 min at 120 C^0 under 15Ib pressure. Twenty minutes after treatment with the standard drug and fractions, the rats were anesthetized with thiopental sodium (25 mg/kg, i.p.) and subcutaneous tunnel was made aseptically using blunted forceps in both sides of previously shaved groin region of each rats. Two sterilized cotton pellets weighing 10±1 mg each were then implanted bilaterally in the subcutaneous tunnel and sutured with chromic catgut (0/4metric-1/2 Circle). Treatment with the standard drug (dexamethasone) and fractions continued for seven consecutive days (p.o., once a day). On the 8th day, the rats were sacrificed with ether anesthesia, thereafter; the pellets surrounded by granuloma tissue were dissected out carefully and freed from extraneous tissue. The wet weight of the cotton was taken immediately after removal and then dried up to constant weight at 60^0C for 24hrs and the net dry weight, that is, after subtracting the weight of the cotton pellets, was determined .

The exudate amount (mg), granulation tissue formation (mg), and percent inhibition of exudate and granuloma tissue formation were calculated according to the formula given by Aziz *et al*., (2014):

$$\text{Exudate inhibition (\%)} = (1 - \frac{\text{Exudate in treated group}}{\text{Exudate in controls}})\text{X}100$$

$$\text{Granuloma inhibition (\%)} = (1 - \frac{\text{Granuloma in treated group}}{\text{Granuloma in controls}})\text{X } 100$$

Where:

Measure of exudate formation = immediate wet weight of pellet - Constant dry weight of pellet

Measure of granuloma tissue formation = Constant dry weight - Initial weight of cotton pellet.

3.10 Preliminary phytochemical screening

The preliminary phytochemical screening of secondary metabolites of 70EE, and chloroform, n-butanol, and aqueous fractions of leaves of *Z.scabra* were carried out using standard tests (Debela, 2002; Sasidharan *et al*., 2011).

Test for saponins

To 0.25 g of 70EE and each fractions (AF, BF and CF), 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of a stable froth that persists for about half an hour indicated the presence of saponins.

Test for terpenoids

To 0.25 g of 70EE and each fraction, 2 ml of chloroform was added. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

Test for tannins

About 0.25 g of 70EE and each fraction was boiled in 10 ml of water in a test tube and then filtered with filter paper (Whatman No. 1). A few drops of 0.1% ferric chloride were added to the filtrate. A brownish green or a blue-black precipitate indicated the presence of tannins.

Test for flavonoids

About 10 ml of ethyl acetate was added to 0.2 g of 70EE and each fraction, and heated on a water bath for 3 min. The mixture was cooled and filtered. Then, about 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammonia layer indicated the presence of flavonoids.

Test for cardiac glycosides

To 0.25 g of 70EE and each fraction diluted with 5 ml of water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides.

Test for steroids

Two ml of acetic anhydride was added to 0.25 g of 70EE and each fraction with 2 ml sulfuric acid. The color change from violet to blue or green in some samples indicated the presence of steroids.

Test for alkaloids

0.5 g of 70EE and each fraction was diluted to 10 ml with acid alcohol, boiled, and filtered. To 5 ml of the filtrate, 2 ml of dilute ammonia and 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Dragendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Dragendorff's reagent) was regarded as positive for the presence of alkaloids.

3.11 Statistical analysis

The data were analyzed using SPSS version 16.0 for Windows. The experimental results are expressed as mean ± standard error of the mean (S.E.M), and statistical significance was carried out by employing one way analysis of variance (ANOVA) followed by Tukey post Hoc test for multiple comparisons to compare results among groups, where p values < 0.05 were considered statistically significant. The analyzed data were then presented using tables. Linear regression was also used where appropriate.

4. RESULTS

4.1 Acute oral toxicity test

The acute oral toxicity test of 70EE and fractions (AF, BF and CF) of leaves of *Z.scabra* showed that neither the 70EE nor the solvent fractions caused gross behavioral changes, toxic effects or mortality within 24 h and in the next 14 days. According to the "Limit Test at 2000 mg/kg" of OECD guideline 425 (2008), it can be concluded that the oral LD_{50} of both the crude 70EE and solvent fractions are greater than 2000 mg/kg in mice.

4.2 Carrageenan induced paw edema

Subplantar injection of 0.05 ml of 1% carrageenan to the mice hind paw produced a progressive increment of paw thickness that reached its maximum value after 3h of induction in both distilled water and 2% tween vehicle controls (Table 2). All tested doses of the aqueous fraction (100, 200 and 400mg/kg of AF) showed a significant inhibition of paw edema that starts from 1 h ($p < 0.01$) and the effect lasted till 5 h post induction ($p < 0.001$ from 2^{nd} - 5^{th} h) as compared to the distilled water vehicle control.

Maximum anti-inflammatory effect (%A) by the 100, 200 and 400 mg/kg of AF was observed at 5 h post induction, with respective values of 50.97%, 72.02 %, and 76.25 %, and the effect at this hour was found to increase in dose dependent manner ($R^2 = 0.721$). Intergroup comparison among doses of the AF also showed a statistically significant different effect in both 200 versus 100mg/kg ($p < 0.05$ at 4h and 5h), and 400 versus 100mg/kg ($p < 0.05$ at 4 h, and $p < 0.01$ at 5 h).

Unlike the AF, only the higher doses of butanol fraction (200mg/kg and 400mg/kg of BF) showed statistically significant inhibition of paw edema at 1h post induction as compared to distilled water vehicle control, with $p < 0.05$ and $p < 0.01$, respectively. The effect in both dose levels then persisted from the 2^{nd} - 5^{th} h post induction with $p < 0.001$, except at the 3^{rd} h where $p < 0.01$ for 200mg/kg as compared to the negative control. However, the 100mg/kg of BF failed to demonstrate a statistically significant inhibition of paw edema as compared to the negative control except at 2 h post induction where $p < 0.05$.

Like the AF, maximum percent inhibition by the 100, 200, and 400 mg/kg of BF was observed at 5 h post induction, with respective values of 19.93%, 43.29%, and 51.33%, and the anti-inflammatory effect was found to increase in dose dependent manner (R^2 = 0.799). Intergroup comparison among doses of the BF also showed a statistically significant different effect in both 200 versus 100 mg/kg (5h, $p < 0.05$) and 400 versus 100 mg/kg (2 h, $p < 0.05$ and 3 - 5 h, $p < 0.01$).

The higher doses of chloroform fraction (CF), on the other hand, significantly inhibited paw edema as compared to the 2% tween vehicle control only late at the 4^{th} h ($p < 0.05$ for 200mg/kg; $p < 0.01$ for 400mg/kg), and 5^{th} h ($p < 0.01$ for 200mg/kg; $p < 0.001$ for 400mg/kg) post induction. The 100mg/kg of CF, however, did not show significant inhibition of paw edema as compare to the negative control throughout the observation period. Interestingly, no significant difference was noted among the doses of CF, except at 5h post induction where 400mg/kg showed a statistically significant inhibition ($p < 0.05$; %A = 39.31) as compared to 100mg/kg (%A = 12.63), and the effect at this hour was found to increase dose dependently (R^2 = 0.931).

A significant inhibition of paw edema occurred with 200mg/kg of aspirin from the 1^{st} h ($p < 0.01$) till the 5^{th} h after carrageenan injection ($p < 0.001$ from 2^{nd} - 5^{th} h) as compared to the negative control. Moreover, no difference in onset and duration of action was observed among all tested doses of the AF, 200 and 400mg/kg of the BF, and 200mg/kg of aspirin; as all showed significant inhibition of paw edema from the 1^{st} h till the 5^{th} h post induction. Nevertheless, 200 and 400mg/kg of the AF showed higher anti-inflammatory effect (%A) than that of 200mg/kg of aspirin throughout the observation period, whereas 100mg/kg of AF had shown a comparable anti-inflammatory effect with 200mg/kg of aspirin (Table 2).

