In-vivo Anti-inflammatory Effect of the Roots of *Indigofera spicata Forssk* in mice

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A thesis submitted to Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Addis Ababa University in Partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology

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ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

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ABSTRACT

In-vivo anti-inflammatory effect of the roots of Indigofera spicata Forssk in mice

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Addis Ababa University, 2014

The search for new anti-inflammatory agents from the huge array of medicinal plant resources is intensifying because currently available anti-inflammatory drugs pose a major problem including induction of gastric or intestinal ulcers as well as cardiovascular side effects during their clinical use. A previous study indicated that the aqueous and methanol extracts of the root of the *I. spicata* possessed anti-inflammatory and analgesic activity. In an effort to further the research, the present study was aimed to investigate the anti-inflammatory effects of chloroform, absolute methanol and aqueous fractions of the roots of the plant. To this effect, different doses of the fractions (50,100 and 200 mg/kg) were investigated for anti-inflammatory activity using acute (carrageenan induced mouse paw edema) and sub acute (formalin induced mouse paw edema) model of inflammation. The methanol and aqueous fractions of *I. spicata* significantly inhibited carrageenan induced edema ($p < 0.001$) with maximal percent inhibition of 45.9 and 39.6 respectively, observed at dose of 200 mg/kg. Similarly, these fractions significantly inhibited formalin induced edema ($p < 0.001$) with maximal effect (41.5 vs. 40.4%) observed at the same dose. The chloroform fraction did not show significant inhibition of paw edema at all dose levels in both models. Phytochemical analysis revealed differential distribution of secondary metabolites. These findings suggest that the plant indeed is endowed with anti-

inflammatory activity, which could be probably attributed to active principles that are semi-polar to polar in nature.

Keywords: *I. spicata*, anti-inflammatory activity, Indomethacin, Carrageenan, Formalin

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ACRONYMS

AAU	Addis Ababa University
ANOVA	Analysis of Variance
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DMARDs	Disease-Modifying Anti-rheumatic Drugs
EHNRI	Ethiopian Health and Nutrition Research Institute
ELAM-1	Endothelial-Leukocyte Adhesion Molecule-1
EPHARM	Ethiopian Pharmaceuticals Manufacturing Share Company
ICAM-1	Intercellular Adhesion Molecule-1
NSAIDs	Non Steroidal Anti-inflammatory Drugs
OECD	Organization for Economic Co-operation and Development
PAF	Platelet Activating Factor
SEM	Standard Error of Mean
SPSS	Statistical Package for Social Sciences
TNF	Tumor Necrosis Factor
WHO	World Health Organization

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1. INTRODUCTION

1.1 Definition and Classification of Inflammation

The term inflammation is derived from the Latin "inflammare" meaning to burn. It is a physiological response of living tissues to injury. It is not, in itself, a disease, but is usually a manifestation of disease. Diseases in which an inflammatory reaction is a major component are classified accordingly. They are usually named from the organ affected followed by the suffix '-itis'. Thus, acute inflammation of the meninges is called meningitis. However, like any rule, it has its own exceptions such as in case of pneumonia, typhoid fever, etc. (Bezabeh, *et al*., 2004; Underwood, 2004). Inflammation is basically a protective response intended to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult. It accomplishes its protective function by diluting, destroying, or otherwise neutralizing harmful agents (e.g., microbes or toxins). It then sets into motion the events that eventually heal and reconstitute the sites of injury. Thus, inflammation is intimately interwoven with repair processes whereby damaged tissue is replaced by the regeneration of parenchymal cells, and/or by filling of any residual defect with fibrous scar tissue (Kumar, *et al*., 2007).

Cardinal signs of inflammation include, among others, swelling (tumour), redness (rubor), heat (calor), pain (dolor), and loss of function (functeo laesa) (Murugan, *et al.*, 2012). Inflammation requires the participation of various types of cells expressing and reacting to diverse mediators along a very precise sequence (Gouwy, *et al.*, 2005).

Inflammation is crudely classified based on duration of the lesion and histological appearances into acute and chronic inflammation. However, these basic forms of

inflammation can overlap, and many factors modify their course and histological appearance (Kumar, *et al*., 2007).

Acute inflammation, which is the initial and often transient series of tissue reactions to injury, is of relatively short duration, lasting from a few minutes up to a few days, and is characterized by fluid and plasma protein exudation and a predominantly neutrophilic leukocyte accumulation. It is associated with increased vascular permeability, capillary infiltration and emigration of leukocytes (Kumar, *et al.*, 2013; Underwood, 2004). The classical symptoms of redness, heat, edema and pain are associated with acute inflammation (Kumar, *et al*., 2007; Villarreal, *et al.*, 2001).

Chronic inflammation is of longer duration (days to years) due to the persistence of the initiating stimulus, interference of the normal healing process, repeated bouts of acute inflammation or low-grade smoldering due to continued production of immune response mediators (Whicher & Chambers, 1984). It is typified by influx of lymphocytes and macrophages with associated vascular proliferation and scarring (Kumar, *et al*., 2007).

1.2 Mediators of Inflammation

A substance that causes one of the component events in inflammation through a specific receptor is called a mediator of inflammation. Both endogenous and exogenous substances may act as mediators (Chauhan, *et al.*, 2006).

Mediators originate primarily from blood plasma (e.g. complement proteins, kinins), white blood cells (basophils, neutrophils, monocytes, and macrophages), platelets, mast cells, endothelial cells lining the blood vessels, and damaged tissue cells (e.g. histamine,

prostaglandins,cytokines). The production of active mediators is triggered by microbial products or by host proteins, such as proteins of the complement, kinins and coagulation systems that themselves are activated by microbes and damaged tissues. Generally mediators of inflammation are histamine, serotonin, nitric oxide (NO), proteins, lipids, lipoxins, prostaglandins (PGs), bradykinin, cytokines, leukotrienes (LTB_4), interleukins, tumor necrosis factors (TNFs), platelet-activation factor (PAF), endotoxin, growth factors etc (Kim, *et al.,* 2009; Posadas, *et al*., 2004; Suralkar, *et al.*, 2008).

1.3 Physiological Role of Inflammation

Inflammation is an important physiological reaction, which occurs in response to a wide variety of injurious agents (bacterial infection or physical trauma) ultimately aiming to perform the dual function of limiting damage and promoting tissue repair (Nathan, 2002). It has beneficial effects, such as the destruction of invading micro-organisms and the walling-off of an abscess cavity, thus preventing spread of infection (Underwood, 2004). The endogenous biochemical pathways activated during defense reactions can counter-regulate inflammation and promote resolution. Hence, resolution is an active process, which promises novel approaches for the treatment of inflammation-associated diseases based on endogenous agonists of resolution (Serhan *et al.,* 2007).

Inflammatory Response

The inflammatory response is a critical protective reaction to irritation, injury, or infection, characterized by redness, heat, swelling, loss of function and pain (Gautam & Jachak, 2009). Redness and heat result from an increase in blood flow, swelling is

associated with increased vascular permeability, and pain is the consequence of activation and sensitization of primary afferent nerve fibers (Calixto, *et al.,* 2003)

Inflammatory response occurs in three distinct phases. The first phase is caused by an increase in vascular permeability resulting in exudation of fluids from the blood into the interstitial space. The second phase involves the infiltrations of leukocytes from the blood into the tissue and the third phase is characterized by granuloma formation and tissue repair (Suralkar, *et al.,* 2008).

First phase: The vascular response which occurs in this phase includes changes in permeability, blood flow and adhesiveness (Villarreal, *et al.,* 2001). Increase in vascular permeability means that large molecules, such as proteins, can escape from vessels (Underwood, 2004). The increased permeability triggered by inflammatory mediators causes contraction of endothelial and perivascular cells, and, as these cells contract, fluid escapes between them (Villarreal, *et al.,* 2001). The exudate fluid has a high protein content of up to 50 g/l. The proteins present include immunoglobulins, which may be important in the destruction of invading micro-organisms, and coagulation factors, including fibrinogen, which result in fibrin deposition on contact with the extravascular tissues (Underwood, 2004). The movement of fluids plays an important role in diluting toxic factors generated at sites of trauma and infection, and allows the influx of important serum proteins, including components of the complement system and immunoglobulins (antibodies) which promote antimicrobial activity (Villarreal, *et al.,* 2001).

Second Phase: This phase is characterized by the infiltration of leukocytes to the site of injury, which is the most important feature of inflammation (Vergnolle, 2003). The accumulation of neutrophil polymorphs within the extracellular space is the diagnostic histological feature of acute inflammation. In normal circulation, cells are confined to the axial stream in blood vessels, and do not flow in the peripheral plasmatic zone near to the endothelium However, loss of intravascular fluid and an increase in plasma viscosity with slowing of flow at the site of acute inflammation allow neutrophils to flow in this plasmatic zone (Figure 1). Neutrophils randomly contact the endothelium in normal tissues, but do not adhere to it. However, at sites of injury, pavementing occurs early in the acute inflammatory response and appears to be a specific process occurring independently of the eventual slowing of blood flow. A variety of factors collaborate to bring leucocytes from the circulation to the inflamed site (Vergnolle, 2003). Increased leukocyte adhesion results from interaction between paired adhesion molecules on leukocyte and endothelial surfaces. Leukocyte surface adhesion molecule expression is increased by component C5a, leukotriene B4 and tumour necrosis factor. Endothelial cell expression of selectins, such as endothelial-leukocyte adhesion molecule-1 (ELAM-1), which establishes the first loose contact between leukocytes and endothelium (resulting in 'rolling'), integrins, and intercellular adhesion molecule-1 (ICAM-1), to which the leukocytes' surface adhesion molecules bond, is increased by interleukin-1, endotoxins and tumour necrosis factor (Underwood, 2004; Vergnolle, 2003).

