Sudan University of Science and Technology

College of Graduate Studies

ANTI-INFLAMMATORY AND ANALGESIC ACTIVITES OF *HIBSCUS SABDARIFFA* AND *BALANITES AEGYPTIACA* SEEDS EXTRACTS IN RATS

النشاطات المضادة للإلتهاب والمسكنة للألم في مستخلصات بذور اللالوب والكركدى فى الجرزان

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B.V.Sc, M.V.Sc

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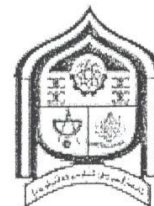
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إقرار

أنا الموقع أدناه أُقر بأنني المؤلف الوحيد لرسالة الدكتوراه المعنونة النشاطات المضادة للإلتهاب والمسكنة للألم في مستخلصات بذور الكركدي والبلانيتس في الجرذان.

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DEDICATION

I lovely dedicate this thesis to my...

Dearly loved parents

Precious brothers & sisters

Great teachers

,,,,,

For all my lovers

&

For all those searching for knowledge

,,,,,,,,,

,,,,,

SUMAIA

PUBLICATIONS

Parts of this study have been published as papers entitled:

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ACKNOWLEDGEMENTS

Abstract

Background: *H. sabdariffa* (Linn) and *Balanites aegyptiaca* (L.) are multipurpose plants used widely in the Sudan for their nutritional, cosmetic, industrial and medicinal values. The seeds oil has been used by natives to treat rheumatism; so this study was designed to investigate the efficacy of seed extracts of *H. sabdariffa* and *B. aegyptiaca* in the treatment of inflammation.

Materials and methods: The anti-inflammatory effect of petroleum ether and ethanolic extracts of *H. sabdariffa* and *Balanites aegyptiaca* seeds was assessed on acute and chronic models by using carrageennan induced paw oedema and cotton pellet granuloma in rats, respectively. The analgesic activity of the petroleum ether extract of seeds was evaluated by acetic acid induced writhes in rats. The acute and sub-chronic toxicities were performed to explore the safety of the extracts. Gas Chromatography was also done for petroleum ether extracts for both plants.

Results: In acute anti-inflammatory models, oral administration of the petroleum ether extracts of *H. sabdariffa* and *B. aegyptiaca* seeds significantly inhibited the hind paw oedema induced by carrageenan compared with the control. The inhibition of oedema by *B. aegyptiaca* seeds petroleum ether extract was better than that observed in rats treated with *H. sabdariffa*. The anti-inflammatory effect of *B. aegyptiaca* and high dose of *H. sabdariffa* seeds petroleum ether extract was comparable to indomethacin used as a standard anti-inflammatory drug. Moreover, the ethanolic extracts of both plants seeds failed in reducing paw oedema compared with the control. The petroleum ether extract of *H. sabdariffa* seeds exhibited significant inhibition in vascular permeability in rats induced by intraperitoneal injection of 0.6% acetic acid compared with the control but less than that observed in diclofenac sodium used as a standard anti-inflammatory drug. In cotton pellet granuloma method, *H. sabdariffa* and *B.*

aegyptiaca seeds petroleum ether extracts produced significant inhibition in granuloma at a dose of 4 and 8 ml/kg when compared with the control, the inhibition degree by *B. aegyptiaca* seeds was superior than that by *H. sabdariffa* seeds but inferior to the inhibition caused in diclofenac sodium used as a standard anti-inflammatory drug. The petroleum ether extract of *H. sabdariffa* and *B. aegyptiaca* significantly reduced the abdominal constrictions produced by injection of 0.7% acetic acid especially at higher doses compared with the control group. The acute oral toxicity study of *H. sabdariffa* and *B. aegyptiaca* seeds petroleum ether extracts proved the safety of the extracts up to 20 ml/kg for 24 hours. Oral administration of the *B. aegyptiaca* petroleum ether extract for 21 days did not have any signs of toxicity, which was evidenced by insignificant change in haematological parameters especially in rats treated with 4 ml/kg as well as the activities of ALT, AST, ALP and total proteins which are taken as markers for liver function tests and the levels of urea and creatinine as indicators for renal function tests compared with the control. Sections of different organs showed insignificant histopathological changes, with exception of two kidneys which showed focal degenerative changes in rats that given high dose of *B. aegyptiaca* petroleum ether extract. Gas chromatography analysis of *H. sabdariffa* seeds oil revealed the presence of linolelaidic acid, palmatic acid and arachidic acid, whereas, *B. aegyptiaca* seeds oil contains capric acid, palmitic acid and oleic acid.

Conclusion: This study confirmed the traditional uses of *H. sabdariffa* and *B. aegyptiaca* seeds as anti-inflammatory and analgesic agents, which may be attributed to their fatty acid contents.

Arabic abstract
ملخص الأطروحة

الخلفية: الكركدى والهجليج من النباتات متعددة الاستخدامات والتى تستعمل بصورة واسعة فى السودان للتغذية، التجميل، التجارة ولقيمتها العلاجية. زيت البذور يستخدم فى الطب الشعبى لعلاج الروماتزم، لذا صممت هذه الدراسة للتقصى عن فعالية مستخلصات البذور لنباتى الكركدى والهجليج لعلاج الالتهابات.

المواد والطرق: تم تقييم تأثير الالتهاب لمستخلصات البتروليوم ايثر والايثانول لبزور الكركدى واللالوب باستخدام نموذج للالتهاب الحاد بواسطة الكراجنان لاحداث الوزمة وحمض الخليك لاحداث التسرب الوعائى، ونموذج للالتهاب المزمن باستخدام كرات القطن فى الجرزان على التوالى. النشاط المسكن تم تقييمه بواسطة حمض الخليك لاحداث الانقباضات فى الجرزان. السمية الحادة وتحت المزمنة تم انجازها لاكتشاف سلامة المستخلصات النباتية. تحليل الغاز اللونى لمستخلص البتروليوم ايثر ايضا انجز للنباتين.

النتائج: فى نموذج الالتهاب الحاد، التجريع الفومى لمستخلصات البتروليوم ايثر لنباتى الكركدى والهجليج ثبط معنوياً الوزمة المحدثة بالكراجنان مقارنة بالمجموعة الضابطة. تثبيط الوزمة بواسطة مستخلص البتروليوم ايثر لنبات الهجليج كان افضل من الذى لوحظ فى الجرزان المعالجة بمستخلص البتروليوم ايثر لنبات الكركدى. الاثر المضاد للالتهاب لنبات الهجليج والجرعة العالية لنبات الكركدى شبيهة للاثر المضاد للالتهاب لعقار الاندوميثازين المستخدم كعقار معيارى لعلاج الالتهاب. ايضاً المستخلص الايثيلى لنباتى الكركدى والهجليج فشلت فى خفض الوزمة مقارنة بالمجموعة الضابطة. مستخلص البتروليوم ايثر لنبات الكركدى احدث اثر مثبط للتسرب الوعائى فى الجرزان بواسطة الحقن البريتونى لحمض الخليك 0.6% مقارنة بالمجموعة الضابطة، لكنه أقل من ذلك الملاحظ فى ديكلوفيناك الصوديوم المستخدم كعقار معيارى مضاد للالتهاب. فى نموذج الورم الحبيبى المحدث بكرات القطن، مستخلص البتروليوم ايثر لنباتى الكركدى والهجليج احدث تثبيطاً معنوياً للورم الحبيبى بجرعة 4 و 8 مل/كجم مقارنة بالمجموعة الضابطة. درجة التثبيط اعلى فى بذور الهجليج من بذور الكركدى ولكنه أقل من التثبيط المسبب بديكلوفيناك الصوديوم. مستخلص البتروليوم ايثر لنبات الكركدى والهجليج احدثا انخفاضاً معنوياً للانقباضات البطنية المحدثة بحمض الخليك 0.7% خصوصاً بالجرعات العالية مقارنة بالمجموعة الضابطة. الدراسة السمية الحادة الفومية لنباتى الكركدى والهجليج اثبتت سلامة المستخلصات حتى جرعة 20 مل/كجم لمدة 24 ساعة. ايضاً اعطاء مستخلص البتروليوم ايثر لنبات الهجليج فومياً لمدة 21 يوم لم يحدث اى علامات سمية والتى تم الدلالة عليها بعدم التغير المعنوى فى مكونات الدم وانزيمات ALT، AST ، ALP والبروتين الكلى والتى تم قياسها كعلامات لوظائف الكبد ومستويات البولينا

والكرياتنين كعلامات لوظائف الكلى خصوصاً فى الجرزان المعطاة 4 مل/كجم مقارنة بالمجموعة الضابطة. بالاضافة لذلك مقاطع الاعضاء المختلفة أوضحت عدم تغير معنوى فى الانسجة المرضية باستثناء مقطعين لكليتين أوضحت تغيير تنكسى بؤرى فى الجرزان المعالجة بالجرعة العالية من مستخلص البتروليوم ايثر لنبات الهجليج. تحليل الغاز اللونى لزيت بذورنباتى الكركدى دل على وجود لينوليديك اسد، بالمتيك اسد و اراشدك اسد بينما احتوى زيت بذور الهجليج على كابريك اسد، بالمتيك اسد و اوليك اسد.

الخلاصة: هذة الدراسة أكدت صحة الاستخدام التقليدى لبذور نباتى الكركدى والهجليج كمضادات للالتهاب ومسكنات للألم والذى يمكن ان يعزى لمحتواهما من الاحماض الدهنية.

Introduction

The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (Hoareau and DaSilva, 1999). The world health organization (WHO) has estimated that approximately 80% of the world's population depends on traditional medicines for meeting their primary health care needs (Deore and Khadabadi, 2010).

Inflammation is a protective reaction by the body in response to some physical or chemical injury; acute inflammatory response begins immediately after cellular injury (Sivaraman *et al.*, 2010). Drugs which are in use presently for the management of fever, pain and inflammatory conditions are either narcotics e.g. opioids or non-narcotics e.g. salicylates and corticosteroids e.g. hydrocortisone. All of these drugs show well known side and toxic effects. Moreover, synthetic drugs are very expensive to develop, since for the successful introduction of a new product approximately 3000-4000 compounds are to be synthesized, screened and tested whose cost of development ranges from 0.5 to 5 million dollars (Ahmed *et al.*, 1992). Non-steroidal anti-inflammatory drugs (NSAIDs) are widely employed as analgesic and anti-inflammatory agents, but their use is significantly limited by their propensity to induce ulceration and bleeding in the gastrointestinal tract (Schoenfeld *et al.*, 1999). On the contrary many medicines of plant origin had been used since long time without any adverse effects. It is therefore, essential that efforts should be made to introduce new medicinal plants to develop cheaper drugs. Plants represent still a large untapped source of structurally novel compounds that might serve as lead for the development of novel drugs (Ahmed *et al.*, 1992).

Various herbal medicines derived from plant extracts are being used in the treatment of a wide variety of clinical diseases, though relatively little

knowledge about their mechanisms of action is known (Ratheesh and Helen, 2007). Many herbal preparations are being prescribed widely for the treatment of inflammatory conditions (Bagul *et al.*, 2005). There is a need for research and developmental work in herbal medicine because apart from the social and economic benefits, it has become a persistent aspect of present day healthcare in developing countries. Furthermore, the use of steroidal and non-steroidal drugs as anti-inflammatory agents is becoming highly controversial due to their multiple side effects. Therefore, developing new agents with more powerful anti-inflammatory and analgesic activities and with lesser side effects is, at present, of great interest. The selected Sudanese plants seeds which of putative anti-inflammatory and analgesic effects are scantly mentioned in literature. The present study is an attempt to evaluate scientifically these seeds of *Hibiscus sabdariffa* and *Balanites aegyptiaca* as step towards their formulation in acceptable dosage form.

Objectives:

1. To evaluate the anti-inflammatory and analgesic activities of *Hibiscus sabdariffa* and *Balanites aegyptiaca* seed extracts using various experimental animal models.
2. To determine the phytochemical constituent(s) of the active plant extracts.
3. To assess the acute and sub-chronic toxicities of the active plant extracts.

CHAPTER ONE

1. Literature Review

1.1 Inflammation

The word "inflammation", derived from the Latin word "inflammare", (to set on fire), is a complex biological process including several chemical mediators which are induced by vascular tissue of the body. Inflammations are generally characterized by certain regular events such as redness, swelling, heat, pain, and at certain times lead to exudation and loss of function (Beg *et al.*, 2011). There are various components to an inflammatory reaction that can contribute to the associated symptoms and tissue injury. Oedema formation, leukocyte infiltration and granuloma formation represent such components of inflammation (Yadav *et al.*, 2011 and Sokeng *et al.*, 2013).

The process of inflammation involves several events and mediators which are potent chemical substances found in the body tissues, such as prostaglnadins, leukotrienes, prostacyclins, lymphokines, and chemokines like interferon-α (IFN-α), γ, interleukin (IL)-1, IL-8, histamine, 5-hydroxytryptamine (5-HT), and tissue necrosis factor-α. These mediators produce several chemical pathways and events to evoke a complementary response against external stimuli (Beg *et al.*, 2011). During the inflammatory process, large amounts of the pro-inflammatory mediator's nitric oxide (NO) and prostaglandin E2 (PGE2) are generated by the inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively (Yadav *et al.*, 2011).

The enzyme, phospholipase A_2, is known to be responsible for the formation of mediators of inflammation such as prostaglandins and leukotrienes which by attracting polymerphonuclear leucocytes to the site of inflammation would lead to tissue damage probably by the release of free radicals. Phospholipase A_2 converts phospholipids in the cell membrane into arachidonic acid, which is highly reactive and is rapidly metabolized by cyclooxygenase to prostaglandins,

which are major components that induce pain and inflammation (Paschapur *et al.*, 2009). Kinins cause vasodilatation, increase vascular permeability and WBC migration in the early stages of the inflammation and are also involved in, collagen formation in the later stages of inflammation. Kinins degranulate mast cells to release histamine as well as other mediators of inflammation and cause plasma extravasation by contraction of vascular endothelial cells. Kinins are potent algogenic substances, which induce pain by directly stimulating nociceptors in skin joint, and muscles (Shaikh, 2011).

Chronic inflammation develops from unresolved symptomatic acute inflammation with or without any clinical manifestations. This may activate macrophages and lymphocytes which release inflammatory mediators. Neutrophils are the first blood leukocytes to arrive to an inflammatory site. The process of trans-endothelial migration from the blood to the tissue is complex and involves response to chemotactic factors and binding to adhesion molecules at the endothelium surface. These events permit the extravasation of blood neutrophils to the tissue where they can perform different functions. Major roles of neutrophils include phagocytosis, production of reactive oxygen and nitrogen species that damage DNA, cell membranes and production of chemo-attractants such as IL-8 which recruit further competent cells. Neutrophil response usually terminates when neutrophils undergo apoptosis which are phagocytosed by arising macrophages. Inflammatory cells release prostaglandins with concomitant increase in the expression of key enzyme cyclooxygenase which in turn can activate several transcription factors including NF-B. Inflammation activates a variety of inflammatory cells, which induce, activate oxidant generating enzymes like NADPH oxidase, xanthine oxidase, myeloperoxidase, etc.; which produce superoxide anion, other reactive nitrogen species like nitric oxide through activation of inducible nitric oxide synthase (iNOS). Free radicals play major role in persistence of inflammation. During the process of inflammation, inflammatory cells secrete chemically reactive oxidants, radicals

and electrophilic compounds that facilitate the elimination of the infectious agents. These inflammatory mediators can damage the surrounding host tissue (Shaikh, 2011).