Therefore, it can be concluded that, the AF was the most active fraction in terms of anti-inflammatory effects on carrageenan induced mice paw edema. This is evidenced by the higher percent inhibition (%A) values of all tested doses of AF throughout the observation period as compared to the equivalent doses of the BF and CF.

Table 2: Effects of the solvent fractions of *Z.scabra* on carrageenan-induced mouse paw edema

Treatment Group	**Mean increase in paw Volume ± S.E.M and [% Inhibition (%A)]**				
	1hr	**2hr**	**3hr**	**4hr**	**5hr**
Distilled H_20	0.490 ± 0.037	0.560 ± 0.020	0.598 ± 0.028	0.5167 ± 0.030	0.4350 ± 0.024
2% Tween 80	0.537 ± 0.025	0.563 ± 0.027	0.610 ± 0.025	0.543 ± 0.023	0.475 ± 0.026
ASA200mg/kg	0.343 ± 0.025^{a2} [29.94]	0.355 ± 0.026^{a3} [36.61]	0.388 ± 0.033^{a3} [35.10]	0.282 ± 0.023^{a3} [45.48]	0.180 ± 0.023^{a3} [58.62]
AF 100mg/kg	0.345 ± 0.023^{a2} [29.59]	0.338 ± 0.022^{a3} [39.59]	0.378 ± 0.022^{a3} [36.77]	0.303 ± 0.024^{a3d1e1} [41.3]	0.213 ± 0.020^{a3d1e2} [50.97]
AF 200mg/kg	0.318 ± 0.021^{a2} [35.04]	0.277 ± 0.027^{a3} [50.59]	0.288 ± 0.028^{a3} [51.81]	0.185 ± 0.024^{a3} [64.2]	0.122 ± 0.012^{a3} [72.02]
AF 400mg/kg	0.317 ± 0.024^{a2} [35.37]	0.272 ± 0.015^{a3} [51.48]	0.267 ± 0.034^{a3b1} [55.42]	0.176 ± 0.026^{a3b1} [65.8]	0.103 ± 0.014^{a3} [76.25]
BF 100mg/kg	0.428 ± 0.023 [12.59]	0.465 ± 0.018^{a1b1e1}[16.96]	0.493 ± 0.022^{b1e2}[17.55]	0.433 ± 0.023^{b2e2} [16.14]	0.348 ± 0.026^{b3d1e2} [19.93]
BF 200mg/kg	0.377 ± 0.022^{a1} [23.12]	0.403 ± 0.030^{a3} [27.98]	0.425 ± 0.028^{a2} [28.97]	0.333 ± 0.035^{a3} [35.49]	0.247 ± 0.030^{a3} [43.29]
BF 400mg/kg	0.350 ± 0.018^{a2} [28.57]	0.357 ± 0.015^{a3} [36.30]	0.363 ± 0.011^{a3} [39.28]	0.272 ± 0.016^{a3} [47.42]	0.212 ± 0.013^{a3} [51.33]
CF 100mg/kg	0.495 ± 0.023^{b2} [7.77]	0.518 ± 0.026^{b2} [7.99]	0.567 ± 0.029^{b2} [7.10]	0.490 ± 0.028^{b3} [9.81]	0.415 ± 0.024^{b3e1} [12.63]
CF 200mg/kg	0.480 ± 0.031^{b1} [10.60]	0.512 ± 0.038^{b2} [9.16]	0.537 ± 0.044^{b1} [12.02]	0.427 ± 0.025^{f1b2} [21.50]	0.343 ± 0.025^{f2b2} [27.73]
CF 400mg/kg	0.482 ± 0.031^{b1} [10.20]	0.500 ± 0.022^{b1} [11.20]	0.488 ± 0.025 [19.95]	0.403 ± 0.036^{f2b1} [25.80]	0.288 ± 0.030^{f3b1} [39.31]

Values are expressed as Mean ± S.E.M.; n = 6; Values in parenthesis shows % inhibition of paw edema; [a] compared with distilled H_20, [b] compared with ASA 200mg/kg, [d] compared with 200 mg/kg of respective fraction, [e] compared with 400 mg/kg of respective fraction, [f] compared with 2% tween 80; [1]*p<0.05*, [2]*p<0.01*, [3]*p<0.001*; AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction.

4.3 Formaldehyde induced Arthritis

4.3.1 Effects of crude 70% ethanol extract of *Z.scabra*

Subplantar injection of 0.05 ml of 2 % v/v formaldehyde to the mice hind paw on the 1st and 3rd day of treatment led to development of arthritis which reached a peak edema on the 5th day from the first induction in both distilled water and 2% tween 80 received negative controls (Table 3). The highest tested dose, 400mg/kg of 70EE, produced a significant inhibition of paw edema starting from day 1 of treatment ($p < 0.05$) and the effect persisted till day 10 ($p < 0.01$ at day2; $p < 0.001$ from day 3 - 10) as compared to the negative control. The middle dose, 200mg/kg of 70EE, also showed a significant inhibition of paw edema that began from day 2 of treatment ($p < 0.05$) and the effect persisted from days 3 - 10 of treatment with $p < 0.001$ except at day 7 where $p < 0.01$. The lowest dose (100mg/kg) of 70EE, on the other hand, demonstrated a significant inhibition of paw edema at days 3 ($p < 0.01$), 5 ($p < 0.05$), 8 ($p < 0.01$), 9 ($p < 0.05$) and 10 ($p < 0.01$) as compared to the negative control.

Maximum anti-inflammatory effect by the 100mg/kg (%A = 25.58) and 200mg/kg (%A= 42.95) of 70EE was observed at day 3 of treatment, right before the second induction dose of formaldehyde. However, 400mg/kg of 70EE showed maximum effect (%A = 60.5) at day 10 of treatment. Intergroup comparison among doses of the 70EE showed significantly different effect in 400 versus 100 mg/kg (days 3 - 5, $p < 0.01$ and days 6 - 10, $p < 0.001$), 400 versus 200 mg/kg (days 6 - 10, $p < 0.05$; except at day 8 where $p < 0.01$), and 200 versus 100mg/kg (day 3, $p < 0.05$). Moreover, the anti-inflammatory effect on the last day of treatment (day 10) was found to increase in dose dependent manner ($R^2 = 0.997$).

The standard drug, 200mg/kg aspirin, produced a significant inhibition of paw edema from day 1 ($p < 0.05$) till day 10 of treatment ($p < 0.001$ from day 2 - 10) as compared to the negative control. Moreover, 400mg/kg of 70EE was comparable with 200mg/kg of aspirin with regard to both the onset and magnitude of anti-inflammatory effect, as both showed statistically significant percent inhibition starting from the 1st day of treatment.

Table 3: Effects of crude 70EE of *Z.scabra* on formaldehyde-induced arthritis in mice

Treatment group	**Mean increase in paw Volume (ml) ± S.E.M and [% Inhibition (%A)]**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Distilled H_20	0.450 ± 0.029	0.533 ± 0.023	0.645 ± 0.024	0.878 ± 0.025	0.887 ± 0.028
ASA 200mg/kg	0.347 ± 0.029^{a1} [22.89]	0.295 ± 0.031^{a3} [44.65]	0.238 ± 0.025^{a3} [63.10]	0.597 ± 0.037^{a3} [32.00]	0.550 ± 0.028^{a3} [37.99]
70EE 100mg/kg	0.367 ± 0.024 [18.44]	0.436 ± 0.027^{b2} [18.20]	0.480 ± 0.024^{a2b3d1e2}[25.58]	0.767 ± 0.029^{b2e2} [12.64]	0.748 ± 0.020^{a1b3e2} [15.67]
70EE 200mg/kg	0.353 ± 0.010 [21.56]	0.398 ± 0.016^{a1} [25.33]	0.368 ± 0.025^{a3b1} [42.95]	0.666 ± 0.031^{a3} [24.15]	0.656 ± 0.030^{a3} [26.04]
70EE 400mg/kg	0.332 ± 0.026^{a1} [26.22]	0.363 ± 0.033^{a2} [31.89]	0.307 ± 0.031^{a3} [52.40]	0.607 ± 0.023^{a3} [30.87]	0.578 ± 0.032^{a3} [34.84]