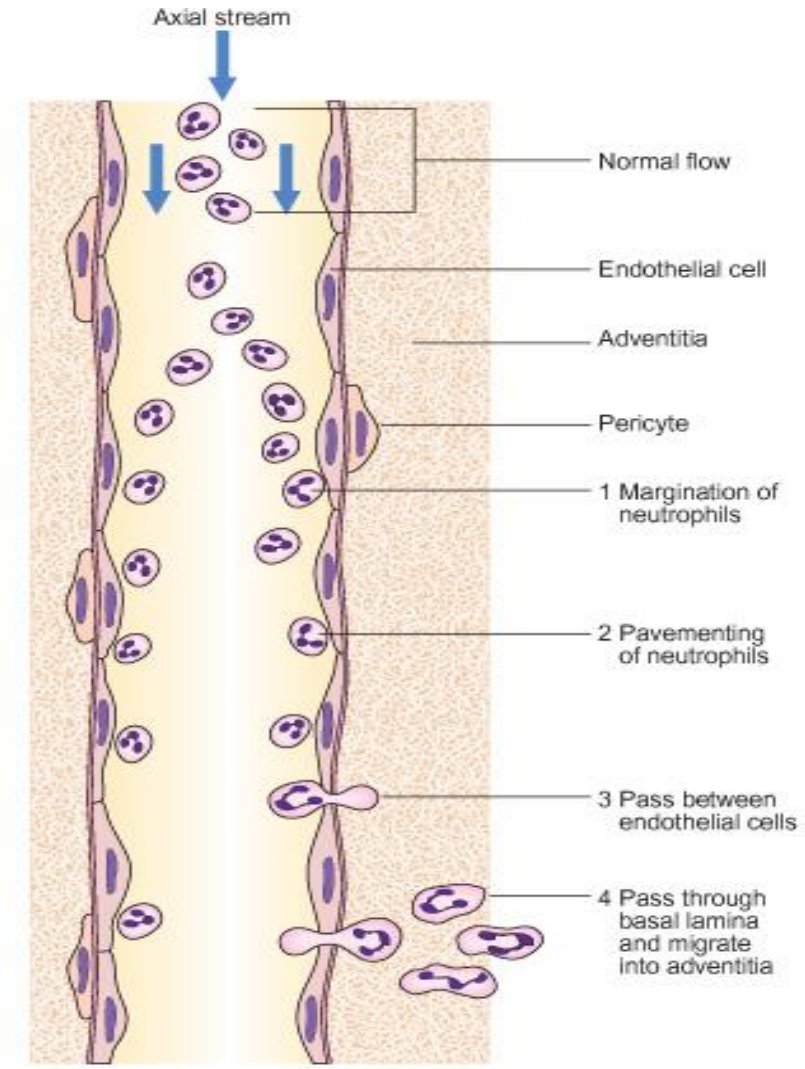

Fig 1: Steps in neutrophil polymorph emigration (Underwood, 2004)

Third Phase (Granuloma formation and tissue repair): One of the results of inflammation is to `wall-off' the area of injury from the remaining tissues. This is done by fibrinogen clots that block the tissue space and the lymphatic in the inflamed area so that after a while fluid rarely flows through the spaces. As a result, the walling-off process delays the spread of bacteria or toxic product (Guyton & Hall, 2006). Clearance of the offending microbes or other stimuli, tissue debri and recruited cells from the

inflammatory site marks the beginning of the final stage of resolution and return of the tissue to homeostasis (Villarreal, *et al.,* 2001).

1.4 Pathological Role of Inflammation

Although inflammation helps clear infections and, along with repair, makes wound healing possible, both inflammation and repair have considerable potential to cause harm (Kumar, *et al*., 2007; Serhan, *et al.,* 2007). In many diseases such as arthritis, obesity induced insulin resistance, multiple sclerosis, inflammatory bowel disease, and asthma, the inflammatory process is not appropriately regulated contributing to the pathogenesis of common chronic inflammatory diseases. As a result, significant tissue dysfunction (leading to the generation of the symptoms that typify these diseases), and tissue re-structuring occur (e.g., fibrosis) that can further impair tissue function (Hanauer, 2006; Vergnolle, 2003; Wellen & Hotamisligil, 2005). Thus, inflammatory responses are the basis of life-threatening anaphylactic reactions to insect bites or drugs, as well as of certain chronic diseases such as rheumatoid arthritis and atherosclerosis. Other harmful examples include inflammation in the peritoneum leading to fibrous bands that cause intestinal obstruction, or pericardial inflammation resulting in dense encasing scar that impairs cardiac function (Kumar, *et al*., 2007).

1.5 Treatment of Inflammation

1.5.1 Conventional Agents

Non Steroidal Anti-inflammatory Drugs

The non steroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed and used drugs in the community for rheumatologic as well as non rheumatologic conditions, which include acute and chronic pain; biliary, ureteric colic; dysmenorrhoea; fever; and other applications that derive from the suppression of prostaglandin synthesis (Bhuvana & Hema, 2013). There are more than 50 different NSAIDs on the global market (Rang & Dale, 2008) and everyday about 30 million people consume NSAIDs (Maity, *et al*., 2009)

They are a chemically heterogeneous group of compounds, which nevertheless share certain therapeutic actions and adverse effects. The class includes derivatives of salicylic acid (e.g., aspirin, diflusinal), propionic acid (e.g., naproxen, ibuprofen, flurbiprofen, ketoprofen), acetic acid (e.g., indomethacin, etodolac, diclofenac, ketorolac), enolic acid (e.g., piroxicam, phenylbutazone), fenamic acid (e.g., mefenamic acid, meclofenamic acid), alkanones (nabumetone), and diaryl heterocyclic compounds (e.g., celecoxib, valdecoxib, rofecoxib, etoricoxib) (Brunton, *et al*., 2011)

They act primarily by inhibiting the cyclooxygenase enzymes that catalyze the first step in prostanoid biosynthesis. This leads to decreased prostaglandin synthesis with both beneficial and unwanted effects (Harvey, *et al.,* 2012). The development of selective-COX-2 inhibitors as anti-inflammatory agents without gastric toxicity is based on the fact

that COX-1 predominates in the stomach, yielding protective prostaglandins, while COX-2 is induced in inflammation, giving rise to pain, swelling, and discomfort. However, selective COX-2 inhibitors decrease vascular prostacyclin (PGI_2) production and may disrupt the homeostatic mechanisms that limit the effects of platelet activation (Mukherjee, 2002). Moreover, detection of serious cardiovascular events associated with COX-2 inhibitors have led to withdrawal of rofecoxib and valdecoxib from the market (celecoxib is still available for use in patients with rheumatoid arthritis) (Harvey, *et al.*, 2012).

Acetaminophen

Acetaminophen inhibits prostaglandin synthesis in the CNS. This explains its antipyretic and analgesic properties. Acetaminophen has less effect on cyclooxygenase in peripheral tissues, which accounts for its weak anti-inflammatory activity. Acetaminophen does not affect platelet function or increase blood clotting time (Harvey, *et al.*, 2012).

Disease-Modifying Anti-rheumatic Drugs (DMARDs)

These drugs are usually employed in treating rheumatoid arthritis when NSAIDs are insufficient to control symptoms and/or when evidence of joint destruction becomes evident and they have been shown to slow the course of the disease, induce remission, and prevent further destruction of the joints and involved tissues (Daoud, *et al*., 1999; Harvey, *et al.*, 2012). These include methotrexate, sulfasalazine, leflunomide, hydroxychloroquine, and cyclosporine; methotrexate is the most widely used (Scott & Kingsley, 2006)

1.6 The Experimental Plant; *Indigofera spicata Forssk*

Indigofera is a large genus of about 700 species of flowering plants most of them found in tropical and subtropical areas but absent from mediterranean region and they belong to the family Fabaceae. The species are herbs or shrubs with biramous hairs. Leaves are usually imparipinnate, sometimes 3-folioate or 1-foliolate. Flowers are usually in axillary racemes. Corolla is usually red or pink, falling soon, or rarely the standard persistent; standard pubescent or glabrous, usually indistinictly veined. Vexillary filament is free and anthers are almost always apiculate. Pod is cylindrical, 4-angled or flattened, usually dehiscent, 1-many-seeded (Hedberg & Edwards, 1989). Several species, particularly *I.arrecta, I.articulata, I. coerulea and I.tinctoria* were once of international importance as the source of the blue-black dye indigo, but now they are only used locally. Presently species of the *Indigofera* are being studied for use as browse and inflammatory control (Hedberg & Edwards, 1989).

I.spicata (Fig 2) is prostrate (meaning growing along ground) or ascending perennial; stems, leaf and inflorescence rachides more or less sparingly appressed strigose. Stipules are with a broad scarious base, glabrescent at least at the margins. Leaf reaches up to 3 cm long including a petiole of 1-3 mm. leaflets (3-)5-11, alternate, cuneate -obovate or cuneate-oblong, 3-30 mm long, strigulose on both sides or glabrous above. Racemes are dense, many-flowered; peduncle 1-4 cm. Calyx is 2-4.5 mm long, divided almost to the base. Stamens are 3-4 mm long. Pod is 11-18 x 2 mm, straight, deflexed, strigulose, 5-8 seeded (Hedberg & Edwards, 1989). Common names of *I. spicata* include yeayit misir (Amharic) (Abate, 1989), sherit or kursi-ashit (Omotic) (Giday, *et al*., 2009) and creeping indigo (English) (Ossedryver, *et al.*, 2013).