1.2 Anti-inflammatory drugs

Inflammatory diseases are currently treated with steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs exert their effects by inhibiting the metabolism of arachidonic acid by both cyclooxygenase and lipoxygenase enzyme pathways (Sivaraman *et al.*, 2010).

Recent research has shown that there are at least two cyclooxygenase (COX) isoenzymes. COX-1 is constitutive and makes prostaglandins (PGs) that protect the stomach and kidney from damage. COX-2 is induced by inflammatory stimuli, such as cytokines, and produces prostaglandins (PGs) that contribute to the pain and swelling of inflammation. COX-2 is mainly an inducible enzyme and is involved primarily in the regulation of inflammation. Prostaglandins and in particular PGE2 are regarded as a potent pro- inflammatory molecule; however, increasing evidence has indicated that they can also exert anti-inflammatory functions which are important for the resolution of the inflammatory response (Yadav *et al.*, 2011).

Despite their widespread use, NSAIDs are often associated with severe adverse effects; the most common being gastro-intestinal ulcers, bleeding and renal disorders due to their non-selective inhibition of both constitutive (COX-1) and inducible (COX-2) isoforms of the cyclooxygenases enzymes (Nonato *et al.*, 2009 and Sivaraman *et al.*, 2010). On the other hand, fully selective cyclooxygenase (COX)-2 inhibitors produce less gastrointestinal bleeding and ulceration than conventional NSAIDs, but this benefit may be offset by significant increases in renal and cardiovascular adverse events associated with their use (Cha´vez-Pin˜a *et al.*, 2007 and Nonato *et al.*, 2009).

1.3 Importance of medicinal plants

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects (Gupta *et al.*, 2006). The medicinal plants find applications in various important sectors like in pharmaceutical, cosmetic, agricultural and food industry. The use of the medicinal herbs for curing diseases has been documented in history of all civilizations (Das *et al.*, 2013).

Due of the deleterious side effects attributed to the prolonged use of NSAIDs and their ineffectiveness in some cases, the control of inflammation is still a major challenge (Nonato *et al.*, 2009). Moreover, synthetic drugs are very expensive to develop and whose cost of development ranges from 0.5 to 5 million dollars. On the contrary many medicines of plant origin had been used since long time without any adverse effects. Exploring the healing power of plants is an ancient concept. For centuries people have been trying to alleviate and treat disease with different plant extracts and formulations (Yadav *et al.*, 2011). It is therefore essential that efforts should be made to introduce new medicinal plants to develop cheaper drugs. Plants represent still a large untapped source of structurally novel compounds that might serve as lead for the development of novel drugs (Ahmed *et al.*, 1992).

The herbal medicines are getting more importance in the treatment of inflammation because of the toxic effect of the current therapy used to treat those inflammation using synthetic drugs. Herbal medicines are less toxic and less costly when compared to the synthetic drugs (Ravi *et al.*, 2009).

There is a need for research and developmental work in herbal medicine because apart from the social and economic benefits, it has become a persistent aspect of present day healthcare in developing countries. Therefore, the development of potent anti-inflammatory drugs with fewer side effects is necessary (Saha and Ahmed, 2009).

1.4 Anti-inflammatory and analgesic plants

Recently, many natural medicines derived from plants were considered effective and safer for the treatment of various diseases including inflammation and pain (Sokeng *et al.*, 2013).

The analgesic and anti-inflammatory effects of ethanolic bark extract of *Plumeria rubra* were evaluated on experimental animal models (Das *et al.*, 2013). The extract produced a highly significant analgesic activity in a dose dependent manner on hot plate method, acetic acid induced writhing test and on both the early and late phases of formalin test. The oral administration of the extract significantly ($P < 0.001$) inhibited inflammatory response induced by carrageenan in a dose dependent manner. The analgesic and anti-inflammatory effects of *P. rubra* bark ethanolic extract may be due to the presence of various chemical constituents specially flavonoids, tannins, alkaloids or terpenoids.

The anti-inflammatory and antipyretic activities of the aqueous extract of the leaf, root and saponin fraction from *Vernonia amygdalina* were investigated by Adiukwu *et al.* (2013). Ear thickness measurement in xylene induced inflammation and anal temperature readings in *Saccharomyces cerevisiae* induced pyrexia in rats were used as standard procedures. Results indicated significant anti-inflammatory and antipyretic activities of the extracts which may partly clarify its use in traditional medicine as a remedy for similar disorders.

Acacia nilotica (L.) Del. (Fabaceae) is traditionally used in Northern Cameroon to treat various inflammatory affections. The anti-inflammatory activity of *A. nilotica* pods aqueous extract was evaluated using acute and chronic models for inflammations. The aqueous extract of *A. nilotica* pods elicited a significant inhibition (44.16%) of xylene-induced ear swilling in mice compared with untreated control. Moreover, the high dose (100 mg/kg) of *A. nilotica* extract produced a maximum inhibition of 64.41 and 25.62%, respectively for the carrageenan induced paw oedema and the cotton pellet induced granuloma in rats. Phytochemical analysis of *A. nilotica* aqueous extract revealed the presence

of flavonoids, anthraquinones, saponins, tannins, polyphenols and alkaloids. These results indicated that the aqueous extract of *A. nilotica* pods may contain orally effective anti-inflammatory principles, justifying its use in folklore medicine for related conditions (Sokeng *et al.*, 2013).

Acute and chronic anti-inflammatory activity of *Clerodendrum phlomidis* root bark aqueous extract was investigated in rats. Carrageenan induced rats' paw oedema and acetic acid induced peritonitis in mice were used to assess acute anti-inflammatory. The activity of *C. phlomidis* was compared with aspirin and Dashamoolarishta (a multi-ingredient plant formulation containing *Clerodendrum phlomidis*) which served as positive controls. *C. phlomidis* at a dose of 21.6 ml/kg showed significant anti-inflammatory activity in carrageenan induced paw odema, acetic acid induced peritonitis and cotton pellet granuloma. These results provide a scientific basis for inclusion of *C. phlomidis* in the Dashamoolarishta formulation (Parekar *et al.*, 2012).

The anti-inflammatory effects of phenolic compounds from *Emblica officinalis* were carried out in carrageenan and cotton pellet induced acute and chronic inflammatory animal models. Fractions of *E. officinalis* containing free (FPEO) and bounded (BPEO) phenolic compounds were studied at dose level of 20 and 40 mg/kg. The results indicated FPEO and BPEO have significant reduction in acute and chronic inflammation only at high doses of both fractions which was comparable to the reference drug, diclofenac sodium. Therefore, phenolic compounds of *E. officinalis* may serve as potential herbal candidate for the treatment of acute and chronic inflammation due to their modulatory action of free radicals (Muthuraman *et al.*, 2011).

Yadav *et al.*, (2011) investigated the anti-inflammatory activity of hydroalcoholic extract of *Quisqualis indica* in Wistar rats. Oral administration of the extract at a dose of 100 and 150 mg/kg exhibited a significant anti-inflammatory activity in acetic acid-induced vascular permeability and cotton

pellet granuloma in a dose dependent fashion, which support the folklore use of *Q. indica* L. as anti-inflammatory agent.

Anti-inflammatory activity of the methanolic extract of *Indigofera oblongifolia* Forsk root was studied in Wistar rats using the carrageenan induced left hind paw edema. Oral administration of methanolic extract of *I. oblongifolia* Forsk at a dose of 250 mg/kg and 500 mg/kg reduced the oedema induced by carrageenan by 32.19 % and 40.97 %, respectively compared with the untreated control. Diclofenac sodium at a dose of 10 mg/kg inhibited the oedema volume by 39.02%. The results indicated that the methanolic extract of 500 mg/kg body weight shows more significant ($p< 0.05$) anti-inflammatory activity when compared with the standard and untreated control (Upwar *et al.*, 2011).

The anti-inflammatory effect of ethanolic extract of *Hemidesmus indicus* (EEHI) was investigated in rats and mice using acute anti-inflammatory models *viz:* carrageenan induced paw edema, leukocyte emigration, acetic acid-induced vascular permeability, mast cell degranulation in rats, subchronic anti-inflammatory model *viz*: cotton pellet granuloma in mice and chronic anti-inflammatory models *viz*: formailn induced arthritis and delayed type hypersensitivity using egg white lysozyme as an antigen. EEHI (100, 200 mg/kg, p.o) exhibited a dose-dependent and significant inhibition ($P <0.05$) in all the experimental models. Preliminary phytochemical screening revealed the presence of flavonoids, steroid, terpenoid, saponins and tannins. The results obtained suggest marked anti-inflammatory activity of the EEHI and support the traditional use of this plant in inflammatory conditions (Shaikh, 2011).

The anti-microbial and anti-inflammatory effects of *Ipomoea aquatica* Forsk (IAF) were investigated by Sivaraman *et al.*, (2010). The antimicrobial property of the IAF was evaluated against Gram-positive and Gram negative microorganisms using the agar disc diffusion method, in which methanolic leaf extract of IAF has shown bigger zone of inhibition than aqueous leaf extract (IAF). The anti-inflammatory effect was evaluated in carrageenan-induced paw

oedema model. The methanolic and aqueous extracts of IAF were administered orally at a dose of 200 mg/kg. Pretreatment with a single dose of IAF produced significant dose dependent anti-inflammatory effects on carrageenan-induced rat hind paw edema. Crude methanolic and aqueous extract (200 mg/kg) and indomethacin (5 mg/kg) inhibited significantly ($p < 0.05$) the formation of the carrageenan-induced rat paw edema, measured in third hour of experiment (peak of edema formation). These results demonstrated that IAF possesses anti-microbial and anti-inflammatory effects and has no obvious acute toxicity, which advanced our understanding of the folk use of IAF in treating various inflammatory disorders.

Ravi *et al.*, (2009) investigated the anti-inflammatory activity of methanolic extract of berries of *Solanum nigrum* Linn. Dried pulverized berries of *S. nigrum* were extracted with methanol by using soxhlet apparatus. The effect of methanolic extracts of berries of *S. nigrum* was studied on carrageenan induced paw edema. The results showed that the methanolic extract of *S. nigrum* (375 mg/kg) decreased significantly the oedema induced in hind paw by carrageenan. It has been concluded that the methanolic extract of berries of *S. nigrum* Linn has good anti-inflammatory activity against carrageenan induced paw oedema.

Anti-inflammatory effect of the bark extract of *Albizia lebbeck* Benth. was carried out in rats' paw oedema model induced by carrageenan. The extract at a dose of 400 mg/kg showed 36.68% ($p<0.001$) inhibition of oedema volume after 4 hours of carrageenan injection. In the acetic acid-induced writhing test, the extract at the 200 and 400 mg/kg showed 39.9 % and 52.4 % inhibition of writhing, respectively. In radiant heat tail-flick method the crude extract produced 40.74% ($p<0.001$) and 61.48% ($p<0.001$) elongation of tail flicking time 30 minutes after oral administration at a dose of 200 and 400 mg/kg, respectively (Saha and Ahmed, 2009).

Borassus flabellifer L. (Arecaceae) had been widely used for its biological activities in indigenous system of medicine. The anti-inflammatory activity of

ethanolic extract of male flowers (inflorescences) of *B. flabellifer* in rodents was evaluated by Paschapur *et al.* (2009). The anti-inflammatory activity was evaluated using acute inflammatory models like; carrageenan induced paw oedema and chronic models like; cotton-pellet induced granuloma and carrageenan induced air-pouch model in rats. The biochemical parameters like serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), lipid per oxidation and alkaline phosphatase (ALP) were also estimated as supportive studies. Oral administration of the extract at the doses of 150 and 300 mg/kg exhibited dose dependent and significant ($p < 0.0001$) anti-inflammatory activity in acute and chronic models of inflammation. The extract also showed significant ($p < 0.0001$) results for biochemical parameters. Therefore, the present investigations support the folkloric use of *B. flabellifer* L. as anti-inflammatory agent.

The hydroalcohol extracts of *Achillea millefolium* L. and *Artemisia vulgaris* L., were evaluated by the hot plate, writhing, formalin and intestinal transit tests in an attempt to confirm their folk use as analgesic, anti-inflammatory and antispasmodic agents. *A. millefolium* at a dose of 500 and 1000 mg/kg significantly inhibited abdominal contortions by 65% and 23%, respectively, whereas, *A. vulgaris* at a dose of 500 and 1000 mg/kg inhibited them by 48% and 59%, respectively. None of the extracts produced differences in the intestinal transit in mice, nor in the response time in the hot plate or in the immediate or late responses in the formalin test (Pires *et al.*, 2009).

The 50% ethanolic extract of *Fumaria indica* was investigated for its anti-inflammatory and antinociceptive potential in animal models. Oral administration of *F. indica* dry extract at a dose of 100, 200 and 400 mg kg^{-1} exhibited dose dependent and significant anti-inflammatory activity in carrageenean and histamine induced hind paw oedema as acute model and chronic cotton pellet granuloma as chronic model of inflammation. The extract (400 mg kg^{-1}) exhibited maximum anti-inflammatory effects of 42.2 and 42.1%

after 3 hours with carrageenean and histamine, respectively. The same dose of extract showed 38.9% reduction in granuloma mass in a chronic condition. Antinociceptive activity of the extract was also confirmed in mice by mechanical, thermal induced pain and acetic acid induced writhing in mice (Rao *et al.*, 2007).

The whole plants of *Sida acuta* and *Stylosanthes fruticosa*, the heart wood of *Toona ciliate,* the leaves of *Bougainvilla spectabilis* and *Polyalthia longifolia* and the bark and leaves of *Ficus glomerata* were studied as analgesic. The different plants were used in folklore medicine in the treatment of toothache and strengthening of gums, anthelmintic, kidney diseases, analgesic, anti-inflammatory, hepatoprotective, antihyperglycaemic and anticancer. The extracts were prepared using powdered material with ethanol and were evaluated for analgesic activity by analgesiometer at three dose level (100, 300 and 500mg/kg). Analgesic activity was significant with *T. ciliata* (heart wood) ethanolic extract when compared with other extracts and its activity was confirmed by tail immersion method (Malairajan *et al.*, 2006).

The anti-inflammatory effects of crude (CSE) and purified (SPE) extracts of *Hedera colchica* in carrageenan and cotton pellet-induced acute and chronic inflammation models in rats were assessed. Both CSE and SPE of *H. colchica* were found to have anti-inflammatory effects. Indomethacin used as a reference drug exert higher activity (90%) at 4th hour pst carrageenan injection, while SPE (83%) and CSE (55%) of *H. colchica* at a dose of 100 mg/kg were found to have quite potent effects in acute phase of inflammation, in respect to control values. Moreover, SPE was found to be the most potent drug in chronic phase of inflammation with an effect of 64.32%. Indomethacin was found more potent than the CSE of *H. colchica,* and their potency was found as 61.2 and 58%, respectively (Gepdiremen *et al.*, 2004).