Treatment group	**Mean increase in paw Volume (ml) ± S.E.M and [% Inhibition (%A)]**				
	Day 6	**Day 7**	**Day 8**	**Day 9**	**Day 10**
Distilled H_20	0.808 ± 0.031	0.693 ± 0.031	0.642 ± 0.033	0.566 ± 0.035	0.477 ± 0.034
ASA 200mg/kg	0.512 ± 0.025^{a3} [36.63]	0.452 ± 0.026^{a3} [34.85]	0.400 ± 0.025^{a3} [37.69]	0.310 ± 0.029^{a3} [45.23]	0.232 ± 0.028^{a3} [51.40]
70EE 100mg/kg	0.713 ± 0.030^{b3e3}[11.76]	0.610 ± 0.031^{b2e3} [12.02]	0.502 ± 0.030^{a2e3} [21.81]	0.455 ± 0.026^{a1b2e3} [19.61]	0.382 ± 0.015^{a1b2e3}[19.92]
70EE 200mg/kg	0.607 ± 0.018^{a3e1}[24.88]	0.518 ± 0.014^{a2e1} [25.24]	0.460 ± 0.016^{a3e2} [28.35]	0.383 ± 0.009^{a3e1} [32.33]	0.308 ± 0.016^{a3e1} [35.33]
70EE 400mg/kg	0.487 ± 0.033^{a3} [39.73]	0.407 ± 0.027^{a3} [41.34]	0.322 ± 0.018^{a3} [49.84]	0.257 ± 0.017^{a3} [54.59]	0.188 ± 0.019^{a3} [60.50]

Values are expressed as Mean ± SEM; n = 6; Values in parenthesis shows % inhibition of paw edema (%A); [a] compared with distilled H_20, [b]compared with 200mg/kg ASA, [d]compared with 200 mg/kg of 70EE, [e]compared with 400 mg/kg of 70EE; [1]*p<0.05*, [2]*p<0.01*, [3]*p<0.001*; 70EE: 70% Ethanol crude extract

4.3.2 Effect of solvent fractions of *Z.scabra*

As shown in Table 4, the aqueous fraction (AF) significantly ($p < 0.001$) inhibited paw edema at all tested doses (100, 200 and 400mg/kg) starting from the 2nd day of treatment till the 10th day as compared to the negative control. The 100mg/kg of AF showed maximum anti-inflammatory effect (%A = 69.41; $p < 0.001$) on the 3rd day of treatment, i.e. right before the second induction dose of formaldehyde. On the other hand, 200mg/kg and 400mg/kg doses of the AF produced maximum anti-inflammatory effect on the 10th day of treatment with respective values of 82.60 % ($p < 0.001$) and 88.51% ($p < 0.001$).

Intergroup comparison among doses of the AF revealed a statistically significant different effect in both 200 versus 100 mg/kg ($p < 0.01$, on days 5 - 9; $p < 0.05$ on day 10) and 400 versus 100 mg/kg ($p < 0.001$, on days 5 - 8; $p < 0.01$ on days 4, 9 and 10). Moreover, the anti-inflammatory effet on the last day of treatment (day 10) was also found to increase in dose dependent manner ($R^2 = 0.729$).

The higher tested doses of butanol fraction (BF), 200mg/kg and 400mg/kg, also demonstrated a significant inhibition of paw edema as compared to the negative control starting from the 2nd day of treatment till the 10th day with $p < 0.001$, except on days 4, 9 and 10 where $p < 0.01$ for 200mg/kg. But, the 100mg/kg of BF showed a significant inhibition of paw edema only on day 2 ($p < 0.01$) and day 3 ($p < 0.001$) as compared to the negative control. Maximum anti-inflammatory effect (%A) was observed on day 3 of treatment for all doses of BF as compare to the negative control.

Intergroup comparison among doses of the BF revealed a statistically significant different effect in 200 mg/kg versus 100 mg/kg ($p < 0.05$, on days 5, 7, and 8), 400 mg/kg versus 200 mg/kg ($p < 0.05$, on days 5 and 7), and in 400 mg/kg versus 100 mg/kg ($p < 0.05$ on day 4, $p < 0.001$ on days 5 - 8, and $p < 0.01$ on days 9 and 10). Moreover the anti-inflammatory effet of the BF on day 10 of treatment was also found to increase in dose dependent manner ($R^2 = 0.974$).

The chloroform fraction (CF) at dose of 400mg/kg exhibited a significant inhibition of paw edema as compared to 2% tween vehicle controls from days 2 - 10; $p < 0.05$ on day 2, $p < 0.01$ on day 4, and $p < 0.001$ from days 5 - 10 and on day 3. The CF at 200mg/kg also showed a significant inhibition of paw edema as compared to the negative control; $p < 0.05$ on day 4; $p < 0.01$ on days 3, 5, 6, 8 and 9; and $p < 0.001$ on days 7 and 10. On the other hand, 100mg/kg of CF showed a significant inhibition of paw edema on day 3 ($p < 0.01$) and lately on day 8 ($p < 0.05$), day 9 ($p < 0.05$) and day 10 ($p < 0.01$).

Intergroup comparison among doses of the CF disclosed a statistically significant different effect in both 400 mg/kg versus 200 mg/kg ($p < 0.05$ on days 6, 8 and 9; and $p < 0.01$ on day 10) and 400 mg/kg versus 100 mg/kg ($p < 0.01$ on days 5 - 7 and $p < 0.001$ on days 8 - 10). In addition, maximum percentage inhibition by all tested doses of CF was noted at the 10^{th} day of treatment on which the effect was found to increase in dose dependent manner ($R^2 = 0.987$).

The reference drug, 200mg/kg of aspirin, showed significant inhibition of paw edema beginning from day 2 of treatment and the effect lasted till day 10 with $p < 0.001$, but at day 4 $p < 0.01$ as compared to the negative control. Maximum percentage inhibition (57.44%) by 200mg/kg of aspirin was noted on day 3 of treatment. Moreover, no significant difference in onset of action was observed among aspirin, all the three doses of AF, 200 and 400mg/kg of BF, and the highest dose (400mg/kg) of CF as all showed a statistically significant inhibition starting from the 2^{nd} day of treatment. Furthermore, 100mg/kg of AF and the BF at 200 and 400mg/kg showed a comparable anti-inflammatory effect (%A) with 200mg/kg of aspirin. The 200 and 400mg/kg of AF, on the other hand, exhibited a significantly higher anti-inflammatory effect than 200mg/kg aspirin throughout days 3 - 10 as shown in Table 4.

Even though all the three fractions, of course at different doses, showed a significant inhibition of paw edema as compared to their respective negative controls, the AF was found to be the most active fraction in terms of anti-inflammatory effect on formaldehyde induced arthritis in mice. This is evidenced by the higher percent inhibition value of the AF throughout the observation period as compared to the equivalent doses of BF and CF.