Fig 2: Photograph of *Indigofera spicata Forssk* (Hyde, *et al.,* 2013)

It usually grows in grassland or woodland, often on waste or cultivated ground; (380-) 1300-2700 m. It is commonly found in Tigray, Gondar, Welo, Gojam, Welega,Shewa, Arsi, Harerge, Bale, Ilubabor, Kefa, Gamo Gofa, Sidamo region of Ethiopia. It is also common throughout tropical Africa, Transvaal, Natal, Madagascar, and also in Yeman, India, Ceylon, South East Asia and it is introduced in America (Hedberg & Edwards, 1989).

Planting of *I. spicata* has been recommended for the control of erosion and it is also a valuable fodder plant. If eaten in large amounts, however, it is reported to cause abortion in cattle and sheep (Hedberg & Edwards, 1989). In Ethiopian folklore medicine, the roots of *I. spicata* have been used in the treatment of stomachache, cough, diarrhea, malaria,

toothache, evil eye, headache, retained placenta, intestinal parasite, boils, tinea nigra, meningitis and external wounds (Birhane, 2013; Giday, *et al*., 2009).

1.7 Rationale for the Study

Since time immemorial, indigenous plants have been a major source of medicine. In folk medicine, they are used, in single or in combined forms for treating different types of inflammatory and arthritic conditions (Mohan, *et al.,* 2009)

Herbal medicine is the mainstay therapy for about 75 - 80% of the population in developing countries for primary health care (Parekh, 2005). This is because of better cultural acceptability, affordability, effectiveness, low cost, better compatibility with the human body and fewer side effects. World Health Organization (WHO) encourages the inclusion of herbal medicines of proven safety and efficacy in the healthcare programs of developing countries because of the great potential they hold in combating various diseases (Amos, *et al.,* 2001).

Many medicinal plants are used in developing countries for the management of pain and inflammatory conditions. The validation of the folkloric claims of these medicinal plants will provide scientific basis for the conservation of tropical medicinal resources, the deployment of the beneficial ones as phytomedicine in the primary healthcare and the development of potential bioactive constituents. These could provide novel compounds or precursors in drug development, and utilization of isolated compounds as investigative, evaluative and other research tools in drug development and testing processes (Musa, *et al.,* 2009).

Studies have been continuing on inflammatory disease, since the side effects of the currently available anti-inflammatory drugs pose a major problem during their clinical use (Vane & Bolting, 1995). The most common symptoms associated with conventional NSAIDs are gastrointestinal, including anorexia, nausea, dyspepsia, abdominal pain, and diarrhea. These symptoms may be related to the induction of gastric or intestinal ulcers, which is estimated to occur in 15-30% of regular users (Brunton, *et al*., 2011). Selective COX-2 inhibitors were developed as a response to the gastrointestinal side effect of conventional NSAIDs. However, COX-2 inhibitors decrease vascular prostacyclin (PGI_2) production and may disrupt the homeostatic mechanisms that limit the effects of platelet activation. Basic and clinical data raise concerns about the cardiovascular side effects (a potential prothrombotic effect) of this class of drugs (Mukherjee, 2002). Therefore, development of newer and more anti-inflammatory drugs with lesser side effects is necessary (Vasudevan, *et al.*, 2006).

Screening of the plants for their biological activity is done on the basis of either their chemotaxonomic investigation or ethnobotanical knowledge for a particular disease (Prakash, *et al.*, 2011). Even though identification of a particular compound against a specific disease is a challenging long process, since the importance of the plant lies in their biologically active principles, plants should be subjected to successive extraction and purification procedures to isolate the compounds of interest, which can themselves be active or they can be used as precursors in hemisynthetic process (Prakash, *et al.*, 2011, Rates, 2001). This study was intended to evaluate which solvent fraction(s) of *I. spicata* were responsible for its anti-inflammatory activity.

2. OBJECTIVES OF THE STUDY

2.1 General Objective

- The general objective of this study was to evaluate the anti-inflammatory activity of the solvent fractions of the root of the plant *I. spicata* in mice models.

2.2 Specific Objectives

- To evaluate the effect of the solvent fractions on acute model of inflammation using carrageenan-induced mouse paw edema
- To evaluate the effect of solvent fractions on sub acute model of inflammation using formalin-induced mouse paw edema
- To perform preliminary phytochemical screening for secondary metabolites of the solvent fractions of the roots of *I. spicata.*

3. MATERIALS AND METHODS

3.1 Drugs, Chemicals and Solvents

Drugs, chemicals and solvents that were used in this study includes; Chloroform (Carlo Erba, France), Methanol (Carlo Erba, France), Indomethacin (Cadila Pharmaceuticals, India), Normal saline (EPHARM, Addis Ababa), Distilled Water, Tween 80 (BDH Laboratory Supplies, England), Formalin (Grace Trading PLC, Addis Ababa), and Carrageenan Lambda (SIGMA CHEMICAL CO. ®, St Louis, USA). Carrageenan and distilled water were kindly donated by Traditional & Modern Medicine Research Directorate of EHNRI and EPHARM respectively, Addis Ababa, Ethiopia. Others were purchased from local vendors.

3.2 Experimental Animals

Swiss Albino mice (25 – 30 g, 8 weeks old) of either sex were used in the study. Animals were purchased from Ethiopian Health and Nutrition Research Institute (EHNRI) as well as bred in the animal house of Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Addis Ababa University. The animals were maintained under standard condition and were fed with a commercial rodent pellet diet and water *ad libitum*. The animals were acclimatized for a week before the experiment and the care and handling was in accordance with the internationally accepted standard guidelines for use of animals (OECD, 2008). A total of 174 mice were used in this study.

3.3 Plant Collection

The roots of *I. spicata* were collected in March 2013 from Gorgora, around Gondar area, Northwest Ethiopia 724 km from the capital, Addis Ababa. The plant material was authenticated by taxonomist (Mr. Assefa Hailu) and voucher specimen (*js001*) was deposited for future reference at the National Herbarium, College of Natural Sciences, Addis Ababa University.

3.4 Extraction and Fractionation

3.4.1 Crude Extract

Crude extract of the plant material was prepared by maceration with 80% methanol as described elsewhere Birhane, (2013) and the yield was 8.8%. Preliminary evaluation of anti-inflammatory effect of the roots of *I. spicata* was performed with this crude extract at a dose of 400 mg/kg using carrageenan induced mouse paw edema model for confirmation of previous report by Birhane, (2013) before proceeding to fractionation. The percentage inhibition of paw edema was found to be 47.7%, which was almost similar to the previous report (48.5%).

3.4.2 Fractionation

Roots of *I. spicata* were dried at room temperature and finely grounded by a cutting mill (FRITSCH, Germany). Solvent fractionation was done using successive soxhlet extraction. A suitable amount (600g) of the powdered plant material was weighed with a balance (Mettler Toledo, Switzerland) and placed in a thimble. The solvent was allowed to extract continuously by heating the solvent in a round bottom flask with electrical

heater. The liquid condensate that drips into the sample performs the extraction which then passes through the thimble and back into the round bottom flask. Chloroform was used as initial solvent for extraction. The marc (residue) which was found after extraction by chloroform was dried and subjected for fractionation with absolute methanol. Fractions, found following fractionation with chloroform and methanol were filtered with Whatman filter paper No. 1 (Whatman, England) separately. The fractions were then evaporated using rotary evaporator (Buchi Rota vapor, Switzerland). The remaining residues were further dried in an oven (< 40 ^{o}C). The aqueous fraction was prepared by taking the dried marc after methanol fractionation and subjected for maceration with distilled water. It was then filtered by using muslin cloth followed by filter paper and lyophilized by using a lyophilizer (Operon, Korea vacuum limited, Korea). The solvents used for fractionation; chloroform, absolute methanol and water were selected according to ascending order of polarity so that those constituents which are more of non polar would fall in the chloroform fraction (polarity index, 4.1), those of semi-polar to the absolute methanol (polarity index, 5.1), and those of most polar would fall to the aqueous (polarity index, 10.2) fraction.

3.5 Grouping and Dosing of Animals

Mice were randomly allocated into five groups of six mice in each (three male and three female). The first group (CONT) served as negative control and given 12.5 ml/kg Tween 80 (2% v/v in water). The second, third and fourth group was given 50,100 and 200 mg/kg of each fraction. The fifth group (INDO) was a positive control and given Indomethacin, 10 mg/kg. In case of the chloroform fraction, firstly 50, 100, 200 mg/kg was tested but there was no significant effect. Further study was done by increasing the

doses to 500, 750, and 1000 mg/kg; unfortunately significant effect was not observed at these high doses too. These dose levels were selected based on pilot study and on previous study made on the crude extract (Birhane, 2013). The fractions were made up for dosing the animals as follows:-The stock solution was prepared by assuming the weight of mouse to be 40 g and the maximum volume to administer as 0.5 ml because OECD states that the maximum volume administered to rodents should not normally exceed 1 ml/100g of body weight (OECD, 2008). Then the mass of fraction in milligram each mouse would take was calculated by using weight of each mouse and the dose in milligram per kilogram. Finally the volume of fraction each mouse would take was calculated by using the mass of fraction obtained above and the stock solution. Administration was performed orally and maximum volume administered was 0.5 ml.