The analgesic properties of aqueous and ethanolic extracts of four Ethiopian plants namely *Ocimum suave, Ocimum lamiifolium, Lippia adoensis,* and *Ajuga*

remota were carried out. All extracts were observed to possess analgesic properties with varying potencies in tail-flick and hot-plate tests. Analgesic activity however, was not observed with tail-pinch test. The analgesic potencies also varied with concentrations and time after administration. From the present findings, it can be concluded that the extracts of all the plant materials have got analgesic properties with fast onset of action whose mechanisms need to be investigated further (Makonnen *et al.*, 2003).

Numerous Sudanese medicinal plants were investigated as anti-inflammatory and analgesic agents such as *Capparis deciduas, Cannabis sativa* and *Blumea aurita*. Chloroform and methanol extracts of *Capparis decidua* aerial parts have shown potent anti-inflammatory activity evaluated by carrageenan induced paw edema in Swiss albino rat model. In Sudan this plant may be used as natural source for pharmaceutical purpose designed for anti-inflammatory activity and may provide a new lead or pharmacophore for more potent analogues (Mohammed *et al.*, 2012).

Cannabis sativa seeds petroleum oil extract was investigated for anti-inflammatory activity using carrageenan induced paw oedema in albino rats (Musa *et al.*, 2011). *C. sativa* seeds oil extract exhibited a significant decrease in oedema size compared with standard drug indomethacin.

Abdulla *et al.* (2013) investigated the anti-inflammatory and analgesic effects of *Blumea aurita* using formalin induced oedema, cotton pellet induced granuloma and hot plate method. Results indicated that the ethanolic extract of *Blumea aurita* have significant anti-inflammatory and analgesic activities in rats which may attributed to its phytoconstituents.

1.5 Phytoconstituents as anti-inflammatory

Medicinal plants are considerably useful and economically essential. They contain active constituents that are used in the treatment of many diseases (Das *et al.*, 2013). The number of chemical compounds, called phytochemicals, found within the plant kingdom is truly vast and their range of activity is equally as

great. Some of the phytochemicals found in certain herbs and plants are reported to demonstrate pain and inflammation reducing properties (Sivaraman *et al.*, 2010).

Many bioactive compounds from plants are known to prevent or mitigate numerous inflammatory processes and diseases. Some of these compounds are flavonoids which are believed to function as antioxidants and possess anti-inflammatory action (Mensah *et al.*, 2011). Das *et al.*, (2013) reported that flavonoids which are also known as nature's tender drugs; possess abundant biological and pharmacological activities. Flavonoids such as rutin, quercetin and luteolin produced significant antinociceptive and anti-inflammatory activities. Certain flavonoids possess strong inhibitory activity against a wide range of enzymes such as protein kinase C, protein tyrosine kinases, phospholipase A2, phosphodiesterases and others. Other flavonoids potently restrain prostaglandins, a group of powerful proinflammatory signaling molecules. Inhibition of these key enzymes provides the mechanism by which flavonoids inhibit inflammatory processes. Steroids and saponins have also been reported to possess anti-inflammatory activities (Mungole and Chaturvedi, 2011 and Sokeng *et al.*, 2013). Studies have demonstrated that terpenoids produced significant analgesic and anti-inflammatory activities. They are known to exert their anti-inflammatory effect by inhibiting phospholipase A2, a key enzyme of arachidonic acid metabolism, thereby stopping prostaglandin synthesis (Das *et al.*, 2013).

Recently, several experimental studies have revealed biological and pharmacological properties of phenolic compounds especially their anti-inflammatory activity. Phenolic are active in curing some problems as well as helpful as anti-inflammatory in action (Muthuraman *et al.*, 2011 and Mungole and Chaturvedi, 2011). Strong occurrence of tannins in extract has been shown to possess potent anti-inflammatory and anti-nociceptive properties. Fatty acids content of the plants could be responsible for the anti-inflammatory action

(Chouhan *et al.*, 2011). Alkaloids have also been shown to possess anti-inflammatory activity by inhibiting the action of arachidonic acid metabolism via the cyclooxygenase and 5-lipoxygenase pathways (Das *et al.*, 2013). On the other hand it has been reported that free radicals are involved in the inflammatory process. Therefore the antioxidant property of the plants may have a beneficial role in its anti-inflammatory activity (Sokeng *et al.*, 2013). Antioxidants are documented in several publications to mitigate the inflammatory processes and some of the antioxidant activity of plants have been ascribed to the phenolic compounds present in the plant particularly flavonoids (Mensah *et al.*, 2011).

1.6 Models used to study new anti-inflammatory agents

1.6.1 Acute anti-inflammatory models

1.6.1.1 Carrageenan indced oedema in rats

It is well known that to investigate the effects of drugs on the acute phase of inflammation, models induced by pro-inflammatory agents such as carrageenan, dextrane, formaldehyde, serotonin, histamine and bradykinin in the rats' paw are employed (Sivaraman *et al.*, 2010).

The most widely used primary test to screen new anti-inflammatory agents to measure the ability of a compound to reduce local oedema induced in the rats' paw is by injection of an irritant agent. Carrageenan is a mixture of polysaccharides composed of sulfated galactose units and is derived from Irish Sea moss, *Chondrous crispus*. Its use as an oedemogen was introduced by Winter *et al*. (1962). Carrageenan induced oedema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1 – 2 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and

prostaglandins produced by tissue macrophages (Ravi *et al.*, 2009 and Parekar *et al.*, 2012).

Shaikh, (2011) reported that the carrageenan-induced paw oedema model in rats is known to be sensitive to cyclooxygenase inhibitors and has been used to evaluate the effect of non-steroidal anti-inflammatory agents, which are known to inhibit the cyclooxygenase thereby, decrease prostaglandin synthesis. The time course of oedema development in carrageenan-induced paw oedema model in rats is generally represented by a biphasic curve. The first phase of inflammation occurs within an hour of carrageenan injection and is partly due to the trauma of injection and also to histamine and serotonin component. Prostaglandins (PGs) play a major role in the development of the second phase of inflammatory reaction which is measured at 3 h. The presence of PGE2 in the inflammatory exudates from the injected foot can be demonstrated at 3 h and period thereafter.

1.6.1.2 Acetic acid induced vascular permeability in rats

Acetic acid induced vascular permeability method is widely used to determine the inflammatory response of vascular tissue origin. In this model, injection of 0.6% acetic acid interaperitonealy caused dilatation of blood vessels and increased vascular permeability by releasing inflammatory mediators such as histamine, prostaglandins and leukotrienes which are released following stimulation of mast cells and also by increased leakage of fluids, including Evans blue dye (used as a peritoneal capillary permeability marker) across the blood vessel epithelial walls. The absorbance of the cavity fluids was measured using spectrophotometer (Shaikh, 2011 and Anosike *et al.*, 2012).

1.6.2 Chronic anti-inflammatory models

1.6.2.1 Cotton pellet granuloma model

Chronic inflammation models produced by implanting a foreign body under the skin are used to study the effects of a drug on the proliferation phase of inflammation (Gepdiremen *et al.*, 2004). The inflammatory granuloma is a

typical feature of an established chronic inflammatory process. The cotton pellet granuloma method has been widely employed to evaluate the transudative, exudative, and proliferative components of chronic inflammation, because the dried weight of the pellets correlates well with the amount of granulomatous tissue (Kaushik *et al.*, 2012).

Angiogenesis, nitric oxide synthesis and kinin release are found to be the main causes of granuloma formation. Angiogenesis in chronic inflammatory state facilitates migration of inflammatory cells into the inflammatory site and supplies nutrients and oxygen to tissue. Hence by suppressing angiogenesis in granulation tissue, it is possible to reduce or / and control the development of chronic granulation tissue (Shaikh, 2011).

1.7 Models to study new analgesic plants

1.7.1 Acetic acid induced writhes in rats

In this model the abdominal constrictions were induced according to the procedure described by Nwafor and Okwuasaba, (2003). This resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an intraperitoneal (i.p) injection of 0.6% aqueous acetic acid.

1.8 Plants under study

1.8.1 *Hibiscus sabdariffa* Linn.

Family: Malvaceae.

Vernacular names: (Arab.), karkadeh. (Engl.) roselle or red sorrel.

1.8.1.1 Description

Roselle is an annual erect, bushy, herbaceous sub shrub, with smooth or nearly smooth, cylindrical, typically red or red coloured stalk, and a red or pale yellow calyx that is edible (Ismail *et al.*, 2008).

1.8.1.2 Distribution

H. sabdariffa is widely grown in Central and West Africa, South East Asia, and elsewhere in parts of West India, Jamaica and Central America (Ali *et al.*, 2005).

1.8.1.3 Traditional uses

It has been traditionally used as antiseptic, astringent, cholagogue, aphrodisiac, demulcent, diuretic, emollient, purgative, digestive, stomachic, sedative and tonic. It has also reported to be used for high blood pressure, liver diseases, fever, ulcers, abscesses and anaemia (Mahadevan *et al.*, 2009).

1.8.1.4 Phytochemistry

There are many published reports on the constituents of different parts of *H. sabdariffa* (Ali *et al.*, 2005; Mungole and Chaturvedi, 2011). Using TLC and HPLC fingerprint analysis of an extract of *H. sabdariffa* flowers, showed the presence of quercetin, luteolin, a luteolin glycoside and chlorogenic acid, in addition to other compounds previously recorded, such as the anthocyanins delphinidin-3- sambubioside and cyanidin-3-sambubioside, the flavonoids gossypetin, hibiscetin and their respective glycosides, protocatechuic acid, eugenol and the sterols β -sitosterol and ergosterol. Quercetin had also been recorded in roselle colour. *Hibiscus* protocatechuic acid was also isolated from the dried flowers of *H. sabdariffa* (Salah *et al.*, 2002). Three water-soluble polysaccharides were extracted from the flower buds of *H. sabdariffa* (Muller, 1992).

Mahadevan *et al.*, (2009) reported that the leaf of *H. sabdariffa* contains protein, fat, carbohydrate, fibre, ash, calcium, phosphours, iron, thiamine, ß-carotene, riboflavin, niacin and ascorbic acid. Analysis of calyces revealed the presence of crude protein and minerals such as iron, phosphorus, calcium, manganese, aluminium, magnesium, sodium and potassium. Mucilage, calcium citrate, ascorbic acid, gossypetin and hibiscin chlorideare.

More than 37 compounds were characterized from the volatile constituents of roselle tea, which were classified into four groups: fatty acid derivatives, sugar derivatives, phenolic derivatives and terpenoids (Ali *et al.*, 2005). More than 25 volatile compounds were detected from the analysis of the seed oil of *H. sabdariffa*. The compounds were mainly unsaturated hydrocarbons, alcohols and

aldehydes, predominantly from C8 to C13. Steroids and tocopherols have been reported in the seeds oil (Mahadevan *et al.*, 2009). The sterols of the seed oil of *H. sabdariffa* were indicated the presence of cholesterol, campasterol, stigmasterol, β -sitosterol, α-spinasterol and ergosterol (Ali *et al.*, 2005). The seeds contain protein (18.8-22.3%), fat (19.1-22.8%) and dietary fibre (39.5-42.6%) content were found to be high. The seeds were found to be a good source of minerals like phosphorus, magnesium, calcium, lysine and tryptophan contents. Seed oil is rich in unsaturated fatty acids (70%), of which linoleic acid constituted 44%. Seeds contain nitrogen, fatty oil, cellulose, pentosans and starch (Mahadevan *et al.*, 2009).

Mungole and Chaturvedi. (2011) studied the phytochemical screening of *H. sabdariffa* for various medicinally important compounds and their quantification. They reported that *H. sabdariffa* have phenols, alkaloids, tannins, flavanoids, saponins and steroids in leaves, stems and roots of the plant. Triterpenoids were found to be present in all the parts of the plant with the exception of seeds.TLC analysis also confirmed these results.

1.8.1.5 Pharmacological properties

There are many published reports on the pharmacological effects of different plant part extracts of *H. sabdariffa*. The aqueous extract of calyces inhibited the tone of various isolated muscle preparations that included rabbit aortic strip and rat ileal strip. The extract also rhythmically contracted rat uterus, guinea-pig tracheal chain and rat diaphragm. The same extract stimulated quiescent rat uterus and frog *rectus abdominus* muscle. The tonic effects on rat uterus were partially reduced by hydrocortisone and indomethacin. The mechanism of action of *H. sabdariffa* aqueous extract on smooth muscles is not certain. But it was suggested that the relaxant response was related to endothelium-dependent and endothelium-independent mechanisms or mediated through calcium channels, possibly generated by constituents such as quercetin and eugenol. However, the presence of stimulatory substance(s) in the extract has also been demonstrated

using the frog *rectus abdominus* preparation (Ali *et al.*, 2005). Intravenous injection of aqueous extracts of *H. sabdariffa* calyx to anaesthetized cats and anaesthetized rats lowered blood pressure in a dose-dependent manner. This effect was resistant to a number of standard receptor blocking agents, but the hypotensive effect was partially blocked by atropine, and antihistamine (H1 blockers) (Adegunloye *et al.*, 1996; Ali *et al.*, 1991). It was also postulated that the hypotensive action of *H. sabdariffa* could be ascribed to a direct vaso-relaxant effect (Adegunloye *et al.*, 1996). More recently the hypotensive effects of ethanolic seed extract of *Hibiscus sabdariffa* l. were evaluated in normotensive cats (Bako *et al.*, 2009). The extract produced a significant (P<0.05) reduction in cat blood pressure compared to standard drug Acetylcholine which showed a greater potency than the extract.

Al-Hashimi, (2012) investigated the total content of phenolic compounds, antioxidant activity, reducing power and chelating of ferrous ion in aqueous and alcoholic extracts of *H. sabdariffa* L. The total phenolic content was 77.2 mg/g and 87.7 mg/g for the aqueous and alcoholic extracts, respectively. The antioxidant activity was equal in rates for both, the roselle alcoholic extract and artificial antioxidant BHT (75.67%), alcoholic extract showed the highest reducing ability with a rate of 222.60%, the chelating of ferrous ion in both aqueous and alcoholic extracts were 73.97 and 32.29% at 5 mg/ml concentration, respectively. The antibacterial activity of roselle extracts against *Escherichia coli, Staphylococcus aureus*, *Streptococcus mutans* an*d Pseudomonas aeruginosa*, showed varying degrees of inhibition on the tested organisms. The antioxidant activity of ethanolic seed extract of *H. sabdariffa* L. was investigated in toxicity induced by chronic administration of sodium nitrate in Wistar rats (Bako *et al.,* 2009). The toxicity induced by chronic administration of $NaNO_3$ seems to be alleviated by the antioxidant effect *H. sabdariffa* L. Tocopherol (Vitamin E) and ascorbic acid (Vitamin C) which may be responsible for the antioxidant effect of *H. sabdariffa* L. Since preliminary

phytochemical screening reveal the presences of substantial amount in seed oil. *H. sabdariffa* L. seed extract are characterized by a very low degree of toxicity with LD_{50} of above 5000 mg/kg in rats (Bako *et al.,* 2009).

Aqueous extract of *H. sabdariffa* was found to be effective in inhibiting yeast-induced pyrexia, and in reducing the reaction time in a hot plate, tail-flick assay, indicating that the extract has antipyretic and antinociceptive actions. The extract, however, was without significant effect in the rat paw carrageenan-induced oedema test. It was suggested that the above antipyretic and antinociceptive actions could be attributed to flavonoids, polysaccharides and organic acids (Dafallah and Al-Mustafa, 1996). However, in one clinical trial involving 50 patients, administration of a decoction of dried fruit (3 g/person, three times every day for 7 days to 1 year) was shown to produce antiinflammatory activity (Ali *et al.*, 2005).