Table 4: Effects of the solvent fractions of *Z.scabra* on formaldehyde-induced arthritis in mice

Treatment group	**Mean increase in paw Volume (ml) ± S.E.M and [% Inhibition (%A)]**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Distilled H_20	0.482 ± 0.026	0.558 ± 0.023	0.627 ± 0.025	0.832 ± 0.017	0.855 ± 0.015
2% Tween	0.515 ± 0.031	0.615 ± 0.030	0.640 ± 0.019	0.812 ± 0.024	0.827 ± 0.021
ASA200mg/kg	0.363 ± 0.027 [24.58]	0.308 ± 0.032^{a3} [44.78]	0.267 ± 0.023^{a3} [57.44]	0.643 ± 0.025^{a2} [22.65]	0.563 ± 0.026^{a3} [34.12]
AF100mg/kg	0.383 ± 0.029 [20.43]	0.262 ± 0.028^{a3} [53.13]	0.192 ± 0.031^{a3} [69.41]	0.600 ± 0.045^{a3e2} [27.86]	0.580 ± 0.047^{a3d2e3}[32.16]
AF200mg/kg	0.408 ± 0.045 [15.24]	0.258 ± 0.026^{a3} [53.73]	0.153 ± 0.025^{a3b1} [75.54]	0.492 ± 0.026^{a3b1} [40.88]	0.402 ± 0.046^{a3b1} [53.02]
AF400mg/kg	0.393 ± 0.025 [18.35]	0.235 ± 0.031^{a3} [57.91]	0.138 ± 0.022^{a3b1} [77.93]	0.447 ± 0.027^{a3b2} [46.29]	0.333 ± 0.026^{a3b2} [61.02]
BF100mg/kg	0.425 ± 0.031 [11.77]	0.372 ± 0.034^{a2} [33.42]	0.323 ± 0.037^{a3} [48.41]	0.715 ± 0.038^{e1} [14.03]	0.768 ± 0.043^{b2d1e3} [10.14]
BF200mg/kg	0.400 ± 0.030 [16.96]	0.345 ± 0.029^{a3} [38.21]	0.280 ± 0.025^{a3} [55.32]	0.662 ± 0.026^{a2} [20.44]	0.617 ± 0.026^{a3e1} [27.87]
BF400mg/kg	0.387 ± 0.030 [19.72]	0.288 ± 0.028^{a3} [48.36]	0.222 ± 0.026^{a3} [64.62]	0.578 ± 0.034^{a3} [30.47]	0.492 ± 0.033^{a3} [42.49]
CF100mg/kg	0.455 ± 0.016 [11.65]	0.525 ± 0.023^{b3} [14.63]	0.497 ± 0.026^{f2b3} [22.39]	0.737 ± 0.022 [9.24]	0.720 ± 0.030^{b2e2} [12.91]
CF200mg/kg	0.445 ± 0.032 [13.59]	0.522 ± 0.030^{b3} [15.17]	0.472 ± 0.031^{f2b3} [26.3]	0.695 ± 0.029^{f1} [14.38]	0.672 ± 0.029^{f2} [18.75]
CF400mg/kg	0.423 ± 0.023 [17.81]	0.468 ± 0.029^{f1b2} [23.85]	0.388 ± 0.034^{f3b1} [39.33]	0.627 ± 0.037^{f2} [22.79]	0.567 ± 0.033^{f3} [31.45]

Treatment	Mean increase in paw Volume (ml) ± S.E.M and [% Inhibition (%A)]…Cont…				
	Day 6	**Day 7**	**Day 8**	**Day 9**	**Day 10**
Distilled H_20	0.797 ± 0.022	0.715 ± 0.027	0.610 ± 0.039	0.522 ± 0.034	0.450 ± 0.035
2% Tween	0.758 ± 0.020	0.698 ± 0.016	0.600 ± 0.017	0.553 ± 0.023	0.492 ± 0.031
ASA200mg/kg	0.498 ± 0.026^{a3} [37.45]	0.442 ± 0.022^{a3} [38.22]	0.370 ± 0.019^{a3} [39.34]	0.327 ± 0.027^{a3} [37.38]	0.262 ± 0.024^{a3} [41.84]
AF100mg/kg	0.505 ± 0.048^{a3d2e3} [36.61]	0.423 ± 0.049^{a3d2e3} [40.80]	0.353 ± 0.039^{a3d2e3} [42.08]	0.273 ± 0.036^{a3d2e2} [47.61]	0.201 ± 0.036^{a3d1e2} [55.18]
AF200mg/kg	0.315 ± 0.037^{a3b2} [60.46]	0.227 ± 0.032^{a3b2} [68.29]	0.172 ± 0.027^{a3b2} [71.85]	0.122 ± 0.020^{a3b3} [76.67]	0.078 ± 0.015^{a3b3} [82.60]
AF400mg/kg	0.243 ± 0.027^{a3b3} [69.46]	0.185 ± 0.017^{a3b3} [74.13]	0.132 ± 0.013^{a3b3} [78.41]	0.097 ± 0.011^{a3b3} [81.46]	0.052 ± 0.010^{a3b3} [88.51]
BF100mg/kg	0.675 ± 0.047^{b2e3} [15.28]	0.590 ± 0.041^{b1d1e3} [17.48]	0.518 ± 0.041^{b1d1e3} [15.03]	0.427 ± 0.037^{e2} [18.21]	0.347 ± 0.037^{e2} [22.96]
BF200mg/kg	0.540 ± 0.028^{a3} [32.22]	0.457 ± 0.029^{a3e1} [36.13]	0.373 ± 0.033^{a3} [38.8]	0.316 ± 0.038^{a2} [39.29]	0.262 ± 0.030^{a2} [41.84]
BF400mg/kg	0.408 ± 0.036^{a3} [48.75]	0.330 ± 0.028^{a3} [53.85]	0.265 ± 0.025^{a3} [56.56]	0.220 ± 0.022^{a3} [57.83]	0.168 ± 0.017^{a3} [62.6]
CF100mg/kg	0.657 ± 0.031^{b2e2} [13.40]	0.592 ± 0.038^{b2e2} [15.27]	0.483 ± 0.037^{f1b1e3} [19.45]	0.422 ± 0.034^{f1e3} [23.78]	0.346 ± 0.031^{f2e3} [29.49]
CF200mg/kg	0.597 ± 0.035^{f2e1} [21.31]	0.503 ± 0.034^{f3} [27.92]	0.420 ± 0.034^{f2e1} [30.00]	0.368 ± 0.035^{f2e1} [33.44]	0.302 ± 0.027^{f3e2} [38.64]
CF400mg/kg	0.473 ± 0.028^{f3} [37.58]	0.406 ± 0.026^{f3} [41.76]	0.286 ± 0.020^{f3} [52.22]	0.227 ± 0.021^{f3} [59.03]	0.150 ± 0.015^{f3b1} [69.49]

Values are expressed as Mean ± SEM; n = 6; Values in parenthesis shows % inhibition of paw edema; [a] compared with distilled H_20, [b] compared with ASA 200mg/kg, [d] compared with 200 mg/kg of respective fraction, [e] compared with 400 mg/kg of respective fraction, [f] compared with 2% Tween 80; [1]*p<0.05*, [2]*p<0.01*, [3]*p<0.001*; AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction

4.4 Cotton pellet induced granuloma

Subcutaneous implantation of two pellets of cotton each weighting 10 ± 1 mg in groin region of rats induced granulomatous inflammation with a maximum granuloma weight and exudates observed in distilled water and 2% tween 80 received negative controls as shown in Table 5. The aqueous fraction (AF) at all tested doses significantly inhibited the formation of inflammatory exudates ($p < 0.001$) and granuloma mass ($P < 0.01$ for 100mg/kg; $p < 0.001$ for 200 and 400mg/kg) as compared to the negative control.

Intergroup comparisons among doses of the AF revealed a statistically significant different effect in 400 versus 200mg/kg ($p < 0.05$ in exudates inhibition; $p < 0.001$ in granuloma inhibition), 400 versus 100mg/kg ($P < 0.001$ in both exudates and granuloma inhibition), and 200 versus 100mg/kg ($p < 0.001$ in exudates inhibition). Furthermore, the anti-inflammatory effect of the AF was found to increase in dose dependent manner ($R^2 = 0.829$ for exudates inhibition; $R^2 = 1$ for granuloma inhibition).