3.6 Carrageenan-Induced Paw Edema

Effect of fractions of *I. spicata* on carrageenan induced hind paw mouse edema was carried out as described elsewhere (Winter, *et al.*, 1962) with slight modification {for example paw tissue may be examined immunohistochemically or by *in situ* hybridization for cellular localization and quantification of specific proteins, i.e., growth factors, cytokines, tachykinins, or homogenized and extracted for the measurement of prostaglandin E2 and elastase activity and cyclooxygenases. But this was not performed due to limited resource}. Left 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. Basal paw volume was measured before administration by volume displacement method using Plethysmometer (Ugo Basile Company Cat No 7140, Italy) by immersing the paw up to the marked point. Animals were treated as described in the

grouping and dosing section above and given a subcutaneous injection of (0.05 ml of 1% (w/v)) solution of carrageenan into the sub-plantar tissue of the left hind paw one hour after dosing. The paw volume was measured again on the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} & 5^{th} h after carrageenan injection. The difference of average values between treated animals and negative control group was calculated for each time interval and evaluated statistically. The percent inhibition was calculated using the following formula.

$$\% \text{ edema inhibition} = [1- (Vt / Vc)] X 100$$

Vt and *Vc* are edema volume in the drug treated and control groups respectively.

Inflammation induced by carrageenan, originally described by Winter, *et al* (1962), is acute, non-immune, well-researched, and highly reproducible. Cardinal signs of inflammation develop immediately following subcutaneous injection. The inflammatory response is usually quantified by increase in paw size (edema) which is maximal around 5 h post carrageenan injection and is modulated by inhibitors of specific molecules within the inflammatory cascade. The NSAID, Indomethacin is a clinically useful example. The model, therefore, has had, and will continue to have, a vital role in novel drug development (Winyard & Willoughby, 2003).

3.7 Formalin-Induced Paw Edema

Experimental arthritis was induced and studied in mice. Basal paw volume measurement and treatment was similar to carrageenan induced mouse paw edema except the dosing was done once daily for seven days and formalin (0.02 ml of 2% (v/v)) solution was injected on the first and third day of the experiment (Mehta, *et al.,* 2009; Mohan *et al.,* 2009). The paw volume was measured again on the 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} and 7^{th} day

after injection of formalin. The percent inhibition was calculated using the above formula.

It is well known that inhibition of formalin-induced pedal edema in rats is one of the most suitable test procedures to screen anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis (Banerjee, *et al*., 2000; Mehta, *et al.*, 2009). There fore formalin induced paw edema was used as a subacute model as it resembles heman arthrities whereas the carrageenan induced paw edema was used as acute model as cardinal signs of inflammation develop immediately following injection of carrageenan.

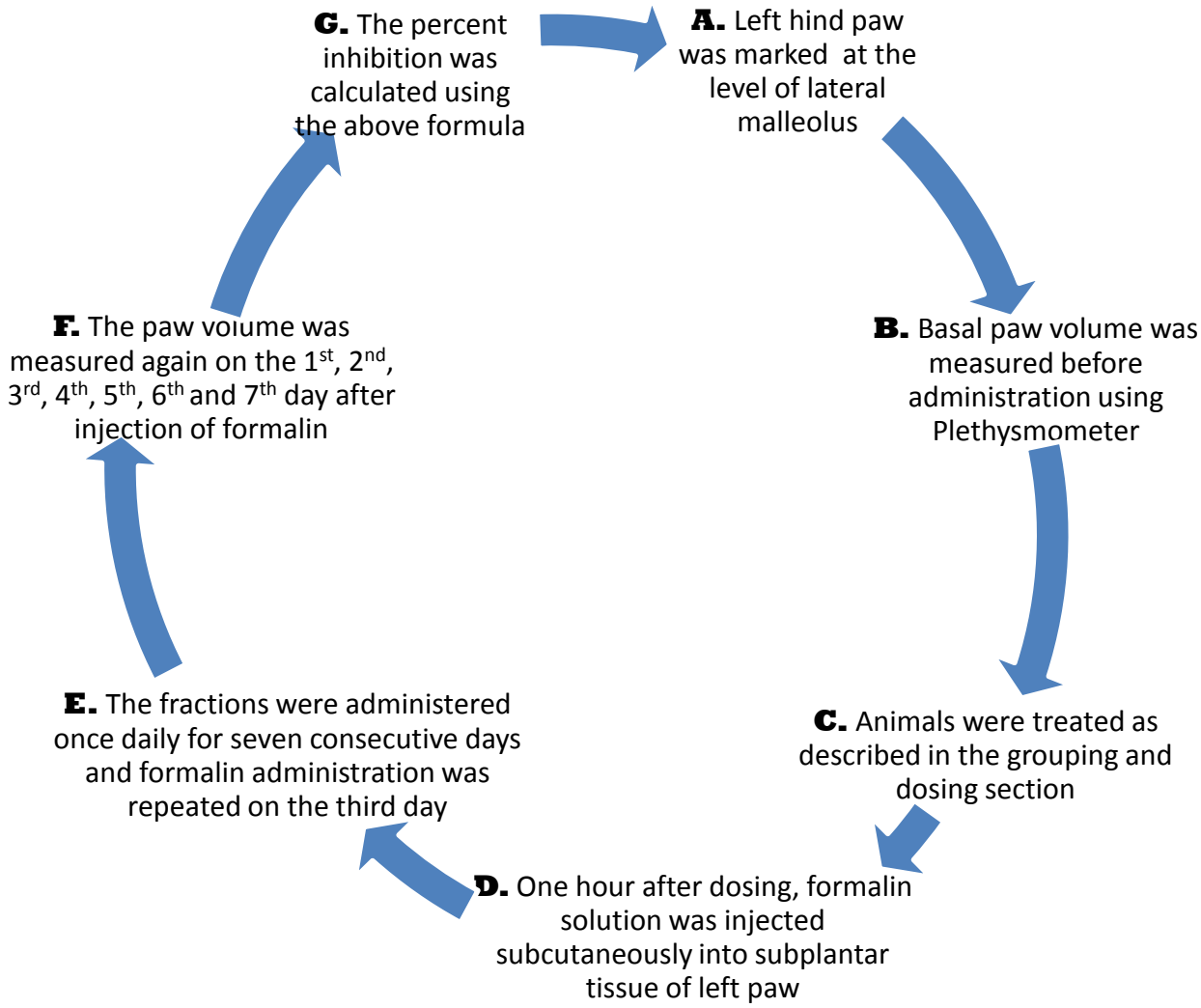

Fig 3: Procedures of formalin induced paw edema

3.8 Preliminary Phytochemical Screening

Chemical tests were carried out on the solvent fractions of *Indigofera spicata* using standard procedures as described elsewhere (Ajayi, *et al.*, 2011; Herborne, 1973; Njoku & Obi, 2009; Rasool, *et al*., 2010; Sofowora, 1993; Trease & Evans, 2010; Yisa, 2009;)

Test for Saponins

Five ml of each fraction was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication for the presence of saponins (Ajayi, *et al.*, 2011; Sofowora, 1993).

Dragendroff's and Mayer's test

Three ml of each fraction was stirred with 3 ml of 1% HCl on a water bath. Mayer's (Potassium Mercuric Iodide) and Dragendroff's (Solution of Potassium Bismuth Iodide) reagents were then added to the mixture separately. The test tubes were observed for colored precipitates or turbidity. In Mayer's test, the acid layer with few drops of Mayer's reagent gives a creamy white precipitate. In Dragendroff's test, acid layer with few drops of Dragendroff's reagent gives reddish brown precipitate. The presence of colored precipitates or turbidity was taken as indication of secondary and tertiary amines (Herborne, 1973; Njoku & Obi, 2009).

Keller-Kiliani test

Two ml of each fraction was dissolved in 2 ml of glacial acetic acid containing one drop of $FeCl_3$ solution. The mixture was then poured into a test tube containing 1 ml of

concentrated $H2SO_4$. A brown ring at the interphase indicates the presence of a deoxy sugar, characteristic of cardenolides (Njoku & Obi, 2009; Trease & Evans, 2010).

Test for Steroids

A red color produced in the lower chloroform layer when 2 ml of solvent fractions of *I. spicata* was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid added indicates the presence of steroids (Njoku & Obi, 2009).

Test for Phenolic compounds

0.2 g of powdered fraction was dissolved in 5ml of ethanol. Then 2 drops of 1M $FeCl_3$ was added. The appearance of intense color indicates the presence of phenolic compounds (Rasool, *et al*., 2010).

Test for Terpenoids

5 ml of each fraction was mixed in 2 ml of chloroform. 3 ml of concentrated H_2SO_4 was then added to form a layer. A reddish brown precipitate coloration at the interface formed indicated the presence of terpenoids (Harborne, 1973; Rasool, *et al*., 2010).

Tests for Anthraquinones

Borntrager's test: 3 ml of each fraction was shaken with 3 ml of benzene, filtered and 5 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of a pink, red or violet color in the ammonical (lower) phase indicates the presence of free anthraquinones (Njoku & Obi, 2009)

For Anthraquinone derivatives: 3 ml of the each fraction was boiled with 3 ml of aqueous sulphuric acid and filtered while hot. 3 ml of benzene was added to the filtrate and shaken. The benzene layer was separated and 3 ml of 10% NH_3 added. A pink, red or violet coloration in the ammonical (lower) phase indicates the presence of anthraquinone derivatives (Njoku & Obi, 2009).