The antinociceptive, anti-inflammatory and antidiarrheal activities of the ethanolic calyx extract of *H. sabdariffa* Linn. in mice were investigated by Ali *et al.* (2011). The dried calyxes of *H. sabdariffa* were subjected to extraction with 95% ethanol and the extract was used to investigate the possible activities. Antinociceptive activity of the extract was evaluated by using the acetic acid-induced writhing test. The anti-inflammatory effect of the extract was tested by using the xylene-induced ear oedema model mice. Castor oil-induced diarrhoeal model mice were used to evaluate the antidiarrheal activity of the extract. In acetic acid-induced writhing test, the extract produced inhibited writhing in mice significantly compared with the control (*P*<0.01). The extract showed significant inhibition of ear oedema formation in xylene-induced ear oedema model mice in a dose-related manner compared with the control (*P*<0.01). The extract demonstrated a significant antidiarrhoeal activity against castor oil-induced diarrhoeal in mice in which it decreased the frequency of defecation and increased the mean latent period at the doses of 250 and 500 mg/kg body weight (*P*<0.01). These findings indicate that the calyx extract of *H. sabdariffa*

possesses significant antinociceptive, anti-inflammatory and antidiarrheal activities that support its uses in traditional medicine.

The changes in urine composition that follow the consumption of *H. sabdariffa* extract at different concentrations and for various periods of time were investigated. The results indicated that consumption of *H. sabdariffa* extract resulted in significant decreases in the urinary concentrations of creatinine, uric acid, citrate, tartrate, calcium, sodium, potassium and phosphate, but not oxalate. It was noted that the low dose of *H. sabdariffa* (16 g/day) caused a more significant decrease in salt output in the urine than a high dose (24 g/day). A significant uricosuric action was noted in rats given a decoction of the dried calyx at an oral dose of 1 g/kg (Ali *et al.*, 2005).

The immunological effects of cold and hot aqueous extracts of *H. sabdariffa* calyces in albino mice were evaluated by Kadri *et al.* (2013). Three oral doses (9.78, 19.56 and 29.34 mg/mouse for cold extract, and 13.72, 27.44 and 41.16 mg/mouse for hot extract) of each extract were evaluated, in addition to the immune suppressive drug etoposide (0.05 mg/mouse/day) and interferon (0.25 mg/mouse/day). Metaphase index, T-rosette formation (bone marrow, thymus, spleen and lymph nodes) and ADA specific activity (serum and thymus) in the investigated organs were significantly decreased in animals treated with etopside as compared with normal controls, while in mice treated with calyx extracts of *H. sabdariffa*, the investigated parameters were significantly increased especially at the third dose of both extracts. These findings suggest the immune enhancement effects of the investigated extract.

The effect of aqueous ethanol (1:1) extract of the calyx of *H. sabdariffa* on carbon tetrachloride (CCl_4) induced liver damage was investigated. Oral administration of the extract following a single CCl_4 dose promoted the healing of oxidative liver damage as determined by serum amino transferases, ALT, AST, levels and liver thiobarbituric acid reactive substances levels. The study

indicated that the extract of *H. sabdariffa* enhances the healing from hepatic damage induced by CCl_4 (Dahiru *et al.,* 2003).

Ali *et al.* (2005) reported that *H. sabdariffa* calyx (5% or 10%) was fed to rats with hypercholesterolaemia for 9 weeks. The treatment progressively lowered the different lipid fractions in plasma, heart, brain, kidney and liver, and also decreased the activities of several plasma enzymes used in tests as markers of tissue function. This treatment, however, slightly raised the content of plasma phospholipids. Although the mechanism of action of *H. sabdariffa* as a cholesterol-lowering agent was not elucidated in this work, it was hypothesized, albeit with no experimental evidence, that the extract may contain some compounds that activate hormonal secretions, such as adrenocortical hormones, which stimulate the metabolic pathway of cholesterol by conversion into other compounds. The anticholesterol action of *H. sabdariffa* (0.5% or 1%) was confirmed in rabbits fed cholesterol for 10 weeks. This treatment was effective in reducing the serum concentrations of triglycerides, total cholesterol and low-density lipoprotein cholesterol, and in mitigating atherosclerosis in the aorta. Histopathologically, it was found that feeding *H. sabdariffa* had reduced foam cell formation and inhibited smooth muscle cell migration and calcification in the blood vessel of treated rabbits (Ali *et al.*, 2005).

1.8.1.6 Toxicological properties

The LD_{50} of *H. sabdariffa* calyx extract in rats was found to be above 5000 mg/kg (Onyenekwe *et al.*, 1999), suggesting that the extract is virtually non-toxic. In spontaneously hypertensive rats, treatment with the extract at doses of 500–1000 mg/kg decreased blood pressure, and also significantly decreased serum creatinine, cholesterol and glucose levels, but significantly increased the serum content of uric acid. The treatment caused no significant effect on either water intake or urine output.

The effect of sub-chronic administration of aqueous extracts of *H. sabdariffa* calyx on the testes have been studied by Orisakwe *et al.* (2004), as the plant is

often claimed in West African folk medicine to be an aphrodisiac. Rats were given 1.15, 2.3 and 4.6 g/kg/day of an aqueous extract of *H. sabdariffa* calyx in the drinking water for up to 12 weeks. At the end of the treatment period there was a steady decrease in body weight, but no changes in the relative or absolute weights of the testes. However, the higher two doses of the extract caused a significant decrease in the epididymal sperm counts, histological distortion of tubules, disruption of normal testicular epithelial organization and disintegration of sperm cells. These effects were related to interference by the extract with spermatogenesis that may have been caused by an oestrogenic action of the extract. Ali *et al.* (1989) have previously alluded to this possibility. It is, however, difficult to ascribe the above testicular effects to an oestrogenic action in the absence of any significant change in testicular weight, as oestrogens are known to reduce the weights of the male reproductive organs. The relevance of the testicular toxicity of *H. sabdariffa* in humans is not certain when the relatively high amount of extract given in the drinking water for 12 weeks is taken into consideration. Akindahunsi and Olaleye, (2003) suggested that, in rats, the average consumption of 150–180 mg/kg/day of an aqueous-ethanol extract of *H. sabdariffa* calyces appeared to be safe, although higher doses might elevate the activity of some plasma enzymes indicative of tissue function (such as alanine aminotransferase and aspartate aminotransferase). However, the activity of some related plasma enzymes (alkaline phosphatase and lactate dehydrogenase) was not significantly affected, nor was there any evidence of histological damage to the heart and liver of the treated rats. Mutagenicity of roselle colour was reported by Takeda and Yasui, (1985) and the compound responsible for this activity was suggested to be quercetin.

Fig.1. *Hibiscus sabdariffa*

Fig.2. *Hibiscus sabdariffa* **seeds**

1.8.2 *Balanites aegyptiaca* (L.) Del.

Synonyms: *Ximenia aegyptiaca* L., Sp. PI. : 1194 (1753).

Vernacular names: (Ar.) Hegleeg, Lal'loub (fruits); (Engl.) Desert date, Soapberry.

Family: Balanitaceae.

Genus: Balanites Delile

Species: *Balanites aegyptiaca* (L.) Delile.

1.8.2.1 Description

It is multi-branched, spiny shrub or tree up to l0 meters tall. Crown spherical, in one or several distinct masses. Trunk short and often branching from near the base. Bark dark brown to grey, deeply fissured. Branches armed with stout yellow or green thorns up to 8 cm long. Leaves with two separate leaflets; leaflets obovate, asymmetric, 2.5 to 6 cm long, bright green, leathery, with fine hairs when young. Flowers in fascicles in the leaf axils, and are fragrant, yellowish-green (Chothani and Vaghasiya, 2011).

1.8.2.2 Distribution

B. aegyptiaea is found in most and to sub humid tropical savannas of African, all over the Sahel and on many sites of the Sudan savanna, extending from the Atlantic coastline of Senegal to the red sea and Indian Ocean and the Arabian Peninsula (Maydell, 1986).

1.8.2.3 Uses

B. aegyptiaca is an extremely useful tree which has been utilized over thousands of years. Balanites wood is pale yellow or yellowish brown, hard and tough, heavy, insect resistant, fire-grained, it saws and planes well and is widely used for tool handles, bowls, posts, mortars and pestles and many household and agricultural implements as well as for handicrafts. Many parts of the plant are used as famine foods in Africa, the leaves are eaten raw or cooked, the oily seed is boiled to make it less bitter and eaten mixed with sorghum and the flowers can be eaten (Wufem *et al.*, 2007). Young leaves, sprouts, green thorns, and

especially the fruits are eaten by all livestock and wildlife. The fruit is eaten fresh or dried as unripe; it has a slightly astringent taste and is a purgative. The fruit can be processed to produce multipurpose intermediate products. The mesocarp of the fruit is a source of fermentation products (e.g. ethanol) and steroidal sapogenins or can be incorporated into animal feed. The cellulosic shell of the fruit is a source of fuel. The kernel is a supplement to food product (e.g. edible, Peanut, butter - like products). The crude Balanites oil is a source of edible vegetable oil or otherwise used in various industries (e.g. Soap -making). The kernel cake, after extraction of oil, is a source of protein and carbohydrate for livestock (Maydell, 1986).

1.8.2.4 Traditional uses

Many medicinal uses are known, these including the bark and roots as laxatives or tranquilizers (for colic). The bark is used against stomachaches, sterility, mental diseases, epilepsy, yellow fever, syphilis and as a vermifuge. Fruit and leaves, and especially the kernel-oil are applied for rheumatism, bark extracts for toothaches (Maydell, 1986).

Fruit is used to treat skin diseases and in whooping cough. Aqueous extract of fruits showed spermicidal activity. Prolonged administration of the fruit pulp extract produced hyperglycemia-induced testicular dysfunction in dogs. Seed is used as febrifuge, expectorant, antibacterial and antifungal activities. The seed oil is used as laxative and to treat tumors, wounds, hemorrhoid, stomachaches, jaundice, yellow fever, syphilis and epilepsy. The seed powder is taken with glass of water in the morning for 10 days for asthma. Bark is used as spasmolytic and root infusion is used as an emetic. Seeds are used as anthelmintic and purgative. Ground seeds are given to camels to cure impaction and colic (Chothani and Vaghasiya, 2011).

In Sudanese folk medicine the aqueous extract of the fruit mesocarp is used in the treatment of jaundice (Sarker *et al.*, 2000). The fruits are also used in Egyptian folk medicine as an oral hypoglycemic (Kamel, 1998). Tablets are

prepared from roots mixed with 'Hing' powder (*Ferula asafoetida*); by adding *Piper betle* leaf, juices are taken once with water for 9 days, soon after the menstruation to avoid unwanted pregnancy (Vijigiri and Sharma, 2010). Root is used in various folk medicines for the treatment of abdominal pain and as purgative, while the bark is employed as a fish poison and also as a remedy for malaria and syphilis. It is also used for the treatment of render pest and anthrax (Khan, 2009). In Senegal, Nigeria, Morocco, and Ethiopia, *B. aegyptiaca* is taken as a purgative for colic and stomach ache. In Chad, fresh twigs are put on the fire in order to keep insects away. For intestinal worm, the fruits are dried and mashed in millet porridge and eaten. In Libya and Eritrea, the leaves are used for cleaning infected wounds.

1.8.2.5 Phytochemical constituents

Leaves contains saponin, furanocoumarin, and flavonoid (Chothani and Vaghasiya, 2011). Balanitoside (furostanol glycoside) and 6-methyldiosgenin, balanitin-3 (spirostanol glycoside) have been reported from fruits (mesocarp) of *B. aegyptiaca* (Hosny *et al.*, 1992 and Kamel, 1998). The kernels contained 45.0 to 46.1% oil and protein (32.4%), oil contains mainly palmitic, stearic, oleic, and linoleic acids which were the main fatty acids (Chothani and Vaghasiya, 2011)**.** Mesocarp of fruit contains 1.2 to 1.5% protein and 35 to 37% sugars, 15% organic acids, other constituents like 3-rutinoside and 3-rhamnogalactoside and diosgenin. It also contains a mixture of 22R and 22S epimers of 26-(O-β-D-glucopyranosyl)-3-β-[4-O-(β-D-glucopyranosyl)-2-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyloxy]-22,26-dihydroxyfurost-5-ene (Chothani and Vaghasiya, 2011). Nine saponin have been reported from kernel cake of *B. aegyptiaca*. The leaves and fruit kernels of *B. aegyptiaca* L. were found to contain six diosgenin glucosides (Chothani and Vaghasiya, 2011)**.** Root is reported to contain steroidal glycosides (Farid *et al.*, 2002). Balanitins 1 to 7 have been reported from root and bark of *B. aegyptiaca*. Bark is reported to contain furanocoumarin bergapten and dihydrofuranocumarin D- marmesin, two alkaloid namely, N-trans-

feruloyltyramine and N-cis-feruloyltyramine, and three common metabolites, vanillic acid, syringic acid; and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, long-chain aliphatic compound, 10-methyl-n-heptacosane, and a new sugar, diglucosyldirhamnoside, have also been reported from the stem-barks. It also contains beta-sitosterol, bergapten, marmesin, and beta-sitosterol glucoside, balanitin-1,-2, and -3; balanitin-1 for example possesses a yamogenin aglycone with a branched glucose and rhamnose side chain (Chothani and Vaghasiya, 2011).

1.8.2.6 Pharmacological activity

The methanolic extract of *B. aegyptiaca* fruits is reported to have anthelmintic action against different stages of *Trichinella spiralis* in rats compared with anthelmintic drug albendazole (Shalaby *et al.*, 2010). The crude aqueous extract of root bark of *B. aegyptiaca* showed a dose-dependent inhibition of spontaneous motility (paralysis) in adult earthworms, and also possesses vermicidal activity (Dwivedi *et al.*, 2009). Koko *et al.* (2000) reported that stem bark water extract (9 g/kg body weight) of *Albizia anthelmintica* and fruit mesocarp water extract (9 g/kg body weight) of *B. aegyptiaca* showed significant anthelmintic activity compared with albendazole (20 mg/kg body weight) against *Fasciola gigantica* adult worm. A single dose of 200 mg/kg body weight of *B. aegyptiaca* fruit mesocarp also showed activity against *Schistosoma mansoni* in infected mice when compared with praziquantel (Koko *et al.*, 2005).

Meda *et al.* (2010) reported that the galls and leaf extracts and fractions of *B. aegyptiaca* showed a significant antioxidant, xanthine oxidase, and acetylcholinesterase inhibitory activities.

The aqueous and organic leaves extracts of *B. aegyptiaca* and *Moringa oleifera* were reported to have antibacterial effect against *Salmonella typhi* isolated from blood clot culture using the disc diffusion method. The extracts of *B. aegyptiaca* plants demonstrated the highest activity than *Moringa*

oleifera. The antibacterial activity appears to increase when extracts of the two plants were used in combination (Doughari *et al.*, 2007).

A mixture of steroidal saponins: balanitin-6 (28%) and balanitin-7 (72%), isolated from *B. aegyptiaca* kernels, demonstrated appreciable anticancer effects in human cancer cell lines *in vitro* by using against A549 non–small-cell lung cancer (IC50, 0.3 µM) and U373 glioblastoma (IC50, 0.5 µM) cell lines (Chothani and Vaghasiya, 2011).