All tested doses of the chloroform fraction (CF) significantly ($p < 0.001$) inhibited the formation of both inflammatory exudates and granuloma mass as compared to the 2% tween negative controls. Comparison among doses of the CF revealed a statistically significant different effect in 200 versus 100mg/kg ($p < 0.01$ in exudates inhibition), 400 versus 200mg/kg ($P < 0.01$ in granuloma inhibition), and 400 versus 100mg/kg ($p < 0.001$ in both exudates and granuloma inhibition). Besides, anti-inflammatory effect of the CF was ascertained to increase in dose dependent manner ($R^2 = 0.928$ for exudates inhibition; $R^2 = 0.998$ for granuloma inhibition). Furthermore, maximum anti-proliferative effect (peak percentage inhibition of granuloma formation, 55.52%) was shown by 400mg/kg of CF as compared to all other doses of the CF, BF and AF.

The butanol fraction (BF), on the other hand, showed a significant inhibition of both inflammatory exudates ($p < 0.001$) and granuloma mass ($p < 0.01$) only at the highest tested dose (400mg/kg) as compared to the distilled water vehicle control. The 200mg/kg of BF showed a significant ($p < 0.05$) inhibition of exudates formation but no significant protection against granuloma formation was noted as compared to the negative control.

The 100mg/kg of BF, on the other hand, failed to demonstrate a significant inhibition of both exudates and granuloma formation as compared to the negative control. Intergroup comparisons among doses of the BF revealed a statistically significant different protection against exudates formation in 400 versus 200mg/kg ($p < 0.05$) and 400 versus 100mg/kg ($p < 0.01$). But, no significant difference was observed among all the three doses of BF regarding inhibition of granuloma mass formation.

The reference drug, 0.5mg/kg of dexamethasone, significantly ($p < 0.001$) inhibited the formation of both exudates (%A = 45.07) and granuloma mass (%A = 65.99) as compared to the negative control. The highest tested dose (400mg/kg) of both the AF and CF showed a comparable inhibition of exudates formation with the reference drug. But a significant ($P < 0.01$ and $P < 0.001$) difference was noted when all doses of the three fractions were compared with dexamethasone in terms of granuloma inhibition (Table 5).

As the results of this model revealed, the AF and CF were comparably effective at all tested doses in inhibiting cotton pellet induced exudates formation. Whereas, the CF was the most active fraction in inhibiting formation of granuloma mass as evidenced by the higher percentage of granuloma inhibition as compare to the respective doses of the AF and BF.

Table 5: Effects of solvent fractions of *Z.scabra* on cotton pellet induced granuloma in rats

Treatment group	**Mean weight of exudates (mg) ± S.E.M and [% inhibition]**	**Mean weight of granuloma (mg) ± S.E.M and [% inhibition]**
Distilled H_20	159.28 ± 1.93	32.61 ± 0.76
2% Tween	152.72 ± 2.08	34.2833 ± 0.44
Dexa 0.5mg/kg	87.50 ± 2.05^{a3} [45.07]	11.0917 ± 0.44^{a3} [65.99]
AF 100mg/kg	$122.02 \pm 2.37^{a3b3d3e3}$ [23.39]	$28.9333 \pm 0.83^{a2b3e3}$ [11.27]
AF 200mg/kg	99.81 ± 1.91^{a3b2e1} [37.34]	$26.3833 \pm 0.66^{a3b3e3}$ [19.09]
AF 400mg/kg	90.63 ± 2.34^{a3} [43.10]	21.3250 ± 0.50^{a3b3} [34.61]
BF 100mg/kg	152.23 ± 1.77^{b3e2} [4.43]	30.6750 ± 1.09^{b3} [5.93]
BF 200mg/kg	150.82 ± 1.58^{a1b3e1} [5.31]	29.6917 ± 0.86^{b3} [8.95]
BF 400mg/kg	142.21 ± 1.29^{a3b3} [10.72]	27.7483 ± 0.65^{a2b3} [14.91]
CF 100mg/kg	$109.07 \pm 3.09^{f3b3d2e3}$ [28.58]	21.01 ± 0.84^{f3b3e3} [38.72]
CF 200mg/kg	97.40 ± 2.38^{f3b1} [36.22]	18.85 ± 0.59^{f3b3e2} [45.02]
CF 400mg/kg	88.61 ± 0.95^{f3} [41.98]	15.25 ± 0.66^{f3b2} [55.52]

Values are expressed as Mean ± S.E.M; n = 6; Values in parenthesis shows % inhibition (%A); [a] compared with distilled H_20, [b] compared with dexa 0.5 mg/kg, [d] compared with 200 mg/kg of respective fraction, [e] compared with 400 mg/kg of respective fraction, [f] compared with 2% Tween 80; [1]*p<0.05*, [2]*p<0.01*, [3]*p<0.001*; AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction; Dexa: dexamethasone.

4.5 Phytochemical screening

Preliminary phytochemical screening for secondary metabolites was carried out on the crude 70EE, and solvent fractions of *Z.scabra*. The result revealed a differential distribution of secondary metabolites into the solvent fractions as shown in Table 6.

Table 6: Preliminary phytochemical screening of 70EE and solvent fractions of *Z.scabra*

Secondary metabolites	**Crude 70EE**	**AF**	**BF**	**CF**	**Distilled water**	**2% Tween 80**
Alkaloids	+	++	+	-	-	-
Tannins	+	+	++	-	-	-
Saponins	+	++	-	-	-	-
Terpenoids	+	++	+	-	-	-
Steroids	+	-	-	++	-	-
Flavonoids	+	-	-	++	-	-
Anthraquinones	-	-	-	-	-	-
Cardiac glycosides	++	+	+	-	-	-

(+, Present); (-, Abscent); (++, relatively concentrated as compared to the crude extract)

5. DISCUSSION

Zehneria scabra has been used in folk medicine of Ethiopia for the management of different inflammatory pathologies. Its use in different inflammatory conditions is recorded in ethnobotanical studies of Ethiopia with high fidelity rate; e.g. FL= 100% for febrile conditions by the ethnic groups of Gondar Zuria district (Birhanu, 2013); 86% for 'mich' by people in Zegie Peninsula (Teklehaymanot and Giday, 2007); 95% for febrile conditions by people of Ankober district, North Shewa Zone (Lulekal *et al*., 2013) and people of Bahirdar Zuria district (Ragunathan and Abay, 2009).

In line with this notion, Akele (2012) has elucidated the anti-nociceptive and acute anti-inflammatory activity of the hydro-alcoholic crude leave extract of *Z.scabra* using acetic acid induced writhing test and carrageenin-induced edema model, respectively. Despite this, no reports about the anti-inflammatory activity of the crude extract and further fractions in experimental models of sub-acute and chronic inflammation were recorded in literatures. Thus, the present study is the first one to demonstrate the anti-inflammatory activity of 70% ethanol leave extract and solvent fractions of *Z.scabra* using a battery of pharmacological inflammatory models ranging from the acute to chronic ones.

The inflammatory response is a polyphasic tissue reaction, which ranges from short lived increase in vascular permeability to a prolonged cellular infiltration and proliferation. So it is important to evaluate the performance of agents claimed for anti-inflammatory effect via a battery of tests valid for various phases of inflammation (Kumar *et al*., 2012).

The carrageenan induced paw edema model is a prototype of exudative phase of acute inflammation (Divakar *et al*., 2010; Sarkar, 2015). Carrageenan as a phlogistic agent is non antigenic and is devoid of apparent systemic effect (Igbe and Inarumen, 2013). Hence, injection of carrageenan induces localized inflammation in two different phases through sequential release of several mediators. The initial phase, which occurs between 0 and 2.5 h after carrageenan injection, has been attributed to the action of mediators such as histamine, serotonin and bradykinin on vascular permeability. Histamine and serotonin

are mainly released during the first 1.5 h while bradykinin is released from 1.5 to 2.5 h after carrageenan injection (Masresha *et al.*, 2012).