3.9 Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM). Statistical evaluations used were t-test, linear regression analysis, one way and two way analysis of variance (ANOVA). T-test was used to compare the maximum effects observed in both models by treatment with each fraction. One way ANOVA followed by post hoc Tukey's comparision was performed for comparison of paw edema among groups. While two way ANOVA followed by post hoc Tukey's comparision was performed to determine whether there was interaction between treatment and duration on paw volume or not. Linear regression analysis was used to check whether the injection of carrageenan or formalin shows a time dependant increase in paw edema or not. Statistical significance was considered at $p < 0.05$, and the computer statistical package, SPSS version 16 was used for data analysis.

4. RESULTS

4.1 Yields of Extraction

The percentage yield and physical description of the solvent fractions of the roots of *I. spicata* is shown in Table 1. The percentage yield of absolute methanol fraction was greater than that of chloroform and aqueous fractions**.**

Table 1: Percentage yield and physical description of the solvent fractions of the roots of *I. spicata*

Extracting solvent	**Physical description of the extract**	**%Yield (w/w)**
Chloroform	Gummy	1.62
Absolute methanol	Hygroscopic Powder	8.61
Aqueous	Gummy	4.12

4.2 Carrageenan-Induced Paw Edema

Subplantar injection of 0.05 ml of 1% carrageenan suspension in mice produced a time dependent increase in paw thickness (Table 2). This was checked by performing linear regression analysis in which, the time elapsed after injection of carrageenan was used as independent variable, whereas the paw edema was taken as dependant variable. The analysis demonstrated that there was a significant correlation between the two variables ($R^2 = 0.702$, $F(1, 28) = 66.103$, $p < 0.05$).

The methanol fraction significantly ($p < 0.001$) inhibited paw edema at all doses used starting from the 2nd h compared to the negative controls. Maximum percent inhibition was observed 4 h following treatment and the values were 33.1%, 41.3%, and 46% for 50 mg/kg, 100 mg/kg and 200 mg/kg, respectively. When doses of methanol fraction were compared with each other, 200 mg/kg showed a significant inhibition of paw edema than 50 mg/kg ($p < 0.001$) 2, 3, 4 & 5 h following treatment. However, no significant difference was observed with that of 100 mg/kg except at 2 h ($p < 0.05$) following treatment. While 100 mg/kg methanol fraction showed a significant inhibition of paw edema than 50 mg/kg on 2 & 3 h ($p < 0.05$), and 4 & 5 h ($p < 0.01$) following treatment.

In contrast to the methanol fraction, the aqueous fraction showed significant inhibition ($p < 0.001$) starting from the 3rd h, with a maximum suppression of 30%, 38.2%, and 40% for 50 mg/kg, 100 mg/kg and 200 mg/kg, respectively, compared to the negative control mice. When doses of aqueous fraction were compared with each other, 200 mg/kg exhibited a significant inhibition of paw edema than 50 mg/kg; 3 h ($p < 0.05$), 4 & 5 h ($p < 0.01$) following treatment. But statistically significant difference was not observed

between 200 mg/kg versus 100 mg/kg or 100 mg/kg versus 50 mg/kg of aqueous fractions at all times. Time at which maximum effect observed was similar to that of the methanol fraction. Unlike both fractions, the chloroform fraction was devoid of any effect on acute inflammation even at doses as large as 1000 mg/kg. Interestingly, no significant difference had been noted between the different doses. Therefore, it can be said that the fractions, especially the methanol fraction, exhibited significant suppression of acute inflammation in a dose dependent manner.

Indomethacin produced maximum inhibition (62%) and the value was significantly greater ($p < 0.001$) than all solvent fractions at all time points except it was not significantly different at 2 h compared with methanol 200 mg/kg, and the significance level at 3 h following treatment was $p < 0.05$ when compared with methanol 200 mg/kg (Table 2).

Table 2: Effect of the solvent fractions of *I. spicata* on carrageenan-induced mouse paw edema

	Mice paw volume in ml measured at different time intervals (Percent Inhibition)					
Group	**0 h**	**1 h**	**2 h**	**3 h**	**4 h**	**5 h**
CONT	0.40±0.015	0.62±0.017	0.73±0.014	0.77±0.014	0.79±0.015	0.82± 0.015
CF500 mg/kg	0.44±0.021	0.61±0.02 e_3 (1.6)	0.7±0.02 e_3 (4.54)	0.71±0.023 e_3 (7.79)	0.76±0.016 e_3 (4.4)	0.79± 0.011 e_3 (3.26)
CF750 mg/kg	0.47±0.011	0.59±0.02 e_3 (4.84)	0.68±0.012 e_3 (7.27)	0.7±0.018 e_3 (9.1)	0.74±0.015 e_3 (6.92)	0.77± 0.016 e_3 (5.72)
CF1000 mg/kg	0.46±0.014	0.56±0.027 e_3 (9.68)	0.68±0.02 e_3 (8.63)	0.7±0.022 e_3 (9.1)	0.73±0.013 e_3 (8.2)	0.76± 0.015 e_3 (6.94)
MF50 mg/kg	0.39±0.028	0.57±0.01 (8.34)	0.57±0.01 a_3 (22.95)	0.55±0.01 a_3 (29.22)	0.53±0.01 a_3 (33.12)	0.57±0.01 a_3 (30.61)
MF100 mg/kg	0.37±0.024	0.63±0.01 (-1.08)	0.51±0.01 $a_3 b_1$ (30.22)	0.49±0.01 $a_3 b_1$ (36.58)	0.47±0.01 $a_3 b_2$ (41.29)	0.49± 0.01$a_3 b_2$ (40)
MF200 mg/kg	0.40±0.011	0.61±0.02 (2.42)	0.46±0.01 $a_3 b_3 c_1$ (37.5)	0.46±0.01 $a_3 b_3 e_1$ (41.13)	0.43±0.01 $a_3 b_3$ (45.9)	0.46± 0.02 $a_3 b_3$ (44.28)
AF50 mg/kg	0.44±0.03	0.66±0.02 (-6.45)	0.72±0.02 (1.81)	0.6±0.01 a_3 (22.08)	0.56±0.01 a_3 (29.56)	0.61± 0.02 a_3 (25.3)
AF100 mg/kg	0.43±0.03	0.65±0.02 (-4.83)	0.7±0.02 (4.54)	0.53±0.01 $a_3 b_1$ (31.17)	0.49±0.01 $a_3 b_1$ (38.17)	0.53 ± 0.01 $a_3 b_1$ (35.1)
AF200 mg/kg	0.38±0.03	0.63±0.03 (-1.61)	0.69±0.01 e_3 (5.9)	0.51±0.01 $a_3 b_3 e_3$ (33.77)	0.48±0.01 $a_3 b_2$ (39.62)	0.51± 0.02 $a_3 b_2$ (37.55)
INDO10 mg/kg	0.34±0.01	0.35±0.02 $a_3 b_3 c_3 d_3$ (43.55)	0.42±0.02 $a_3 b_3 c_3$ (43.17)	0.39±0.02 $a_3 b_3 c_3$ (49.13)	0.33±0.02 $a_3 b_3 c_3 d_3$ (58.08)	0.31±0.02 $a_3 b_3 c_3 d_3$ (62.04)

Values are expressed as Mean ± SEM; n = 6; Values in parenthesis shows % inhibition of paw edema; a = compared with negative control, b = compared with 50 mg/kg of respective fraction, c = compared with 100 mg/kg of respective fraction, d = compared with 200 mg/kg of respective fraction, e = compared with Indomethacin 10 mg/kg; 1 = $p < 0.05$, 2 = $p < 0.01$, 3 = $p < 0.001$, CONT: negative control; CF: chloroform fraction; MF: methanol fraction; AF: aqueous fraction; INDO: Indomethacin

Figure 4 shows the percentage inhibition of mouse paw edema of solvent fractions of *I. spicata* at their maximal dose employed against time for carrageenan induced paw edema. The methanol fraction was superior next to the positive control (62.04%) in inhibiting paw edema with maximal value of (45.9%). The aqueous and chloroform fraction follow with maximal percentage inhibition of 39.6 and 9.7 respectively.