It is reported that *B. aegyptiaca* has potent wound-healing activity, as evident from the wound contraction. The results also indicated that the plant possesses potent antioxidant activity by inhibiting lipid peroxidation, bleaching DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, and protecting against oxidant injury to fibroblast cells (Annan and Dickson, 2008)

The ethanol and petroleum ether extracts of aerial parts of *B. aegyptiaca* have been reported to have significant anti-inflammatory action on carrageenan-induced hind paw oedema in rats, and analgesic activity by using Eddy's hot plate method and tail-flick method in albino rats. The ethanol and petroleum ether extracts showed a greater anti-inflammatory and analgesic effects compared with the standard drugs, indomethacin and diclofenac sodium, respectively. It also indicated that the ethanolic extract of *B. aegyptiaca* exhibited more significant activity than petroleum ether in the treatment of pain and inflammation (Gaur *et al.*, 2008).

Methanolic and butanol (BE) extracts and of two new saponins isolated from *B. aegyptiaca* showed significant anti-inflammatory, antinociceptive activity in the carrageenin-induced oedema in the rat, and acetic acid-induced writhing test in mice and antioxidant action by using *in vitro*, using a method based on the Briggs–Rauscher oscillating reaction. The samples, extracts and pure substances, were intragastrically administered to animals (Speroni *et al.*, 2005).

The whole and extracted pulps of *B. aegyptiaca* fruits have a hypocholesterolemic effect when tested on adult albino rats (Abdel-Rahim *et al.*,

1986). The aqueous extract of the mesocarp of fruits of *B. aegyptiaca* was reported to have antidiabetic effect in streptozotocin-induced diabetic mice (Mansour and Newairy, 2000).

Hepatoprotective effect of alcohol extract of the bark of *B. aegyptiaca* (Linn) was tested against CCl_4 induced liver damage in rats. The extract was effective in preventing damage which was evident by parameters such as SGPT, SGOT, SALP and total bilirubin (Jaiprakash *et al.*, 2003).

The oil exhibited anticancer activity against lung, liver, and brain human carcinoma cell lines. It also had antimutagenic activity against *Fasciola gigantica*-induced mutagenicity besides anthelmintic activity against hepatic worms (*Schistosoma mansoni* and *Fasciola gigantica*). Preliminary screening showed that the oil had antiviral activity against Herpes simplex virus. It also had antimicrobial activity against selected strains of Gram-positive bacteria, Gram-negative bacteria, and *Candida* (Al Ashaal *et al.*, 2010).

1.8.2.7 Toxicological studies

Seed oil of *B. aegyptiaca* has been used in Nigeria as ingredient and substituted to groundnut oil in the preparation of local foods. A four week repeated dose toxicity of crude *B. aegyptiaca* seed oil was performed in Wistar strain rats. The rats were divided in four groups of five animals each and feed diet containing 0, 0.5, 1 and 5% crude *B. aegyptiaca* seed oil. The results showed no significant changes in AST and ALT, except in 5% group where ALT activity was elevated. Moreover, there were no significant changes in total proteins, albumin, A/G ratio, serum urea, creatinine, mean final body weight, food consumption and relative liver and kidney weight were observed. The results showed that dietary exposure of crude *B. aegyptiaca* seed oil in rats did not result in marked changes in the toxicological parameters been assayed, thus, consumption of the crude oil at the present level of exposure may be of no serious safety concern, especially in liver and kidney injury (Wilson *et al.*, 2009).

Fig. 3. *Balanites aegyptiaca* **fruits**

Fig. 4. *Balanites* *aegyptiaca* kernel

CHAPTER TWO

2. Materials and Methods

2.1 Materials

2.1.1 Collection and identification of the plants

2.1.1.1 *Hibiscus sabdariffa* seeds

Hibiscus sabdariffa seeds were purchased from local market, Omdurman, Sudan, in April 2013. The seeds were identified and authenticated by the botanists in Medicinal and Aromatic Plants Research Institute (MAPRI), National Centre for Research (NCR), Khartoum, Sudan.

2.1.1.2 *Balanites aegyptiaca* seeds

Balanites aegyptiaca fruits (lal'loub) were purchased from local market of Omdurman, Sudan in April 2013. The fruits were then identified and authenticated by the botanists in Medicinal and Aromatic Plants Research Institute (MAPRI), National Centre for Research (NCR), Khartoum, Sudan.

2.1.2 Animals:

Adult Wistar albino rats of both sexes were purchased from the Medicinal and Aromatic Plants Research Institute (MAPRI), National Centre for Research (NCR), Khartoum, Sudan, and kept in plastic (propropylene) cages in the Laboratory Animal House in the College of Veterinary Medicine, Sudan University of Science and Technology. The animals were maintained under standard environmental conditions and provided with standard diet containing flour, meat, sodium chloride, edible oil and vitamins and minerals and water provided *ad libitum*. The rats were deprived of food overnight before the commencement of the experiment but allowed free access to water. This study was approved by the Scientific Research Committee of the College of Veterinary Medicine, Sudan University of Science and Technology in accordance with good clinical practice and international guidelines for animal use in experimentations.

2.1.3 Equipment and materials

All materials used during this study are shown in appendix 1.

2.2 Methods

2.2.1 Preparation of the extracts

2.2.1.1 *Hibiscuss sabdariffa*

2.2.1.1.1 Petroleum ether extract

One thousand gram (1000 g) of the seeds of *H. sabdariffa* was powdered by a blender. The coarsely powdered seeds were successively extracted with petroleum ether (40 – 60) °C for about four hours using soxhlet extractor. The solvent was then collected and evaporated under reduced pressure using rotary evaporator apparatus. The residue percentage was calculated relative to the dry matter, the oil extract obtained from petroleum ether was stored at room temperature in dark bottles till used (Harborne, 1984).

2.2.1.1.2 Ethanolic extract

The same sample (1000g of the powdered seeds of *H. sabdariffa*) was then unpacked and left to dry and repacked in soxhlet and ethanol 98% was used as a solvent for about eight hours. The solvent was then collected and evaporated under reduced pressure using rotary evaporator. The yield percentage was calculated; the ethanolic extract was stored at room temperature sheltered from light and humidity (Harborne, 1984).

2.2.1.2 *Balanites aegyptiaca*

2.2.1.2.1 Petroleum ether extract

The fruits of *B. aegyptiaca* were broken manually and the seeds were then collected from opened fruits and ground using a blender. 200 g of *B. aegyptiaca* powdered seeds were successively extracted with petroleum ether (40 – 60) °C for about four hours using soxhlet extractor. The solvent was then collected and evaporated under reduced pressure using rotary evaporator. The residue

percentage was calculated; the oil extract obtained from petroleum ether was stored in dark bottles till used (Harborne, 1984).

2.2.1.2.2 Ethanolic extract

The same sample (200 g of the powdered seeds of *B. aegyptiaca*) was dried and then extracted with ethanol 98% using soxhlet extractor apparatus for about eight hours. The solvent was then collected and evaporated under reduced pressure using rotary evaporator apparatus. The yield percentage was calculated; the ethanolic extract was stored at room temperature sheltered from light and humidity (Harborne, 1984)

2.2.2 Phytochemical screening

2.2.2.1 Fatty acids composition of seed oil of *H. sabdariffa* and *B. eagyptiaca*.

Methyl ester of the seeds oil of *H. sabdariffa* and *B. aegyptiaca* was prepared prior to injection into Gas Chromatography (GC) as follows: 1 ml of seed oil was weighed into screw capped tube and about 6 ml of NaOH (0.5M) dissolved in methanol was added together with 6ml of sulphuric acid 1% in methanol. After a vigorous homogenization the tube was kept overnight to allow the reaction to proceed. Then 2ml of N. hexane was added and mixed well. After that saturated NaCl was added and mixed. The supernatant was recovered filtered with anhydrous Na_2SO_4 and injected in GC (Christie, 1989). Fatty acids composition of the seeds oil of *H. sabdariffa* and *B. aegyptiaca* was performed using Gas Chromatography (GC- 2010, SHIMADZU-Japan) equipped with flame ionization detector (FID) and capillary column (BD-1, Japan/ 30m×0.25mm×0.25mm). The detector temperature was programmed at 300 ºC with flow rate of 1 ml/min. The injector temperature was adjusted at 250 ºC. Nitrogen was used as the carrier gas. The identification of the peaks characteristic and fatty acids composition of *H. sabdariffa* and *B. aegyptiaca* seeds oil was performed by the comparison of the retention times obtained from seeds oil with the retention times of standard fatty acids analyzed under the same condition.

2.2.3 Anti-inflammatory activity evaluation

2.2.3.1Carrageenan induced paw oedema (for acute inflammation)

Anti-inflammatory activity was determined in albino rats of either sex according to the method of (Ramprasath *et al.*, 2004). Four experiments were carried out in this study.

2.2.3.1.1 Anti-inflammatory activity of *H.sabdariffa* seeds petroleum ether extract on carrageenan induced rats׳ hind paw oedema

Twenty five rats (86 – 128 g) divided into 5 groups of 5 rats each were allotted randomly to the different treatments.

Group 1: Control: Animals were kept untreated.

Group 2: Standard anti-inflammatory drug: rats were given indomethacin at a dose of 10 mg/kg orally.

Group 3: Rats were treated with *H. sabdariffa* seeds petroleum ether extract at a dose of 2 ml/kg orally**.**

Group 4: Rats received *H. sabdariffa* seeds petroleum ether extract at a dose of 4 ml/kg orally**.**

Group 5: Rats were given *H. sabdariffa* seeds petroleum ether extract at a dose of 8 ml/kg orally.

2.2.3.1.2 Anti-inflammatory activity of *H.sabdariffa* seeds ethanolic extract on carrageenan induced rats׳ hind paw oedema.

Twenty five rats (80 – 120 g) divided into 5 groups of 5 rats each were selected randomly to the different treatments.

Group 1: Control: Rats were given distilled water (1 ml/100g) orally.

Group 2: Standard anti-inflammatory drug: rats were given indomethacin at a dose of 10 mg/kg orally.

Group 3: Rats were treated with ethanolic extract of *H. sabdariffa* seeds at a dose of 100 mg/kg orally**.**

Group 4: Rats were given ethanolic extract of *H. sabdariffa* seeds at a dose of 200 mg/kg orally**.**

Group 5: Rats received ethanolic extract of *H. sabdariffa* seeds at a dose of 400 mg/kg of orally.

2.2.3.1.3 Anti-inflammatory activity of *B. aegyptiaca* seeds petroleum ether extract on carrageenan induced rats' hind paw oedema.

Twenty five rats (80 – 122 g) divided randomly into 5 groups of 5 rats in each group were divided randomly to the different treatments.

Group 1: Control: Animals were kept untreated.

Group 2: Standard anti-inflammatory drug: Rats were administered indomethacin at a dose of 10 mg/kg orally.

Group 3: Rats were treated with petroleum ether extract of *B. aegyptiaca* seeds at a dose of 2 ml/kg orally.

Group 4: Rats were administered petroleum ether extract of *B. aegyptiaca* seeds at a dose of 4 ml/kg orally.

Group 5: Rats received petroleum ether extract of *B. aegyptiaca* seeds at a dose of 8 ml/kg orally.

2.2.3.1.4 Anti-inflammatory activity of *B. aegyptiaca* seeds ethanolic extract on carrageenan induced rats'hind paw oedema.

Twenty rats (86 – 142 g) divided randomly into 4 groups of 5 rats in each group were allotted to the different treatments.

Group 1: Control: Rats were kept untreated.

Group 2: Standard anti-inflammatory drug: Animals were treated with diclofenac sodium at a dose of 10 mg/kg orally.

Group 3: Rats were administered ethanolic extract of *B. aegyptiaca* seeds at a dose of 200 mg/kg orally.

Group 4: Rats were received ethanolic extract of *B. aegyptiaca* seeds at a dose of 400 mg/kg orally.

2.2.3.1.5 Procedure for the evaluation of the anti-inflammatory activity of the above extracts by carrageenan induced paw oedema

One hour after the administration of the treatments, oedema was induced by injection of carrageenan (0.1 ml, 1% w/v in saline) into the sub planter tissue of the right hind paw of all rats. The paw diameter, up to the tibiotarsal articulation, was measured using a digital vernier calliper. The measurements were determined at 0 h (before carrageenan injection) and 1, 2, 3 and 4 hours after carrageenan injection and the % paw volume inhibition was measured using the following formula:

$$\% \text{ inhibition} = (Pv_f - Pv_O)_{\text{control}} - (Pv_f - Pv_O)_{\text{treated}} / (Pv_f - Pv_O)_{\text{control}} \times 100$$

Where Pv_O = Paw volume before administration of carrageenan (i.e. initial paw volume) and Pv_f= Is the paw volume after administration of carrageenan.

2.2.3.2 Acetic acid induced vascular permeability (for acute inflammation)

Twenty Wistar albino rats weighing between 108-180 g were divided randomly into 4 groups of 5 rats per group and treated as follows:

2.2.3.2.1 Effect of *H. Sabdariffa* seeds petroleum ether extract on acetic acid induced vascular permeability in rats.

Group 1: Control: Animals received Distilled water (1ml/100 g) orally.

Group 2: Standard anti-inflammatory drug: Animals were administered diclofenac sodium at a dose of 10 mg/kg orally.

Group 3: Rats were given petroleum ether extract of *H. sabdariffa* seeds at a dose of 4ml/kg orally.

Group 4: Rats were treated with petroleum ether extract of *H. sabdariffa* seeds at a dose of 8ml/kg.

2.2.3.2.2 Procedure of evaluation of acetic acid induced vascular permeability in rats.

After one hour of administration of distilled water, petroleum ether extract and diclofenac sodium, rats were injected with 0.25 ml of 0.6 % (v/v) solution of acetic acid intraperitoneally (IP). Immediately, 10 mg/kg of 10 % (w/v) Evans

blue was injected intravenously via tail vein. After 30 min of Evan's blue injection, the animals were anaesthetized and sacrificed and their peritoneum washed with 10 ml of normal saline, then the wash solution was collected in tubes and centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant was measured at 620 nm using spectrophotometer. The vascular permeability effects were expressed as the absorbance (A), which represented the total amount of dye leaked into the intraperitoneal cavity (Shaikh, 2011 and Anosike *et al.*, 2012)

2.2.3.3 Cotton pellet granuloma (for chronic inflammation)

In this model, twenty rats of both sexes were divided randomly into 4 groups of 5 rats each per experiment. The animals were anaesthetized with thiopental sodium (40 mg/kg, i.p). The subcutaneous implantations of sterile cotton pellets (20 mg) were done in shaved lumbar region using small incision to induce chronic inflammation. This was done according to the procedure described by (Lalitha and Sethuraman, 2010 and Chouhan *et al.*, 2011).

2.2.3.3.1 Effect of *H. Sabdariffa* seeds petroleum ether extract on cotton pellet granuloma in rats

Twenty rats weighing 85-155g were divided randomly into 4 groups of 5 rats each as follows:

Group 1: Served as control animals.

Group 2: Served as standard anti-inflammatory drug: Rats were administered diclofenac sodium at a dose of 10 mg/kg orally.

Group 3: Animals were given petroleum ether extract of *H. sabdariffa* seeds at a dose of 4ml/kg orally.