The second phase (2.5 - 6 h post-carrageenan injection) is a result of overproduction of COX-2 and its pro-inflammatory PG products, with infiltration of polymorphonuclear leucocytes (neutrophils) (Dawson *et al*., 1991; Coura *et al*., 2015). The peak inflammation is usually seen approximately 2 - 3 h post carrageenan injection and is attributed to PG release (Kumar *et al*., 2012; Silva-Neto *et al*., 2014). Hence, this second phase appears to be the most interesting phase in terms of inflammatory processes. The carrageenan-induced paw edema is known to be sensitive to COX inhibitors but not to 5-LOX inhibitors, and hence has been used to evaluate the effect of NSAIDs which primarily inhibit the COX pathway, i.e. single-action NSAIDs such as aspirin. It has been demonstrated that the suppression of carrageenin-induced hind paw edema after the 3rd h correlates reasonably with therapeutic doses of most clinically effective anti-inflammatory agents (Panthong *et al*., 2007; Mathew *et al*., 2014). Thence, in this study carrageenan-induced hind paw edema was used as an appropriate acute model of inflammation and aspirin was opted as reference drug.

The formaldehyde induced arthritis model, on the other hand, represents a sub-acute phase characterized by increased migration of leucocytes and phagocytes to the area of inflammation (Divakar *et al*., 2010). Hence, the inhibition of formaldehyde-induced edema is one of the most suitable methods to evaluate anti-proliferative activity and screen anti-arthritic agents. Injection of formaldehyde into mice hind paw produces localized inflammation and pain which is biphasic in nature. During the first neurogenic phase (0-5 min), pain is initiated due to the direct chemical stimulation of nociceptors and is thought to be mediated by substance-P and bradykinin (Kaithwas *et al*., 2012; Sanusi *et al*., 2013). The second phase (15 min post induction) appears to be an inflammatory phase during which histamine, serotonin, prostaglandin and bradykinin become key mediators and thus could be inhibited by peripherally acting anti-inflammatory drugs such as aspirin. Leukocyte migration into the inflamed site will commence late in this phase (2.5 - 6 h) and is considered to be the most important process in the inflammatory

response (Silva-Neto *et al*., 2014; Cui *et al*., 2014). In line with this notion, on the 1st day of induction paw edema was determined 3 h following formaldehyde injection.

Cotton pellet induced granuloma model is one of the most commonly employed models in animal research to screen for chronic anti-inflammatory activity of drugs and novel natural products (Roome *et al*., 2014). In this model, the transudative phase causes an increase in the wet weight of the cotton pellet while hosting inflammatory response to the implanted cotton between days 3 - 6 causes granuloma formation due to proliferation of fibroblasts and infiltration of modified macrophages and lymphocytes. Hence, the increase in dry weight is considered as a measure of proliferative component of chronic inflammation (Bagad *et al*., 2013). This model was, therefore, used for further verification of the anti-inflammatory activity of solvent fractions of *Z.scabra* on the transudative and proliferative components of chronic inflammation. Steroidal anti-inflammatory drugs were found to demonstrate higher activity in this model (Andrade *et al*., 2007), and hence dexamethasone was opted as a reference drug.

The anti-inflammatory activity of hydroalcoholic crude leaves extract of *Z.scabra*, in carrageenan induced paw edema, was reported by Akele (2012). In the present study, the anti-inflammatory potential of 70% ethanol crude leaves extract of *Z.scabra* was first tested against formaldehyde induced arthritis, which is one of the most suitable test procedures to screen anti-arthritic and sub-acute anti-inflammatory activity of natural products as it closely resembles human arthritis (Deshpande *et al*., 2011). In this model, the crude 70EE at 200 and 400mg/kg doses demonstrated a statistically significant inhibition of paw edema from day 2 till day 10 of treatment as compared to the negative control (Table 3). In addition, 400mg/kg of 70EE showed a comparable anti-inflammatory effect (%A) with 200mg/kg of aspirin. This could be due to certain alterations in the inflammatory response with possible anti-arthritic potential comparable with the standard drug aspirin.

Furthermore, the result was also in agreement with the anti-inflammatory effects of the hydroalcoholic crude extract on carrageenan-induced acute inflammation, as reported by Akele (2012), and expands the evidence of its anti-arthritic and anti-proliferative efficacy

on sub-acute model of inflammation. The result obtained for the 70EE in formaldehyde induced arthritis model was also in line with those reported elsewhere (Umukoro and Ashorobi, 2006; Deshpande *et al*., 2011; Reddy *et al*., 2015), where extracts of *Momordica charantia*, *Coccinia grandis,* and *Momordica cymbalaria* showed statistically significant inhibition of formaldehyde induced paw edema, signifying the potential anti-arthritic and anti-proliferative activity of plants in the Cucurbitaceae family.

Besides, for further evaluation of the nature of the active constituents and state the possible mechanism(s) of the anti-inflammatory activity of the plant, the 70EE was successively fractionated by partitioning into solvents of differing polarity and the anti-inflammatory activity of the fractions was evaluated by using acute, sub-acute and chronic models of inflammation.

The aqueous fraction (AF), at all tested doses, showed a statistically significant inhibition of inflammatory parameters in all the three employed models of inflammation. In the acute model (carrageenan induced paw edema), all employed doses of the AF showed a significant ($p < 0.01$ and $p < 0.001$) inhibition of paw edema starting from 1h post induction and the effect maintained till the 5^{th} h (Table 2). The significant anti-inflammatory activity of this fraction during the first 2 hours of the initial phase of inflammation could possibly be due to inhibitory effect on mediators like histamine and 5-HT. This is further evidenced by the higher inhibitory effect of all doses of the AF as compared to the respective time inhibitory effect of aspirin, which as most other NSAIDs has less effect on the first phase of carrageenan induced inflammation. No significant increment in percent inhibition values of all tested doses of the AF was noted from the 2^{nd} to the 3^{rd} h. As noted above, this is the time period where release of the mediator bradykinin reaches its peak, and hence it can be generalize that the AF may not have a significant inhibitory effect on the release or activity of bradykinin.

Furthermore, all tested doses of the AF showed more pronounced edema inhibition in the second phase of inflammation, on the 4^{th} and 5^{th} h, as compared to their respective inhibitory values in the first phase (0 - 2.5 h), achieving the maximum anti-inflammatory effect on the 5^{th} h. This indicates that the primary mechanism of anti-inflammatory effect

of the AF could be via inhibition of COX and its pro-inflammatory metabolites, such as PGs. This is substantiated by the fact that, even the lower employed dose of the AF (100mg/kg) showed a comparable inhibitory effect with that of aspirin, which as most other NSAIDs, exert a more pronounced effect on the second phase (Panthong et al., 2007; Mathew et al., 2014). Moreover, the higher employed doses, 200 and 400mg/kg, of the AF even showed a better inhibitory effect than aspirin in this late phase of inflammation which is primarily mediated through products of the inducible COX.

This finding is also in line with the previous report of Akele (2012), where the crude hydro-alcoholic extract of *Z.scabra* showed significant inhibition of carrageenan induced paw edema in the second phase of inflammation with the maximum effect being observed at the 3^{rd} h. In comparison with the report of Akele (2012) on the crude extract, it could be concluded that the phytochemicals responsible for pronounced anti-inflammatory effect on the second phase of acute inflammation could be highly concentrated in the AF. This could be explained since the equivalent doses of the crude extract showed a better percent inhibition value in the first phase (~ up to the 3^{rd} h) than doses of the AF at a given point of time; whereas from the 3^{rd} to 5^{th} h of the second phase, all tested doses of the AF showed a remarkably higher percent inhibition than the equivalent doses of the crude extract at the respective point of time. This could be possibly due to preferential partitioning of the secondary metabolites responsible for a better anti-inflammatory effect on second phase, possibly through COX inhibition, into the AF.