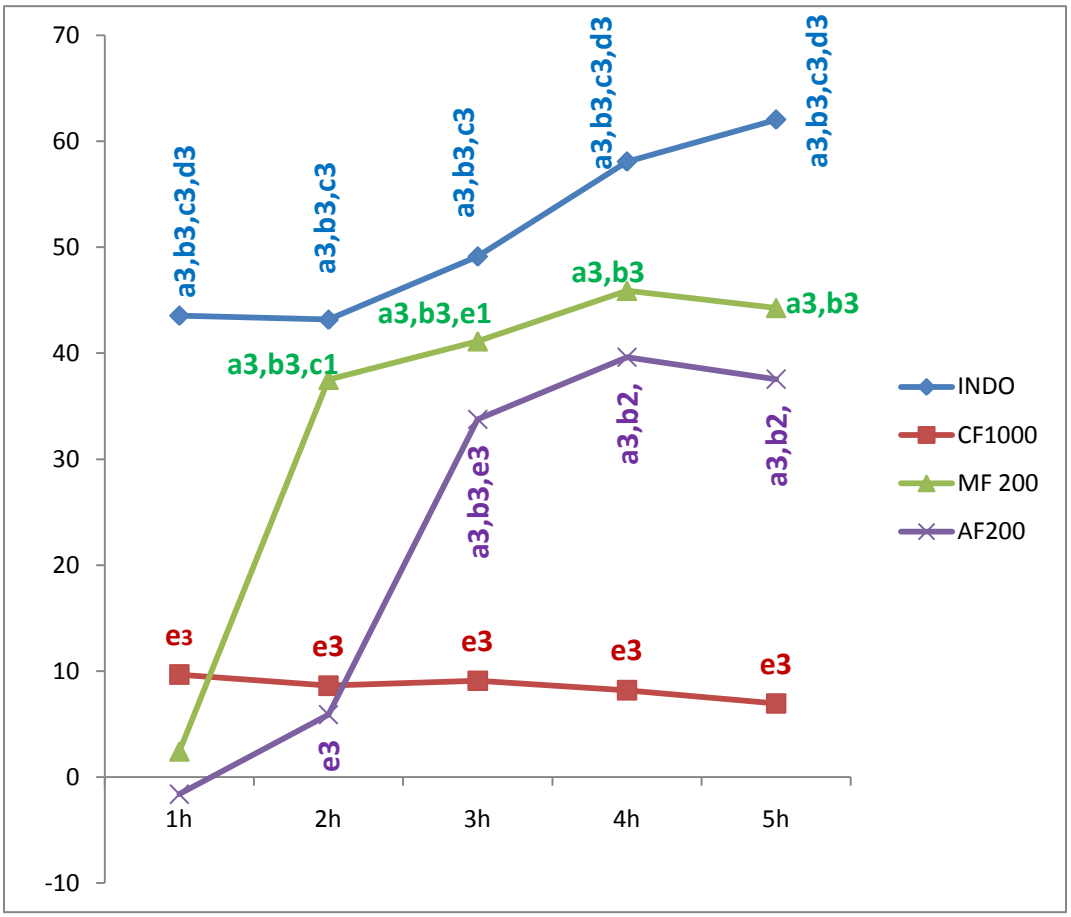

Fig 4: Percentage inhibition of carrageenan induced mouse paw edema by all solvent fractions of *I. spicata* at the maximum dose employed: a = compared with negative control, b = compared with 50 mg/kg of respective fraction, c = compared with 100 mg/kg of respective fraction, d = compared with 200 mg/kg of respective fraction, e = compared with Indomethacin 10 mg/kg; 1 = $p < 0.05$, 2 = $p < 0.01$, 3 = $p < 0.001$, CONT: negative control; CF: chloroform fraction; MF: methanol fraction; AF: aqueous fraction; INDO: Indomethacin

4.3 Formalin-Induced Paw Edema

Subplantar injection of formalin (0.02 ml of 2% (v/v) solution in mice also produced a time-dependent increase in paw thickness (Table 3). Linear regression analysis was also performed to determine the correlation existed between the variables as performed for the acute test. And it was found that significant correlation existed between time and paw thickness (R^2 = 0.615, F (1, 40) = 63.93, P < 0.05). The methanol fraction significantly (p < 0.001) inhibited paw edema at all doses used starting from the 2nd day compared to the negative controls. Maximum percent inhibition was observed 6 day following treatment and the values were 37.8%, 40.2%, and 41.5% for 50 mg/kg, 100 mg/kg and 200 mg/kg, respectively. No significant difference was observed among the different doses at all time points. Moreover, similar to the methanol fraction, the aqueous fraction showed significant inhibition (p < 0.001) starting from the 2nd day with a maximum suppression of 32.4%, 39.4% and 40.4% for 50 mg/kg, 100 mg/kg and 200 mg/kg, respectively, compared to the control mice. No significant difference was observed among the different doses of aqueous fraction similar to methanol fraction except for the dose of 200 mg/kg which showed significant inhibition of paw edema than 50 mg/kg (p < 0.05) that was observed 5 days following treatment. Time at which maximum effect observed was 6 days (for 100 & 200 mg/kg) and 7 days (for 50 mg/kg) following treatment (Table 3). Unlike both fractions, the chloroform fraction was devoid of any effect on subacute inflammation even at doses as large as 1000 mg/kg. Interestingly, no significant difference had been noted between the different doses. Like the acute model indomethacin produced maximum inhibition (58.7%) and the value was significantly greater than the values of the methanol and aqueous fractions.

Table 3: Effect of the solvent fractions of *I. spicata* on formalin-induced mouse paw edema

Group	Mice paw volume in ml measured on different days (Percent Inhibition)							
	0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
CONT	0.48±0.03	0.60±0.02	0.71±0.02	0.84±0.04	0.88±0.04	0.92±0.04	0.94±0.04	0.96±0.04
CF500 mg/kg	0.49± 0.01	0.62±0.02 e_3 (-1.9)	0.67±0.01 e_3 (5.8)	0.78±0.01 e_3 (7.5)	0.82±0.02 e_3 (6.5)	0.85±0.02 e_3 (7.4)	0.88±0.02 e_3 (6.9)	0.90±0.02 e_3 (5.8)
CF750 mg/kg	0.48±0.02	0.59±0.01 e_3 (2.5)	0.62±0.01 e_3 (13.3)	0.76±0.01 e_3 (9.5)	0.80±0.01 e_3 (8.8)	0.84±0.01 e_3 (8.7)	0.87±0.01 e_3 (7.8)	0.89±0.01 e_3 (6.8)
CF1000 mg/kg	0.44± 0.01	0.56±0.01 e_3 (7.7)	0.63±0.01 e_3 (11.7)	0.75±0.01 e_3 (10.1)	0.78±0.01 e_3 (11.6)	0.83±0.02 e_3 (9.1)	0.85±0.02 e_3 (8.7)	0.88±0.01 e_3 (8.5)
MF50 mg/kg	0.49±0.04	0.67±0.03 e_3 (-10.51)	0.51±0.02 a_3 e_2 (28.74)	0.61±0.02 a_3 (27.03)	0.66±0.02 a_3 (25.09)	0.64±0.02 a_3 (30.62)	0.59±0.01 a_3 (37.76)	0.6±0.01 a_3 (37.63)
MF100 mg/kg	0.42± 0.04	0.63±0.01 e_3 (-4.14)	0.46±0.01 a_3 (35.9)	0.54±0.02 a_3 (35.98)	0.61±0.02 a_3 (30.61)	0.59± 0.02 a_3 (36.05)	0.56±0.02 a_3 (40.24)	0.59±0.02 a_3 (38.83)
MF200 mg/kg	0.44±0.02	0.64±0.01 e_3 (-6.63)	0.44±0.01 a_3 (37.4)	0.52±0.01 a_3 (37.97)	0.59±0.01 a_3e1 (32.89)	0.58±0.01 a_3 (37.32)	0.55±0.01 a_3 (41.48)	0.57±0.01 a_3 (40.42)
AF50 mg/kg	0.45±0.04	0.59±0.03 e_1 (1.92)	0.54±0.02 a_3e_3 (24.1)	0.61±0.01 a_3 (26.84)	0.68±0.00 a_3 (22.24)	0.67±0.00 a_3 (26.99)	0.64±0.00 a_3 (31.73)	0.65±0.00 a_3 (32.4)
AF100 mg/kg	0.33±0.00	0.55±0.01 (9.38)	0.52±0.01 a_3e_2 (27.8)	0.56±0.01 a_3 (33.4)	0.62±0.01 a_3 (29.08)	0.59±0.01 a_3 (35.87)	0.57±0.01 a_3 (39.36)	0.58±0.01 a_3 (39.19)
AF200 mg/kg	0.49±0.01	0.57±0.02 (4.97)	0.51±0.01 a_3e_2 (28.96)	0.55±0.02 a_3 (33.99)	0.63±0.02 a_3e_2 (28.52)	0.58±0.02 a_3b_1 (36.95)	0.56±0.02 a_3 (40.43)	0.58±0.02 a_3 (39.37)
INDO 10 mg/kg	0.41±0.01	0.49±0.01 a_2 (17.4)	0.36±0.04 a_3 (49.07)	0.49±0.03 a_3b_1 (40.56)	0.47±0.02 $a_3b_3c_2$ (45.63)	0.45±0.02 $a_3b_3c_2d_2$ (50.18)	0.43±0.02 $a_3b_3c_2d_2$ (53.5)	0.39±0.02 $a_3b_3c_3d_3$ (58.71)

Values are expressed as Mean ± SEM; n = 6; Values in parenthesis show % inhibition of paw edema; a = compared with negative control, b = compared with 50 mg/kg of respective fraction, c = compared with 100 mg/kg of respective fraction, d = compared with 200 mg/kg of respective fraction, e = compared with Indomethacin 10 mg/kg; 1 = $p < 0.05$, 2 = $p < 0.01$, 3 = $p < 0.001$, CONT: negative control; CF: chloroform fraction; MF: methanol fraction; AF: aqueous fraction; INDO: Indomethacin

Figure 5 depicts the percentage inhibition of mouse paw edema of solvent fractions of *I. spicata* at the maximum dose employed against time for formalin induced mouse paw edema. Indomethacin has inhibited the paw edema with maximum of 58.7%. Methanol, aqueous and chloroform fractions followed Indomethacin with 41.48%, 40.43 %, and 11.7 %, respectively.