Group 4: Rats were administered petroleum ether extract of *H. sabdariffa* seeds at a dose of 8ml/kg orally.

2.2.3.3.2 Effect of *B. aegyptiaca* seeds petroleum ether extract on cotton pellet granuloma in rats

Twenty rats weighing from 80 - 166 g were divided randomly into 4 groups of 5 rats each as follows:

Group 1: Served as control animals.

Group 2: Served as standard anti-inflammatory drug: Rats were administered diclofenac sodium at a dose of 10 mg/kg orally.

Group 3: Animals were given petroleum ether extract of *B. aegyptiaca* seeds at a dose of 4ml/kg.

Group 4: Rats were administered petroleum ether extract of *B. aegyptiaca* seeds at a dose of 8ml/kg orally.

2.2.3.3.3 Procedure of evaluation of above extracts by cotton pellet induced granuloma in rats

All treatments were administered orally for 6 consecutive days from the day of cotton pellet implantation. On the 7th day, animals were anaesthized by an over dose of chloroform anaesthesia. The cotton pellets were removed surgically, dried at 60 °C for 24 hours until a constant weight was obtained and weighed. The increment in dry weight of pellets over 20 mg were taken as an index of granuloma formation (Lalitha and Sethuraman, 2010 and Chouhan *et al.*, 2011).

2.2.4 Analgesic study

2.2.4.1 Acetic acid induced writhing test

The peripheral analgesic activities of the petroleum ether extracts of *H. sabdariffa* and *B. aegyptiaca* seeds were measured by the acetic acid induced writhing test as described by Nwafor and Okwuasaba, (2003).

2.2.4.1.1 Effect of *H. sabdariffa* seeds petroleum ether extract on acetic acid induced writhes in rats

Twenty five rats (102 - 146 g) were divided randomly into 5 groups of 5 rats each.

Group 1: Served as control: Rats were given distilled water (1ml/100 g) orally.

Group 2: Served as standard analgesic drug: Animals were administered diclofenac sodium at a dose of 10 mg/kg orally.

Group 3: Rats were given petroleum ether extract of *H. sabdariffa* seeds at a dose of 2 ml/kg orally.

Group 4: Rats were administered petroleum ether extract of *H. sabdariffa* seeds at a dose of 4 ml/kg orally.

Group 5: Rats were received petroleum ether extract of *H. sabdariffa* seeds at a dose of 8 ml/kg orally.

2.2.4.1.2 Effect of *B. aegyptiaca* seeds petroleum ether extract on acetic acid induced writhes in rats

Twenty five rats (90 – 156 g) were divided into 5 groups of 5 rats each.

Group 1: Served as control: Rats were given distilled water (1ml/100 g) orally.

Group 2: Served as standard analgesic drug: Animals were administered diclofenac sodium at a dose of 10 mg/kg orally.

Group 3: Rats were administered petroleum ether extract of *B. aegyptiaca* seeds at a dose of 2 ml/kg orally.

Group 4: Animals were received petroleum ether extract of *B. aegyptiaca* seeds at a dose of 4 ml/kg orally.

Group 5: Rats were received petroleum ether extract of *B. aegyptiaca* seeds at a dose of 8 ml/kg orally.

2.2.4.1.3 Procedure of evaluation of *H. sabdariffa* and *B. aegyptiaca* seeds petroleum ether extracts on acetic acid induced writhes in rats

After 30 minutes of the treatments, the animals were injected intraperitoneally by acetic acid (0.7%) at a dose of 0.1 ml/10g of body weight to create pain sensation. The number of writhing was calculated immediately for 20 min after the application of acetic acid. The inhibition of writhing produced by the plant extract was determined by comparing with the inhibition produced by the control group.

2.2.5 Toxicological studies

2.2.5.1 Acute toxicity study (LD_{50})

The acute toxicity tests were performed according to Organization for Economic Co-operation and Development (OECD/OCDE) guidelines (OECD, 2001). Wistar albino rats of either sex were selected randomly to be employed in this study.

2.2.5.1.1 Acute toxicity of *H.sabdariffa* seeds petroleum ether extract

Twenty rats (100 – 136 g) were divided into 4 groups 5 rats in each group. The animals were fasted for 12 h with free access to water only.

Group 1: Rats were given *H. sabdariffa* seeds petroleum ether extract at a dose of 2 ml/kg orally.

Group 2: Animals were treated orally with *H. sabdariffa* seeds petroleum ether extract at a dose of 4 ml/kg.

Group 3: Rats were administered orally *H. sabdariffa* seeds petroleum ether extract at a dose of 8 ml/kg.

Group 4: Rats were given *H. sabdariffa* seeds petroleum ether extract at a dose of 20 ml/kg.

2.2.5.1.2 Acute toxicity of *B. aegyptiaca* seeds petroleum ether extract

Twenty rats (100 – 120 g) were divided into 4 groups of 5 animals each. The animals were fasted for 12 h with free access to water only.

Group 1: Rats were given *B. aegyptiaca* seeds petroleum ether extract at a dose of 2 ml/kg orally.

Group 2: Animals were treated orally with *B. aegyptiaca* seeds petroleum ether extract at a dose of 4 ml/kg.

Group 3: Rats were administered orally *B. aegyptiaca* seeds petroleum ether extract at a dose of 8 ml/kg.

Group 4: Rats were given *B. aegyptiaca* seeds petroleum ether extract at a dose of 20 ml/kg.

2.2.5.1.3 Clinical signs and mortality

Clinical signs and mortality were observed for 24 hours.

2.2.5.2 Sub-chronic toxicity study

2.2.5.2.1 Toxicity of *B. aegyptiaca* seeds oil in rats

Eighteen adult Wister albino rats of both sexes (97 – 150 g) were used; the animals were randomly divided into 3 groups of 6 rats each.

Group 1: Control; animals received distilled water only for 21 days.

Group 2: Rats were administered 4ml /Kg /day, of the petroleum ether extract of *B. aegyptiaca* seeds orally for 21 days.

Group 3: Rats were given 8ml /Kg /day, of the petroleum ether extract of *B. aegyptiaca* seeds orally for 21 days.

2.2.5.2.1.1 Blood samples:

Blood was obtained by puncturing retroorbital plexus under anaesthesia using capillary tubes. Blood drops were collected gently; serum was separated by centrifugation at 2500 rpm for 15 min and stored at -20°C until analyzed for various biochemical parameters. EDTA was used as an anticoagulant in blood samples for haematological parameters and analyzed immediately. Blood was collected before treatment at day "0" and after slaughtering the animals at day "21".

2.2.5.2.1.2 Haematolgical studies

Haemoglobin concentration (Hb), packed cell volume (PCV), red blood cells count (RBC), white blood cells count (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were measured using Sysmex automatic analyzer (KX-21N, Spain).

2.2.5.2.1.2.1 Principle of haematology analyzers Sysmex (KX-21N):

This instrument perform blood count by DC detection method to measure WBC and differential count ,RBCs, HCT, MCV, MCH, MCHC, Hb and Platelets count. Blood sample is aspirated, measured to predetermined, then diluted at the

specified ratio. Then fed into each transducer. The transducer chamber has a minute hole called the aperture. On both side of the aperture, there are the electrodes, between which flows direct current. Blood cells suspended in diluted sample pass through the aperture, causing direct current resistance to change between the electrodes .As a direct current resistance Changes, the blood cell size is detected as electric pulses. Blood cell count is calculated by counting the pulses, and histogram of blood cell size is blotted by determining the pulse sizes. Also analyzing histogram makes it possible to obtain various analysis data. Haemoglobin is measured by non-cyanide haemoglobin analysis method which rapidly converts blood haemoglobin as oxy haemoglobin method and contains no poisonous substance making it suitable for automated method (Diamond, 1999).

2.2.5.2.1.2.2 Procedure of Sysmex KX-21N:

Measurement of blood cells (RBCs and WBCs), and haematological concentration were obtained by aspiration of small volume of well mixed K_2 EDTA blood by sample probe and mixed with isotonic diluents in nebulizer Diluted mixture aspiration was Delivered to RBCs aperture both for providing information about RBCs and Platelet Based on the cell size . Particles of 2 to 20 fl counted as platelet. Above 36 fl was counted as reamed cell. Some portion of aspiration mixture induced into WBCs both in which Haemolytic reagent (Stromatolyzer) was added automatically to measure hemoglobin Concentration in build calorimeter, based on cyanomethemogolobin method (HICN). Blood cell were counted according to size information was generated in triplicate pulses according to electronic conductivity. Parameters were directly measured and displayed on (LCD) other values of red cell indices, platelets; leukocytes differential and absolute Count were calculated from given information and automated constructed histograms (Dimond, 1999).

2.2.5.2.1.2 .3 Reagents and Materials:

Commercial close system reagents were provided by sysmex KX-21N operators and Consist of: Cell pack and Stromatolyser: diluents and lysing reagent for use in Sysmex. Detergent and cell cleaner: use for cleaning solution to remove lysing reagents, cellular residuals and blood proteins remaining in the hydraulics of sysmex automated haematology analyzers (Dimond,1999).

2.2.5.2.1.3 Biochemical analysis

Biochemical parameters, i.e. alanine amino transeferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), total proteins, creatinine and urea were analyzed using diagnostic kits by Mindray automatic analyzer (BS-200 – China) see appendix 1.

2.2.5.2.1.4 Clinical signs

Rats were observed for signs of toxicity and mortality during the course of experiment.

2.2.5.2.1.5 Histopathological studies

At the end of experiment, animals were sacrificed under anaesthesia. Tissue specimens from liver, lung, heart, kidney, intestine, stomach and spleen were collected immediately after necropsy and were fixed in 10% formal saline for histopathology. Sections were prepared and stained with haematoxcyline and eosin according to Drury and Willington (1967).

2.2.6 Statistical analysis

Data were expressed as the mean ± SEM. Differences between experimental groups were compared by one way analysis of variance (ANOVA) followed by Duncan test. The results were considered statistically significant when $P < 0.05$ (Gomez and Gomez, 1984)

CHAPTER THREE

3. Results

3.1 Phytochemical studies

3.1.1 The yield percentages of *H. sabdariffa* and *B. aegyptiaca* seeds

H. sabdariffa and *B. aegyptiaca* seeds were extracted by petroleum ether and ethanol. The 1000 g of *H. sabdariffa* seeds extracted by petroleum ether and ethanol yielded 168.22 g and 62.30 g, respectively. Extraction of 200 g of *B. aegyptiaca* seeds yielded 79.65 g in petroleum ether extract and 32.50 g in ethanolic extract (Table 1).

3.1.2 Fatty acids composition of *H. sabdariffa* and *B. aegyptiaca* seeds oil

The analysis of seed oil of *H. sabdariffa* petroleum ether extract using Gas chromatography (GC) revealed the presence of high percentage of saturated fatty acids. The major components of fatty acid in *H. sabdariffa* seed oil were linolelaidic acid (26.02%), arachidic acid (20.59%) and palmitic acid (16.05%). Gas chromatography analysis of seed oil obtained from *B. aegyptiaca* petroleum ether extract revealed the presence of oleic acid in high concentation (20.61%), palmitic acid (4.90%) and capric acid (1.49%). The fatty acids composition of *H. sabdariffa* and *B. aegyptiaca* seeds oil is presented in Table 2, Fig.5 and 6.

Table1. The yield percentages of *H. sabdariffa* and *B. aegyptiaca* seeds

Plants	Quantity (gm)	Extract	Yield (gm)	Yield %
H. sabdariffa seeds	1000	Petroleum ether	168.22	16.82
		Ethanol	62.30	6.23
B. aegyptiaca seeds	200	Petroleum ether	79.65	39.83
		Ethanol	32.50	16.25

Table 2. Fatty acids composition of *H. sabdariffa* and *B. aegyptiaca* seeds oil.

Plants	Compound	Concentration (%)
***H. sabdariffa* seeds**	Linolelaidic acid	26.02
	Arachidic acid	20.59
	Palmitic acid	16.05
***B. aegyptiaca* seeds**	Oleic acid	22.61
	Palmitic acid	4.90
	Capric acid	1.49
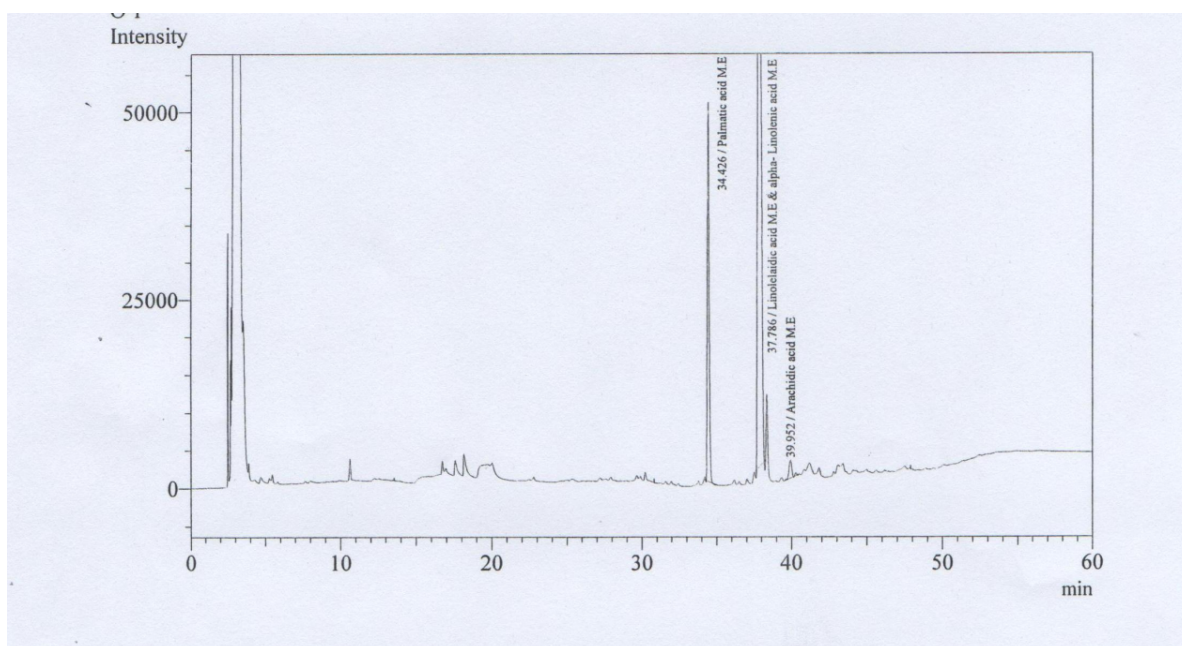
Fig.5. Chromatogram of Gas Chromatography (GC) for *H. sabdariffa* seed oil. Three distinct peaks corresponding to the authentic fatty acids were seen.
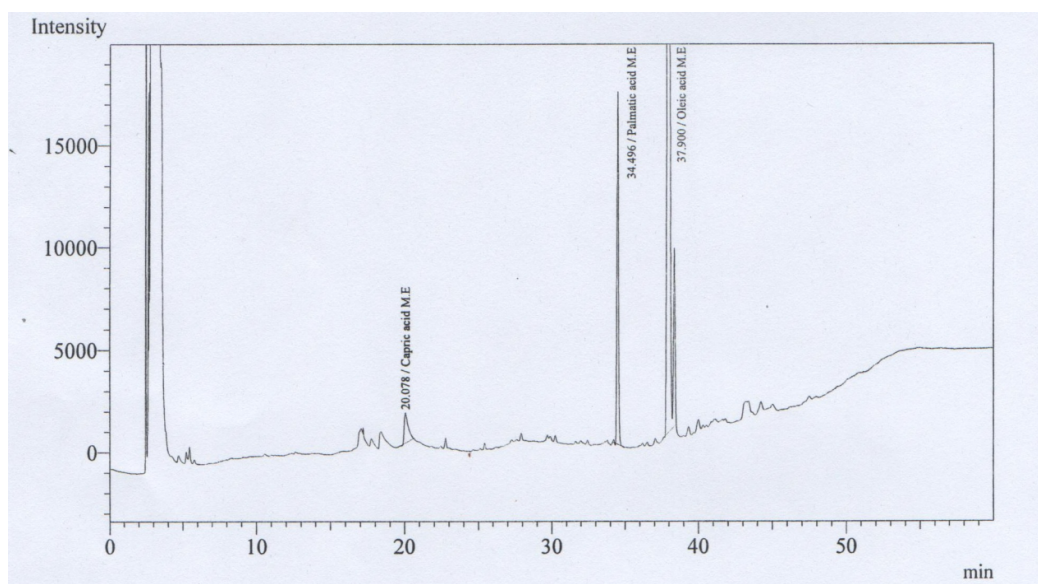
Fig.6. Chromatogram of Gas Chromatography (GC) for _B. aegyptiaca_ seed oil. Three distinct peaks corresponding to the authentic fatty acids were seen.