The results from the sub-acute and chronic models also revealed the anti-inflammatory effectiveness of this fraction. In the cotton pellet induced granuloma model, for example, all tested doses of the AF showed a statistically significant inhibition of both exudates and granuloma formation. In this model, the significant ($P < 0.001$) inhibitory effect of the AF on formation of exudates (Table5) substantiates the finding of the carrageenan induced acute model, i.e. both findings solidify the effectiveness of the AF in inhibiting the exudative and transudative component of inflammation. The statistically significant ($P < 0.01$ and $P < 0.001$) inhibition of granuloma formation, on the other hand, justify the effectiveness of this fraction in inhibiting the proliferative phase of inflammation. This could also be ascertained from the findings of the formaldehyde induced arthritic model,

in which all tested doses of the AF significantly ($p < 0.001$) inhibited development of arthritis from day 2 -10 of treatment (Table 4). Moreover, the AF at all tested doses demonstrated a considerably better inhibitory effect on formaldehyde induced arthritis as compared to equivalent doses of the crude 70EE (Table 3) from day 2 throughout to day 10 of treatment. This could be possibly due to preferential partitioning of the secondary metabolites responsible for a better anti-arthritic activity into the AF.

Phytochemical screening for secondary metabolites revealed the presence of alkaloids, saponins, tannins and terpenoids in the aqueous residue. The anti-inflammatory activity of this fraction could emanate from the presence of these secondary metabolites whose protection against inflammation is also reported elsewhere; alkaloids (Küpeli *et al*., 2002; Souto *et al*., 2011), saponins (Navarro *et al*., 2001; Ahn *et al*., 2005; Chen *et al*., 2014), and terpenoids (Heras and Hortelano, 2009; Bellik *et al*., 2012; Ku and Lin, 2013).

The mechanism of anti-inflammatory effect of these different secondary metabolites is also documented in literatures. Different terpenoids were reported to exert their anti-inflammatory effect through inhibition of PLA2 activity, inhibition of TNF-α production, inhibition of iNOS expression, inhibition of COX-2 expression, and inhibition of NF-κB activation (Bellik *et al*., 2012). Saponins were also reported to exert anti-inflammatory effect through inhibition of iNOS expression, inhibition of COX-2 expression and subsequent production of PGE2 (Ahn *et al*., 2005). The anti-inflammatory activity of different alkaloids was also reported to be mediated through inhibition of COX expression and production of PGE2 (Fukuda *et al*., 1999; Kuo *et al*., 2004), inhibition of pro-inflammatory cytokines production (IL-1β, IL-6, TNF-α) and inhibition of the expression of ICAM-1 and VCAM-1 adhesion molecules (Bellik *et al*., 2012).

From the results of the phytochemical screening, terpenoids were found to be highly concentrated in the AF, less in the BF and none in the CF. Polar solvents such as ethanol and water led to the extraction of highly oxygenated polar triterpenes and triterpenoid glycosides, whereas non-polar solvents such as chloroform and petroleum ether were found to extract most other lipid soluble terpenoids such as Sesquiterpene lactones, diterpenes, and sterols (Citoglu and Acikara, 2012). From this fact and the findings of the

phytochemical screening, highly oxygenated polar triterpenes or triterpenoid glycosides could be the major terpenoids concentrated in the AF and responsible for its anti-inflammatory activity. This could further be substantiated by lack of activity of the petroleum ether extract in the pilot study and the negative terpenoid test result of the CF for the lipid soluble terpenoids.

Other plants in the Cucurbitaceae family such as *Cayaponia tayuya* (Escandell *et al*. 2007), *Cucurbita andreana* (Jayaprakasam *et al*., 2003), *Picrorhiza scrophulariaeflora* (Smit *et al*., 2000), *Wilbrandia ebracteata* (Peters *et al*., 1999), and *Citrullus lanatus* (Abdelwahab *et al*., 2011), were also reported to contain highly oxygenated triterpenoid molecules called cucurbitacins, which were proved to have potent anti-inflammatory and anti-proliferative activities (Wakimoto *et al*., 2008; Duangmano *et al*., 2012).

All in all, from the results of the acute (Table 2) and sub-acute (Table 4) models of inflammation, and from percentage inhibition values of exudates formation in the chronic model (Table 5) the AF was found to be the most active fraction in inhibiting the exudative and transudative component of inflammation. It was also found to exhibit a significant anti-proliferative activity as revealed by the results of the sub-acute model (Table 4) and percentage inhibition values of granuloma formation in the chronic granulomateus inflammatory model (Table 5).

Moreover, the probable mechanism of anti-inflammatory effect of the phytochemicals concentrated in the AF could be explained through inhibition of the mediators like histamine, serotonin and products of COX, such as pro-inflammatory PGs. The later mechanism, i.e. inhibition of COX, could be the primary mechanism of the anti-inflammatory effect of this fraction since it showed greater effect during the second phase of the acute model of inflammation. This is further substantiated by the type of secondary metabolites found in this fraction, most of which were proved to possess COX inhibitory activities.

The butanol fraction (BF), at doses of 200 and 400mg/kg, significantly inhibited mice paw edema in both acute (Table 2) and sub-acute (Table 4) models of inflammation. In

the cotton pellet induced granuloma model, on the other hand, only the highest tested dose (400mg/kg) showed a statistically significant inhibition of both inflammatory exudates ($P < 0.001$) and granuloma formation ($P < 0.01$). Phytochemical test (Table 6) of this fraction revealed the presence of tannins, alkaloids and terpenoids, albeit the last two secondary metabolites with a relatively lower concentration as compared to the AF.

The moderate anti-inflammatory effect of the BF in the acute and sub-acte model, as compared to the AF, could be accounted for the presence of alkaloids and terpenoids, which were actually the main constituents of the most active AF. The anti-inflammatory effect of the BF could also emanate from tannins, the major concentrate. The anti-inflammatory effect of tannins was reported to be mediated through inhibition of leukocyte migration by their well known astringent properties which cause precipitation of cell membrane proteins and hence affecting cellular movements, recruitment and membrane permeability (Mota *et al*., 1985), and/or through inhibition of expression of pro-inflammatory cytokines and chemokines by blocking of transcription factors, NF-κB and AP-1 (Erdèlyi et al., 2005). These different mechanisms underlying tannins may at least partly be responsible for the moderate anti-inflammatory effect observed for higher doses of the BF in inhibition of edema (carragenan and formaldehyde induced edema models) and cellular proliferation in the chronic model.

Unlike the AF and BF, the higher doses of chloroform fraction (200 and 400mg/kg CF) showed a statistically significant anti-inflammatory effect only during the second phase (4^{th} and 5^{th} h) of carrageenan-induced paw edema (Table 2). Similarly the anti-inflammatory effect of this fraction was delayed until the 3^{rd} day of formaldehyde induced arthritis. This could probably be due to the specific inhibitory effect of phytochemicals in the CF on the synthesis or effect of pro-inflammatory PGs which mainly mediate the late phase of carrageenan-induced paw edema.

In the cotton pellet induced granuloma model, on the other hand, the CF at all employed doses showed a comparable inhibitory effect on exudates formation with the equivalent doses of the AF (Table 5). Moreover, in this model the CF at all doses was found to be the most active fraction with regard to inhibition of granuloma formation; the maximum

inhibitory effect (%A = 55.52, $p < 0.001$) being observed at dose of 400mg/kg. From these findings, i.e. the delayed anti-inflammatory effect in the acute and sub-acute model, and its profound inhibitory effect on formation of granuloma in the chronic model, it can be conclude that phytochemicals in the CF may be most effective in inhibiting pro-inflammatory prostanoids and cytokines induced cellular proliferation.

The results of the phytochemical screening revealed the preferential partitioning of steroids and flavonoids into the CF. Different literatures support the anti-proliferative efficacy of these phytochemicals; Loizou *et al.,* (2009) and Bigoniya *et al.,* (2013), for example, showed phytosterols to inhibit TNF-α induced endothelial activation and expression of ICAM-1 and VCAM-1 adhesion molecules which mediate cellular recruitment to sites of inflammation. Hernández-Valle *et al*., (2014); Han *et al*., (2015) and Wagle *et al*., (2016) on the other hand showed phytosterols to inhibit the inflammatory cytokines IL-6, IL-1β, TNF-α and on the contrary induce the production of anti-inflammatory cytokines IL-4 and IL-10.