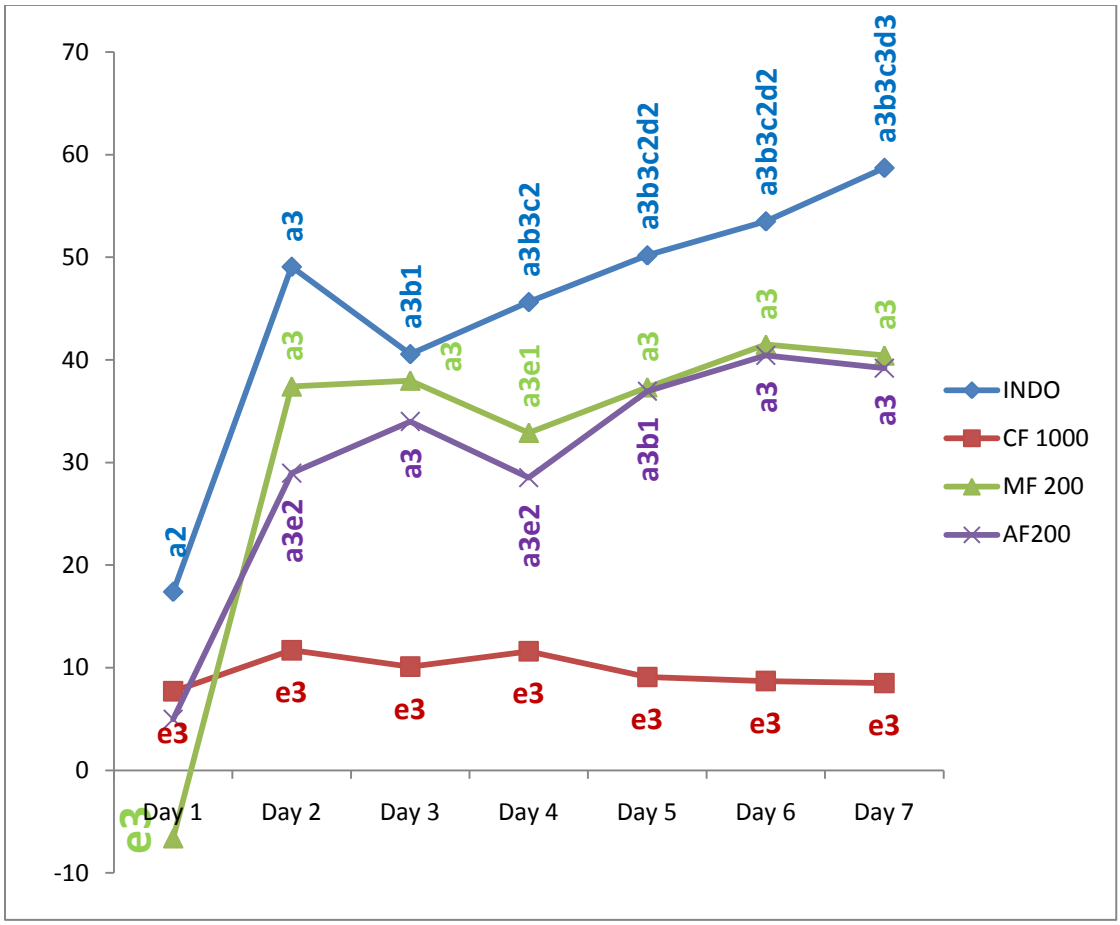

Fig 5: Percentage inhibition of formalin induced mouse paw edema by all solvent fractions of *I. spicata* at the maximum dose employed; a = compared with negative control, b = compared with 50 mg/kg of respective fraction, c = compared with 100 mg/kg of respective fraction, d = compared with 200 mg/kg of respective fraction, e = compared with Indomethacin 10 mg/kg; 1 = $p < 0.05$, 2 = $p < 0.01$, 3 = $p < 0.001$, CONT: negative control; CF: chloroform fraction; MF: methanol fraction; AF: aqueous fraction; INDO: Indomethacin

Maximum effect for methanol and aqueous fractions in both models was compared by using independent sample t-test to have an idea whether there was any difference between the two fractions. Figure 6 shows that the effect of methanol fraction was significantly greater ($p < 0.05$) than the aqueous fraction in the acute model. However, no apparent difference was observed between the two fractions in the subacute model.

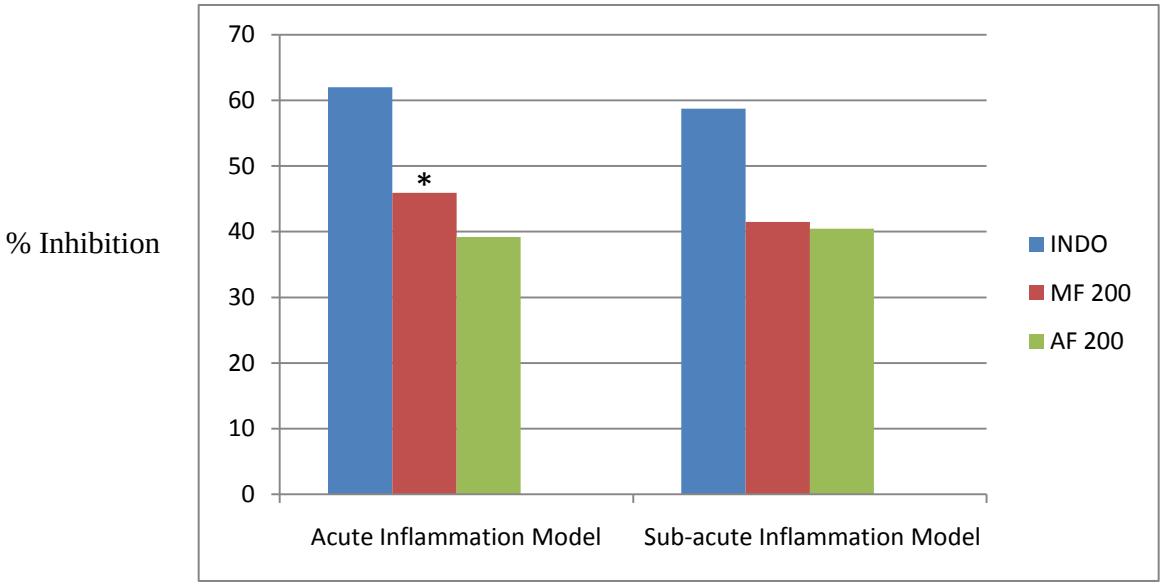

Fig 6: Comparison of the maximal percentage inhibition of paw edema of the methanol and aqueous fractions of *I. spicata* in both models * = $p < 0.05$ INDO: Indomethacin MF: methanol fraction; AF: aqueous fraction

Two way ANOVA was also performed to determine whether there was an interaction between doses administered (treatment) and duration of treatment on the paw volume in Swiss Albino mice, where treatment and duration of treatment were used as independent variables, and paw volume was used as dependant variable. The *treatment-duration interaction* (their combined effect) was found to have statistically significant interaction ($p < 0.05$) with paw volume, in both carrageenan and formalin induced paw edema for both methanol and aqueous fractions. Besides, treatment and duration of treatment

separately showed statistically significant difference in mean paw volume ($p < 0.05$) in both models for both effective fractions.

4.4 Preliminary Phytochemical Screening

Phytochemical screening was carried out for secondary metabolites on the three solvent fractions of *I. spicata*. The analysis revealed that there was a differential distribution of secondary metabolites. Anthraquinone derivatives and phenolic compounds possibly present in the chloroform fraction. The methanol fraction possibly contains anthraquinone derivatives, terpenoids, secondary & teritiary amine containing compounds (like alkaloids), steroids, saponins, deoxy sugar and phenolic compounds. Whereas aqueous fraction possibly contains secondary & teritiary amine containing compounds like (alkaloids), deoxy sugar, anthraquinone derivatives, saponins and phenolic compounds. Phenolic compounds include flavonoids, phenolic acids, and polyphenols (commonly known as hydrolizable and condensed tannins). Note that these phytochemical screening tests are preliminary. Specific phytochemical tests are necessary for further confirmation of these results.

5. DISCUSSION

Carrageenan induced paw edema is a widely used model for determining the acute phase of inflammation and it is a working model of inflammation in the search for new anti-inflammatory agents (Di Rosa, *et al.,* 1970). This model is based on the principle of release of various inflammatory mediators by carrageenan (Suralkar, *et al.,* 2008). Histamine, serotonin and bradykinin are the first detectable mediators in the early phase of carrageenan-induced inflammation. The late phase is sustained by prostaglandins release and mediated by leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Brito, *et al.,* 1988; Di Rosa, *et al.,* 1970; Musa, *et al.,* 2009; Nanda, *et al*., 2010; Salvemini, *et al.,* 1996). The first phase begins immediately after injection of carrageenan and diminishes in two hours. The second phase begins at the end of first phase and remains through the third hour up to five hours. Subcutaneous injection of carrageenan into the mouse paw produces inflammation resulting from plasma extravasation, increased tissue water and plasma protein exudation along with neutrophil extravasation; all due to the metabolism of arachidonic acid (Lo, *et al.,* 1982; Suralkar, *et al.,* 2008).

Formalin induced paw edema is one of the most suitable test procedures used to screen anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis (Banerjee, *et al*., 2000; Mehta, *et al.,* 2009). Injection of formalin subcutaneously into hind paw produces localized inflammation and pain (Greenwald, 1991). In addition, it has been reported that formalin injection produces edema and an increase in vascular permeability (Damas & Liegeois, 1999; Taylor, *et al*., 2000).