3.2 Pharmacological studies

3.2.1 *H. sabdariffa* seeds

3.2.1.1 Anti-inflammatory activity of *H. sabdariffa* seeds petroleum ether extract on carrageenan induced rats' hind paw oedema.

Oral administration of petroleum ether extract of *H. sabdariffa* seeds at a dose of 2ml/kg showed insignificant ($P > 0.05$) decrease in the rats' hind paw oedema after 3 hours compared with control group. *H. sabdariffa* seeds petroleum ether extract at a dose of 4 ml/kg reduced significantly ($P < 0.05$) the rats' hind paw oedema after 3 hours by 27.9% compared with control rats, but the inhibition was less ($P < 0.05$) than that observed in standard drug, indomethacin for the same period. The high dose (8 ml/kg) of *H. sabdariffa* seeds petroleum ether extract inhibited significantly ($P < 0.01$) the hind paw oedema after 3 hours of carrageenan injection by 34.2% compared with indomethacin ($P > 0.05$). After 4 hours the inhibition of hind paw oedema in animals given 8ml/kg was found to be significant ($P < 0.05$) when compared with control, but less than that observed in rats treated with indomethacin ($P < 0.05$). On the other hand, rats given 2 and 4 ml/kg of *H. sabdariffa* seeds petroleum ether extract showed insignificant ($P > 0.05$) inhibition of hind paw oedema after 4 hours of carrageenan injection compared with control. Indomethacin used as standard anti-inflammatory drug decreased significantly ($P < 0.001$) paw oedema from 1 – 4 hours. The inhibition percentage of indomethacin was 57.1% after 3 hours and 64.9% after 4 hours of carrageenan injection (Table 3), Fig.7.

3.2.1.2 Anti-inflammatory activity of *H. sabdariffa* seeds ethanolic extract on carrageenan induced rats' hind paw oedema.

Oral administration of ethanolic extract of *H. sabdariffa* seeds at a dose of 100 mg/kg produced significant inhibition ($P < 0.05$) in rats' hind paw oedema after 1 hour of carrageenan injection compared to the control. This activity was found to be insignificant ($P > 0.05$) after 2, 3 and 4 hours. Animals treated with 200 and

400 mg/kg of *H. sabdariffa* seeds ethanolic extract did not show any significant reduction of paw oedema from 1 - 4 hours compared with the control group. Contrary to that the hind paw oedema was increased significantly ($P < 0.01$) after 3 and 4 hours of carrageenan injection in these rats compared to the control. The hind paw oedema was inhibited significantly ($P < 0.001$) from 1 – 4 hours by indomethacin used as a standard anti-inflammatory drug (Table 4), Fig.8.

Table 3. Anti-inflammatory activity of *H. sabdariffa* seeds petroleum ether extract given orally on carrageenan induced rats' hind paw oedema.

Groups	Dose	Increase in paw volume (mm)				Inhibition %			
		1h	2h	3h	4h	1h	2h	3h	4h
Control	-	2.11 ± 0.13^{a}	2.82 ± 0.24^{a}	2.27 ± 0.20^{a}	1.90 ± 0.09^{a}	-	-	-	
Indomethacin	10mg/kg	1.22 ± 0.09^{b}	1.15 ± 0.23^{c}	0.97 ± 0.23^{d}	0.68 ± 0.12^{c}	42.1	59.9	57.1	64.9
	2ml/kg	1.81 ± 0.12^{a}	2.08 ± 0.14^{b}	2.11 ± 0.05^{ab}	1.76 ± 0.15^{a}	16.3	22.8	7.8	7.5
H. sabdariffa	4ml/kg	1.37 ± 0.21^{b}	1.63 ± 0.13^{bc}	1.85 ± 0.12^{bc}	1.57 ± 0.08^{a}	32.0	38.5	27.9	15.8
	8ml/kg	0.92 ± 0.11^{b}	1.25 ± 0.20^{c}	1.45 ± 0.22^{cd}	1.29 ± 0.27^{b}	55.3	55.7	34.2	32.9

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).
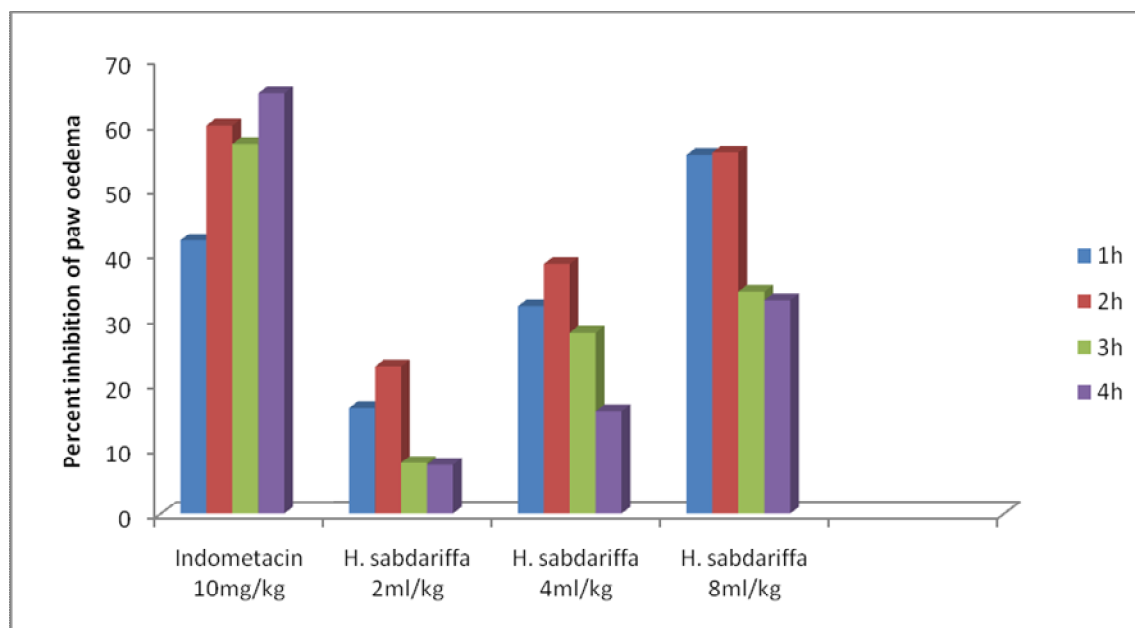
Fig.7. Inhibition of rats' hind paws oedema by *H. sabdariffa* petroleum ether extract and indomethacin.

Table 4. Anti-inflammatory activity of *H. sabdariffa* seeds ethanolic extract on carrageenan induced rats'hind paw oedema.

Groups	Dose	Increase in paw volume (mm)				Inhibition %			
		1h	2h	3h	4h	1h	2h	3h	4h
Control	-	2.52±0.21[a]	3.30±0.21[ab]	3.01±0.20[b]	3.06±0.20[b]	-	-	-	
Indomethacin	10mg/kg	0.57±0.04[c]	1.20±0.08[c]	1.33±0.13[c]	1.41±0.07[c]	76.8	63.6	55.6	53.8
	100mg/kg	1.92±0.16[b]	2.75±0.17[b]	2.66±0.20[b]	2.73±0.14[b]	21.4	15.2	9.8	10.0
H. sabdariffa	200mg/kg	2.46±0.07[a]	3.44±0.08[a]	3.55±0.05[a]	3.47±0.04[a]	0.6	-5.9	-19.9	-14.9
	400mg/kg	2.52±0.11[a]	3.32±0.16[a]	3.58±0.16[a]	3.42±0.12[a]	-1.4	-2.8	-20.1	-12.9

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).
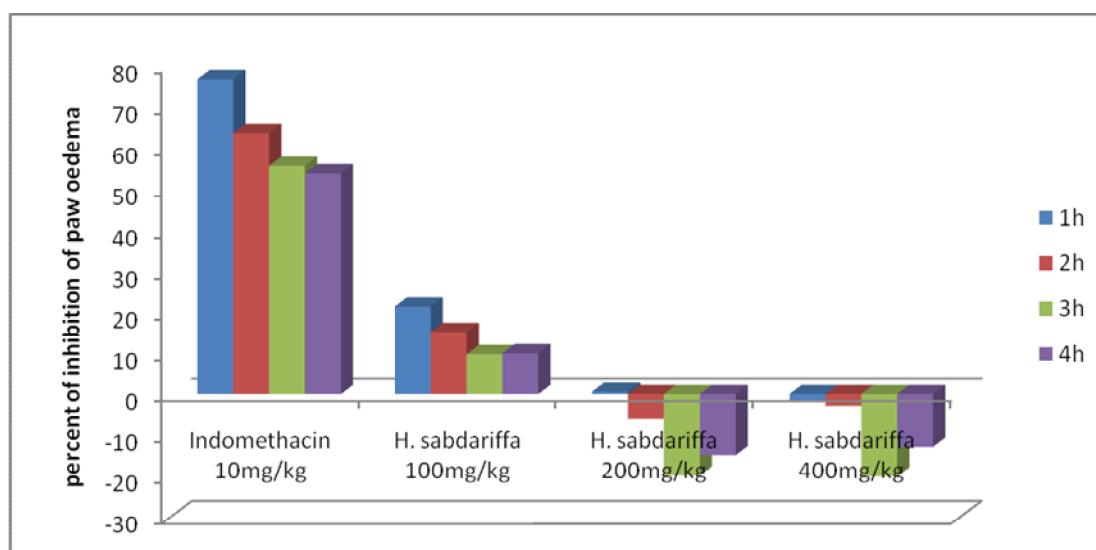
Fig.8. Inhibition of rats' hind paws oedema by _H. sabdariffa_ ethanolic extract and indomethacin.

3.2.1.3 Effect of *H. sabdariffa* seeds petroleum ether extract on acetic acid induced vascular permeability in rats

Oral administration of diclofenac sodium at a dose of 10 mg/kg and petroleum ether extract of *H. sabdariffa* seeds at a dose of 4 and 8 ml/kg significantly ($P < 0.01$) inhibited the dye leakage induced by acetic acid when compared with the control animal. The vascular permeability was inhibited significantly ($P < 0.05$) by 48.5% in the rats treated with 4ml/kg of *H. sabdariffa* seeds petroleum ether extract and 56.5% ($P < 0.01$) in those treated with 8ml/kg of *H. sabdariffa* seeds petroleum ether extract compared with control. The inhibition was found to be more expressed in diclofenac sodium used as a standard anti-inflammatory drug (76.7%) but was similar to that demonstrated in rats treated with *H. sabdariffa* petroleum ether extract at a dose of 4 and 8ml/kg ($P > 0.05$). The results are shown in Table 5, Fig.9.

3.2.1.4 Effect of *H. sabdariffa* seeds petroleum ether extract on cotton pellet granuloma in rats

The petroleum ether extract of *H. sabdariffa* seeds was also screened for cotton pellet induced granuloma in rats. Granuloma formation was inhibited significantly ($P < 0.001$) by the standard anti-inflammatory drug diclofenac sodium and after oral administration of petroleum ether extract of *H. sabdariffa* seeds for 6 consecutive days compared with control group. Petroleum ether extract of *H. sabdariffa* seeds at a dose of 4 and 8 ml/kg showed 30.3% and 27.2 % inhibition of granuloma, respectively as compared with the control group, whereas, diclofenac sodium exhibited the highest inhibition rate 52.7% compared with plant extract treated groups ($P < 0.05$). The results are presented in Table 6, Fig.10.

3.2.1.5 Effect of *H. sabdariffa* seeds petroleum ether extract on acetic acid induced writhes in rats

Treatment of animals with *H. sabdariffa* seeds petroleum ether extract produced a significant inhibition in abdominal writhes induced by (0.7%) acetic acid especially at a dose of 2 ($P < 0.01$), 4 and 8 ($P < 0.001$) ml/kg compared to the control group. The inhibition of abdominal writhes in animals treated with *H. sabdariffa* seeds petroleum ether extract at a dose of 2 and 4ml/kg was found to be less than that recorded in rats treated with diclofenac sodium ($P < 0.001$). Maximum writhing inhibition was found to be 52.6% in diclofenac sodium group used as a standard analgesic drug. Rats received 8ml/kg of *H. sabdariffa* seeds petroleum ether extract showed inhibition in abdominal writhes (45.0%) near to that observed in rats given diclofenac sodium at a dose of 10 mg/kg. The results are shown in Table 7 and Fig.11.

Table 5. Effect of oral administration of petroleum ether extract of *H. sabdariffa* seeds on acetic acid induced vascular permeability

Treatment	Dose	Absorbance ±SE	% Inhibition
Control	-	0.140 ± 0.03^{a}	-
Diclofenac sodium	10mg/kg	0.026 ± 0.00^{b}	76.7
H. sabdariffa	4ml/kg	0.072 ± 0.02^{b}	48.5
	8ml/kg	0.047 ± 0.01^{b}	56.5

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).
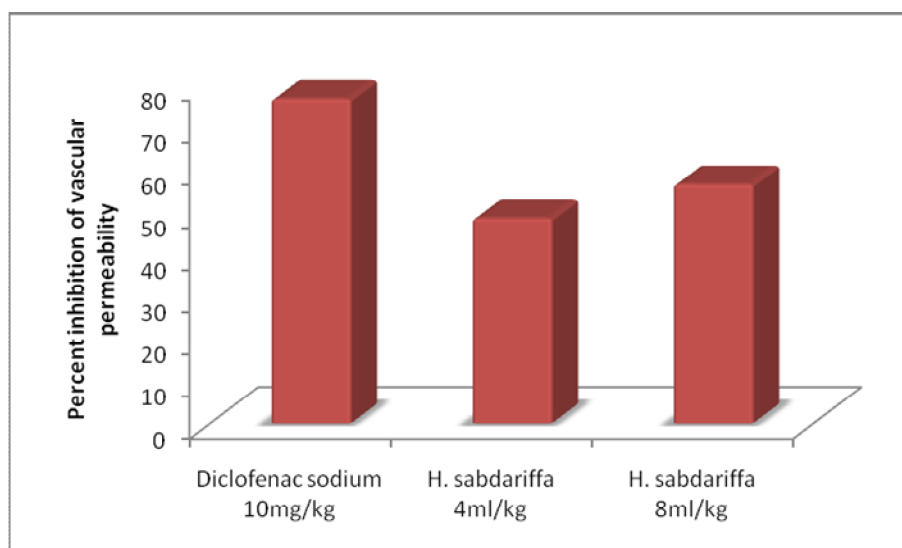
Fig.9. Inhibition of vascular permeability by petroleum ether extract of *H. sabdariffa* seeds in rats.