Flavonoids, on the other hand, were reported to exhibit anti-inflammatory and anti-proliferative effect through selective inhibition of 5-LOX (Schewe *et al*., 2002; Redrejo-Rodriguez *et al*., 2004), COX-2 and/or iNOS (Tunon *et al*., 2009). Aquila *et al*., (2009) further reported the anti-inflammatory effect of flavonoids from *Cayaponia tayuya* (Cucurbitaceae) to be mediated through inhibition of COX-2 and iNOS induction. The possibility of selective COX-2 inhibition by flavonoids in the CF is further evidenced by the delayed anti-inflammatory effect shown on the second phase of the acute model.

The major products of 5-LOX pathway, LTB4 and CysLTs, in concert with the adhesion molecules ICAM-1 and VCAM-1 are known to mediate chemokinesis, firm adhesion and subsequent extravasation of leucocytes to sites of inflammation (Werz *et al*., 2002; Pelletier *et al*., 2003; Weber *et al*., 2007; Afonso *et al*., 2012). On the other hand, products of the COX pathway, especially PGE2 and PGI2, are well known for their potent vasodilatory actions and to increase vascular permeability and leukocyte infiltration (Pelletier *et al*., 2003; Smyth *et al*., 2009). Hence, the effectiveness of the CF in inhibiting the formation of inflammatory exudates and more profoundly granuloma in

the chronic model could emanate from the possible inhibitory effect of the flavonoids and phytosterols on the COX and 5-LOX pathways, and/or inhibition of inflammatory cytokines induced endothelial activation and expression of ICAM-1 and VCAM-1.

All in all, results of this study revealed that the crude extract (70EE) and solvent fractions of *Z.scabra* leaves possess anti-inflammatory activities. The overall order of efficacy in inhibiting the exudative phase of acute inflammation, as evidenced by the percentage inhibition of paw edema in the acute model, was found to be AF > BF > CF. Hence the phytochemicals in the AF could be most effective in inhibiting acute phase of inflammation. While on the contrary, the overall order of effectiveness in inhibiting the proliferative phase of chronic inflammation, as evidenced by the percentage of granuloma inhibition in the chronic model, was found to be CF > AF > BF. This indicates that the phytochemicals concentrated in the CF may be specifically effective in inhibiting the cellular response of proliferative phase of inflammation.

Such disparity in order of effectiveness of the fractions in modulating the acute and chronic phases of inflammation could be due to the differential partitioning of phytochemicals into the three fractions (Table 6) and the associated difference in mechanism of action of the secondary metabolites. Furthermore, the greater efficacy of the AF in comparison with other fractions in inhibiting acute phase of inflammation supports the traditional method of extraction of the leaves of *Z.scabra* in Ethiopia, where the leaves are boiled in water and the vapor inhaled or the leave juice is given orally for treatment of inflammatory conditions (Teklehaymanot and Giday, 2007; Ragunathan and Abay, 2009; Birhanu, 2013).

From the overall results of this study, it can be postulated that *Z.scabra* to be rich in anti-inflammatory secondary metabolites which act by different mechanisms to inhibit acute, sub-acute and chronic inflammatory conditions. Moreover, the strong anti-inflammatory activity of the 70EE and solvent fractions of *Z.scabra* in this study as compared to the standard drug aspirin may be due to cumulative effects of different active constituents in reducing the synthesis, release and/or action of different inflammatory cytokines, chemokines and mediators such as histamine, serotonin, prostaglandins and Leucotrienes.

This notion is in line with the proven concept that medicinal plants possess a combination of phytoconstituents with different anti-inflammatory mechanisms, offering synergistic or additive effects (Liu, 2003; Csaki *et al*., 2009).

The phytochemical screening in the present study revealed the presence of secondary metabolites such as alkaloids, tannins, saponins, terpenoids, steroids, flavonoids and cardiac glycosides in the crude 70EE and their differential partitioning into employed solvents of differing polarity (Table 6). Tesfaye and Alamneh (2014) reported the absence of saponins in the crude 80% methanol extract of the leaves of *Z.scabra,* although the test for other secondary metabolites was in line with the finding of the present study. Tadesse *et al*. (2014), on the other hand, reported the presence of saponins, which is in agreement with results of the present study, while Alkaloids and steroids were tested negative in 80% methanolic leaves extract of *Z.scabra*. Such discrepancy in the presence and/or absence of secondary metabolites could be accounted for seasonal and geographical variations which are known to influence the expression of phytochemical constituents in plants (Jayanthy *et al*., 2013).

Arulappan *et al*. (2015), conversely, showed the presence of phenolic compounds, steroids and glycosides and the absence of tannins, flavanoids, alkaloids and saponins in the ethanolic (absolute) and aqueous tuber extract of *Z.scabra*. Such disagreement with results of the present study could be due to the differential distribution of the secondary metabolites into different parts of the plant, i.e. the tuber and leaves, on top of the geographic variation and the difference in the extraction solvents.

In this study ethanol specifically was chosen as the solvent of extraction as it can extract a wide variety of polar as well as non polar phytochemical constituents in medicinal plants (Herman *et al*., 2013; Tatke and Rajan, 2014). Hydroalcoholic solvents (mixture of alcohol and water in varying proportions) are generally considered to give high extraction yields, owing to their expanded polarity range (Gupta *et al*., 2012). It is also hypothesized that alcoholic solvents efficiently penetrate the cell membranes, permitting the extraction of high amounts of endocellular components including phytochemicals produced in plants. Furthermore, ethanol is widely used to obtain crude extracts of phytochemicals in

the herbal medicine industry for therapeutic applications due to its relative safety (Wendakoon *et al*., 2012). Hence, 70% v/v ethanol was the solvent of choice in the present study for extracting the leaves of *Z.scabra*.

Moreover, male mice and rats were preferably used in this study for all the anti-inflammatory models. The rationale for using male sex in inflammation models is due to the fact that estrogen, the primary female sex hormone, has been confirmed to possess anti-inflammatory activity by a line of evidences (Miyamoto *et al*., 1999; Cuzzocrea *et al*., 2000; Vegeto *et al*., 2002).

6. CONCLUSION

The results of phytochemical tests revealed that leaves of *Z.scabra* are chemically enriched with alkaloids, tannins, saponins, terpenoids, steroids, flavonoids and cardiac glycosides. The results from the pharmacological tests further confirmed the aqueous fraction to be the most efficacious fraction in inhibiting exudative component of acute inflammation while the chloroform fraction showed highest activity in inhibiting the cellular response of proliferative component of chronic inflammation. The butanol fraction, on the other hand, was found to possess a moderate activity in both the acute (carrageenan induced) and sub-acute (formalin induced) paw edema models, and was found to be the least active in the cotton pellet induced granuloma chronic model.

In conclusion, the data obtained in this study demonstrated that the leaves of *Z.scabra* possess different secondary metabolites which, by acting through an array of possibly different mechanism, are effective in treatment of both acute and chronic inflammatory conditions. The current findings also demonstrated scientific rationale for the traditional use of this plant in different inflammatory conditions. It also confirms the presence of biologically active components, which are worth for further investigation and elucidation.

7. RECOMMENDATIONS

The results of this study on both the crude extract and solvent fractions of *Z.scabra* should be taken as a basis for further investigation of the anti-inflammatory action of the plant. Hence, further studies are recommended;

- To determine the sub-acute and chronic safety profile of the plant
- To isolate, purify and identify pharmacologically active anti-inflammatory principles of the AF and CF.
- To determine the exact mechanism of action of the active constituents
- To validate the efficacy of traditionally simulated preparations such as the squeezed juice of aerial part of *Z.scabria*.
- To study other pharmacologically related activities of the fractions claimed by folklore medicine, such as its analgesic and anti-pyretic activities.

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