The chloroform fraction didn't show statistically significant suppression of paw edema in both acute and subacute inflammation models at all dose ranges employed in the present study, suggesting that non-polar constituents are not responsible for the observed anti-inflammatory effect. In addition, phytochemical screening for secondary metabolites of *I. spicata* revealed that the chloroform fraction was possibly devoid of secondary and tertiary amines, terpenoids, deoxy sugars and steroids which are reported to have anti-inflammatory effects (Igwe & Okwu, 2013; Kumar, *et al.,* 2013; Zakaria, *et al*., 2001). This could be one reason why the chloroform fraction did not show any significant anti-inflammatory effect in both inflammation models.

Methanol fraction showed statistically significant inhibition of paw edema in both acute and subacute inflammation models in all dose ranges employed in the present study. The results obtained for the methanol fraction in the acute inflammation model are in line with those reported elsewhere (Choi, *et al.,* 2004; Sarkar, *et al.,* 2013), where methanol fraction of *Clerodendron trichotomum thunberg,* and *Leucas indica* Linn. showed significant inhibition of carrageenan induced paw edema. Similarly, the results obtained for the methanol fraction in the subacute model were similar to the findings of Sarkar, *et al.,* (2013), where methanol fraction of *Leucas indica* Linn. was significantly effective in reducing formalin induced paw edema. The results in subacute model suggest the usefulness of *I. spicata* in the treatment of inflammation associated with diseases like arthritis (Banerjee, *et al*., 2000; Mehta, *et al.,* 2009).

Like the methanol fraction, aqueous fractions showed statistically significant inhibition of paw edema in both acute and subacute inflammation models in all dose ranges employed in the present study. The aqueous fraction results obtained in the acute inflammation

model are similar with those reported elsewhere (Khan, *et al.,* 2011; Okunrobo, *et al*., 2008; Sarkar, *et al.,* 2013), where aqueous fraction of *Betel nut, Anthocleista djalonensis,* and *Leucas indica* Linn., respectively, inhibited carrageenan induced paw edema. Similarly, the results obtained for the aqueous fraction in the subacute model were similar to the finding of others (Jayakumari, *et al.*, 2012; Sarkar, *et al.*, 2013; Zurron, *et al.*, 2010), where aqueous fractions of *Adenanthera pavonina* Linn., *Leucas indica* Linn., *Ipomoea imperati*, respectively, were significantly effective in reducing formalin induced paw edema.

The onset of action of methanol fraction in the acute model was shorter (two hours following treatment) than that of the aqueous fraction (three hours following treatment), but the onset of action in the sub acute model was similar to the aqueous fraction. This could be explained by the absorption of drugs. When delivered by oral route, the onset of drug action will be delayed because of the required transit time in the gastrointestinal tract, the absorption process and enteroheparic circulation (Aulton, 1998). The vast majority of orally administered drugs are absorbed via passive transcellular transport. This necessitates that the drug traverse through a highly lipophilic membrane. Accordingly, diffusion processes are governed by Fick's laws of diffusion and therefore influenced by the compound's lipophilicity. This ability to diffuse through lipids has been found to be highly correlated with the ability of a drug to partition between water and an organic solvent (Martinez & Amidon, 2002). In this case methanol fraction could have achieved the minimum effective plasma concentration with in short period of time than aqueous fraction as it harbors relatively more lipid soluble constituents. However, the duration of administration is relatively longer in case of subacute model, so that both

fractions could have achieved the required therapeutic concentration in similar time. This could be the reason why the onset of action for both fractions in the subacute model was similar.

Phytochemical screening for secondary metabolites revealed that terpenoids and steroids were possibly found in the methanol fraction but not in the aqueous fraction. This could be one reason why the methanol fraction was more effective in suppression of paw edema in both models employed in this study than the aqueous fraction as these phytochemicals were reported to have anti-inflammatory effect in the literatures (Igwe & Okwu, 2013; John & Shobana, 2012; Kumar, *et al.,* 2013; Muthaiah, *et al.*, 1993; Zarkaria *et al*., 2001). Terpenoids were reported to inhibit the development of chronic joint swelling and to affect different mechanism (s) relevant to inflammation arising in response to varied etiological factors (Kumar, *et al.*, 2013). As a result, the activity of *I. spicata* against formalin-induced paw edema, a model which closely resembles human arthritis, could have emanated from the terpenoid constituents.

In general, the core chemical classes of anti-inflammatory agents from natural sources that have been reported to engage a vast range of compounds are flavonoids, terpenoids, steroids, alkaloids, anthraquinones, glycosides, polyphenols, lignans, polysaccharides, saponins and peptides (Igwe & Okwu, 2013; Kumar, *et al.*, 2013; Odyntya, *et al.*,2005; Zarkaria, *et al*., 2001). The phytochemical screening for secondary metabolites of the solvent fractions of *I. spicata* showed that secondary and tertiary amine containing compounds, deoxy sugar, anthraquinone derivatives, phenols and saponins were possibly common to both methanol and aqueous fractions. Therefore, it is possible to say that the

anti-inflammatory effect of solvent fractions in both inflammation models could be due to these phytochemicals.

Since the methanol and aqueous fractions in the acute model reduced mouse paw edema during the late phases (two hours after carrageenan injection), it is possible that the plant could have interacted with polymorphonuclear cells and mediators such as prostaglandins, bradykinin and leukotriens or it could have inhibited the synthesis or release of these mediators (Igwe & Okwu, 2013; Jothi, *et al.*, 2012; Murugan, *et al.*, 2012).

When maximum effects for methanol and aqueous fraction, which are observed at the maximum dose employed in both models were compared, the methanol fraction showed significantly greater effect (45.9%, $p < 0.05$) than the aqueous fraction (39.6%) in acute inflammation model but not in the subacute one (41.5% & 40.4% for methanol and aqueous fractions, respectively). Therefore one can conclude that in case of acute inflammation model the methanol fraction showed statistically different effect than the aqueous fraction due to the absorption and lipid solubility factor discussed above. This possibly suggests the need for loading dose if this fraction is to be used for its anti-inflammatory effect, as loading dose is important to achieve the steady-state plasma drug concentration rapidly and to reduce the time required for onset of the full therapeutic effect (Aulton, 1998, Schie, *et al.*, 2011). However, in the subacute model, even though the effect of methanol fraction was higher than that of the aqueous fraction, statistically significant different effect was not observed. This could be due to the fact that the methanol fraction in acute model could have attained the required therapeutic concentration with-in a short period of time as discussed above. However, in case of sub

acute model, since the duration of administration is relatively longer than the acute, both fractions could attain the required therapeutic concentration to bring effects in a relatively similar time as a relationship exists between pharmacological effect and plasma drug concentration (Brunton, *et al*., 2011). This closeness of effect between methanol and aqueous fractions in the subacute model could be further explained by observing Figure 5 starting from day five. One can observe that in Figure 5, starting from day five, the percentage inhibition of paw edema of methanol and aqueous fractions in the subacute inflammation at their maximal dose employed was almost overlapping to each other. This clearly indicates that the effect of methanol fraction was becoming more similar to that of aqueous fraction in the subacute model of inflammation. Generally, the relative superiority of methanol fraction effect over the aqueous one in both models could be explained as follows: - since successive solvent fractionation by using soxhlet apparatus was employed in the present study; majority of semi-polar and some polar constituents would have been solubilized by methanol, only leaving those more polar constituents. This is due to the fact that in soxhlet extraction, it is possible to extract all of the soluble materials, if enough time is given (Wang & Weller, 2006). Thus, only the more polar constituents will come out with the aqueous fraction. However, almost all semi-polar and some polar constituents would have already fallen in the methanol fraction, which could be responsible for the greater effect of methanol fraction due to synergistic effect. This could be additional reason why the aqueous fraction was with less anti-inflammatory effect than the methanol fraction in both models.

Intergroup comparisons showed that the methanol fraction showed statistically significant different effect in both 200 mg/kg versus 100 mg/kg and 100 mg/kg versus 50 mg/kg in

the acute model, but there was no statistically significant different intergroup difference in suppression of paw edema in the subacute model; whereas the aqueous fraction showed statistically significant different effect only when 200 mg/kg was compared with 50 mg/kg in both inflammation models used in the present study. Therefore, one can conclude that the methanol fraction exhibited significant suppression of acute inflammation in a dose dependant manner.

The result of two way analysis of variance indicated that the treatment-duration of treatment interaction had significant effect on the paw volume in both inflammation models for both methanol and aqueous fractions. In addition to their interaction, the statistically significant effect observed by both treatment and duration of treatment separately indicates that the paw edema is affected by both the treatment and duration of treatment as well as by their combined effect.

Indomethacin showed highly significant anti-inflammatory effect in both models. This indicates that the solvent fractions showed lower anti-inflammatory effect than the Indomethacin.

6. CONCLUSION

In conclusion, the data obtained in this study demonstrated that methanol and aqueous fractions of roots of *I. spicata* have anti-inflammatory activities but the chloroform fraction is devoid of any effect. In addition, the methanol fraction appeared to have a better anti-inflammatory effect in both acute and sub acute anti-inflammatory models, suggesting phytochemical constituents with solubility range from semi-polar to polar were responsible for the observed pharmacological effect.

7. SUGGESTIONS FOR FURTHER WORK

- Isolation and characterization of *I. spicata* components and testing their anti-inflammatory effect will provide a body of knowledge about the components which are more responsible for its anti-inflammatory effect.
- Specific tests for phytochemical would be necessary for further confirmation of the results obtained in the preliminary phytochemical screening tests employed here.
- It would be appropriate to test the anti-inflammatory effect of *I. spicata* on chronic models as well as on other models of acute and sub acute inflammation other than those employed in this study.
- It would have a great value to study other biological activities of the plant claimed by folklore medicine.

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