Table 6. Effect of petroleum ether extract of *H. sabdariffa* seeds on cotton pellet granuloma in rats.

Groups	Dose	Granuloma dry weight (mg)	Inhibition %
Control	-	44.3 ± 3.24^{a}	-
Diclofenac sodium	10 mg/kg	20.4 ± 1.90^{c}	52.7
H. sabdariffa	4 ml/kg	30.2 ± 0.81^{b}	30.3
	8 ml/kg	31.5 ± 0.59^{b}	27.2

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).
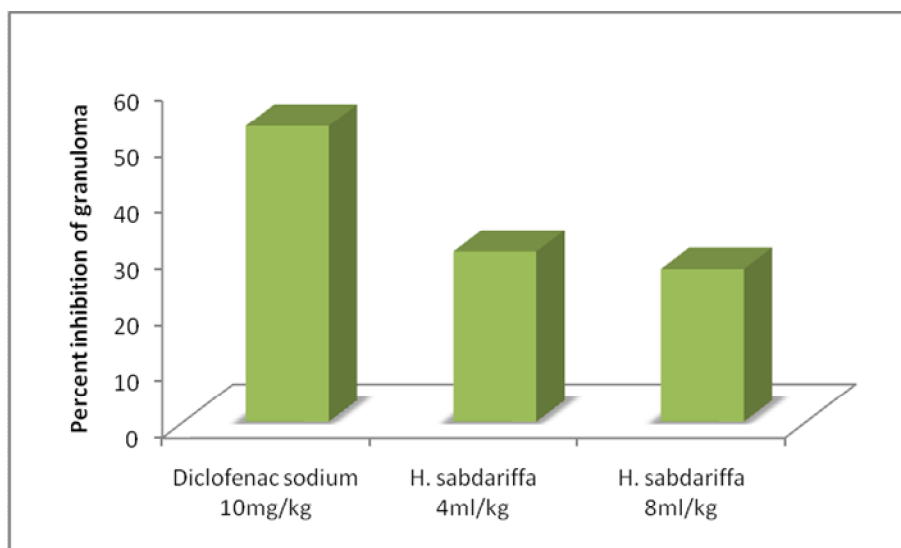
Fig.10. Inhibition of granuloma by petroleum ether extract of *H. sabdariffa* seeds in rats.

Table 7. Effect of petroleum ether extract of *H. sabdariffa* seeds on acetic acid induced writhes in rats

Groups	Dose	Number of writhes	Inhibition rate %
Control	-	77.8±1.80 [a]	-
Diclofenac sodium	10 mg/kg	36.8±1.74 [c]	52.6
	2 ml/kg	66.6±3.04 [b]	14.5
H. sabdariffa	4 ml/kg	61.6±2.23 [b]	20.7
	8 ml/kg	42.8±1.62 [c]	45.0

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).

Fig.11. inhibition of writhes by petroleum ether extract of *H. sabdariffa* seeds in rats

3.2.2 *B. aegyptiaca* seeds

3.2.2.1 Anti-inflammatory activity of *B. aegyptiaca* seeds petroleum ether extract on carrageenan induced rats' hind paw oedema

B. aegyptiaca seeds petroleum ether extract administered orally at a dose of 2, 4 and 8 ml/kg significantly ($P < 0.001$) reduced the hind paw oedema induced by carrageenan from 1 – 4 hours. The inhibition of hind paw oedema in the plant extract treated groups was comparable ($P > 0.05$) to the inhibition seen in rats treated with the standard drug, diclofenac sodium during the period of study. The data are shown in Table 8, Fig.12.

3.2.2.2 Anti-inflammatory activity of *B. aegyptiaca* seeds ethanolic extract on carrageenan induced rats' hind paw oedema

Diclofenac sodium used as a standard anti-inflammatory drug at a dose of 10 mg/kg significantly ($P < 0.001$) inhibited the hind paw oedema from 1 – 4 hours, by 46.3%, 54.5%, 69.8% and 85.7%, respectively. Oral administration of *B. aegyptiaca* seeds ethanolic extract at a dose of 200 mg/kg produce significant ($P < 0.05$) inhibition of paw oedema after 2 and 3 hours compared with the control, but the inhibition was found to be insignificant ($P > 0.05$) after 4 hours of carrageenan injection. Treatment of animals with *B. aegyptiaca* seeds ethanolic extract at a dose of 400 mg/kg did not prove any significant activity in the inhibition of hind paw oedema from 1 – 4 hours after carrageenan injection compared to the control. However, the inhibition of hind paw oedema was found to be similar in animals given *B. aegyptiaca* seeds ethanolic extract at a dose of 200 and 400 mg/kg after 2 and 3 hours. The data are shown in Table 9 and Fig.13.

3.2.2.3 Effect of *B. aegyptiaca* seeds petroleum ether extract on cotton pellet granuloma in rats

Granuloma formation was hindered significantly ($P < 0.001$) by the standard drug, diclofenac sodium and after administration of petroleum ether extract of *B. aegyptiaca* seeds for 6 consecutive days when compared with the control group.

B. aegyptiaca seeds petroleum ether extract at a dose of 4 and 8 ml/kg showed 42.7% and 45.4% of inhibition, respectively, whereas diclofenac sodium exhibited the highest inhibition rate (59.9%) compared to control. *B. aegyptiaca* seeds petroleum ether extract at a dose of 4 and 8 ml/kg produced similar anti-inflammatory effect on cotton pellet granuloma, but the effect is significantly ($P < 0.01$) less compared to diclofenac sodium. The results are shown in Table 10 and Fig.14.

3.2.2.4 Effect of *B. aegyptiaca* seeds petroleum ether extract on acetic acid induced writhes in rats

The animals treated with 2 ml/kg of *B. aegyptiaca* seeds petroleum ether extract showed insignificant ($P > 0.05$) inhibition of writhes induced by acetic acid compared to the control group. Treatment of animals with *B. aegyptiaca* seeds petroleum ether extract produced a significant inhibition in abdominal writhes induced by acetic acid especially at 4 ($P < 0.01$) and 8 ($P < 0.001$) ml/kg doses compared to the control group. The inhibition was found to be 46.8% and 56.0%, respectively. Maximum writhing inhibition was achieved by diclofenac sodium used as a standard analgesic drug (60.8%) and animals given *B. aegyptiaca* seeds petroleum ether extract at a dose of 8 ml/kg ($P > 0.05$). The results are presented in Table 11and Fig.15.

Table 8. Effect of petroleum ether extract of *B. aegyptiaca* seeds on carrageenan induced rats' paw oedema.

Groups	Dose	Increase in paw volume (mm)				Inhibition %			
		1h	2h	3h	4h	1h	2h	3h	4h
Control	-	1.98±0.07 [a]	2.02±0.04 [a]	1.96±0.06 [a]	1.73±0.03 [a]	-	-	-	
Indomethacin	10mg/kg	1.11±0.07 [b]	0.89±0.11 [b]	0.70±0.09 [b]	0.51±0.05 [b]	43.5	55.9	63.5	70.4
	2ml/kg	0.97±0.06 [b]	0.98±0.02 [b]	0.91±0.08 [b]	0.71±0.06 [b]	51.0	51.2	53.4	59.0
B. aegyptiaca	4ml/kg	0.95±0.05 [b]	0.90±0.04 [b]	0.74±0.03 [b]	0.61±0.03 [b]	51.9	55.5	62.3	64.8
	8ml/kg	0.98±0.02 [b]	0.88±0.02 [b]	0.72±0.05 [b]	0.52±0.01 [b]	50.1	56.5	62.8	69.8

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).
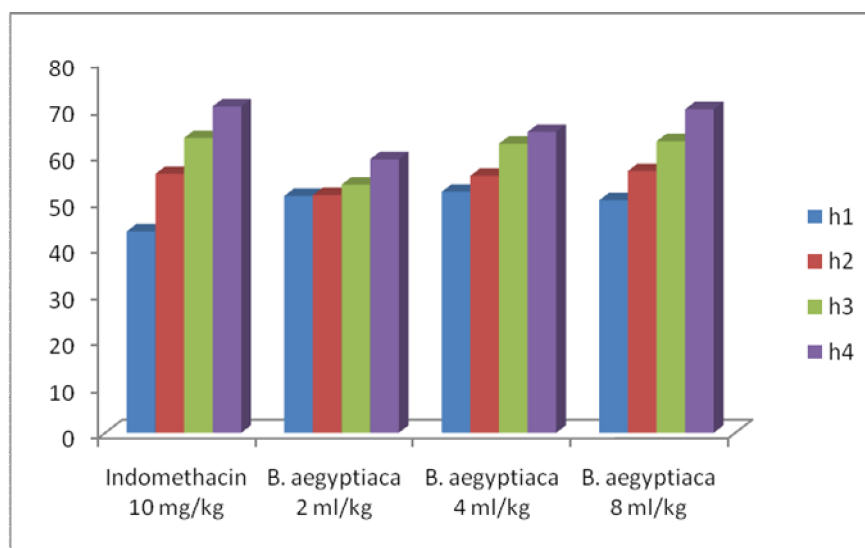
Fig.12. Inhibition rats' hind paws oedema by petroleum ether extract of *B. aegyptiaca* seeds.

Table 9. Effect of ethanolic extract of *B. aegyptiaca* seeds on carrageenan induced rats' paw oedema.

Groups	Dose	Increase in paw volume (mm)				Inhibition %			
		1h	2h	3h	4h	1h	2h	3h	4h
Control	-	1.41±0.07^a	1.68±0.03^a	1.55±0.04^a	1.41±0.04^a	-	-	-	-
Diclofenac sodium	10	0.74±0.09^b	0.76±0.05^c	0.46±0.05^c	0.20±0.05^b	46.3	54.5	69.8	85.7
B. aegyptiaca	200	1.36±0.04^a	1.35±0.06^b	1.25±0.04^b	1.22±0.05^a	2.6	19.5	18.9	13.3
	400	1.40±0.17^a	1.44±0.18^{ab}	1.34±0.16^{ab}	1.25±0.16^a	-1.1	13.7	11.9	10.4

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).

Fig.13. Inhibition of rats' hind paws oedema by ethanolic extract of *B. aegyptiaca* seeds.

Table 10. Effect of *B. aegyptiaca* seeds petroleum ether extract on cotton pellet granuloma in rats

Groups	Dose	Granuloma dry weight (mg)	Inhibition %
Control	-	38.8±2.3 [a]	-
Diclofenac sodium	10 mg/kg	15.4±0.9 [c]	59.9
B. aegyptiaca	4 ml/kg	22.0±2.1 [b]	42.7
	8 ml/kg	20.9±0.9 [b]	45.4

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).
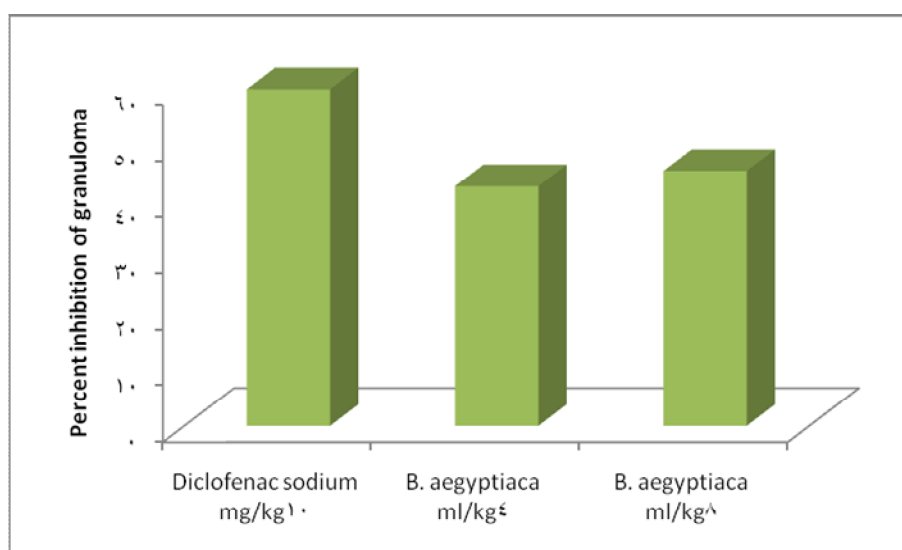
Fig.14. Inhibition of granuloma by petroleum ether extract of *B. aegyptiaca* seeds in rats.

Table 11. Effect of *B. aegyptiaca* seeds petroleum ether extract on acetic acid induced writhes in rats

Groups	Dose	Number of writhes	Inhibition rate %
Control	-	100.2 ± 2.52^{a}	-
Diclofenac sodium	10 mg/kg	37.6 ± 1.94^{c}	60.8
	2 ml/kg	94.2 ± 2.42^{a}	6.0
B. aegyptiaca	4 ml/kg	53.2 ± 2.53^{b}	46.8
	8 ml/kg	44.0 ± 0.89^{bc}	56.0

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).
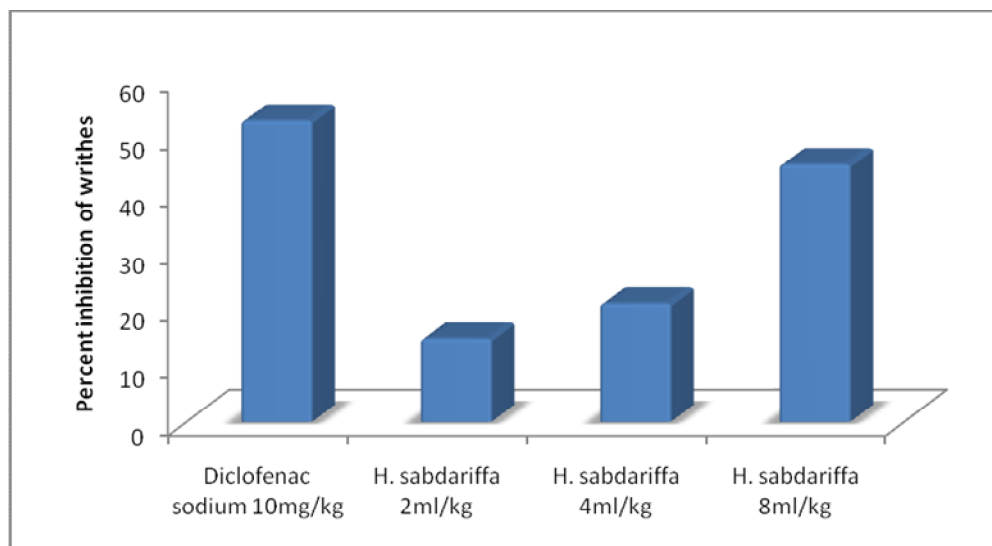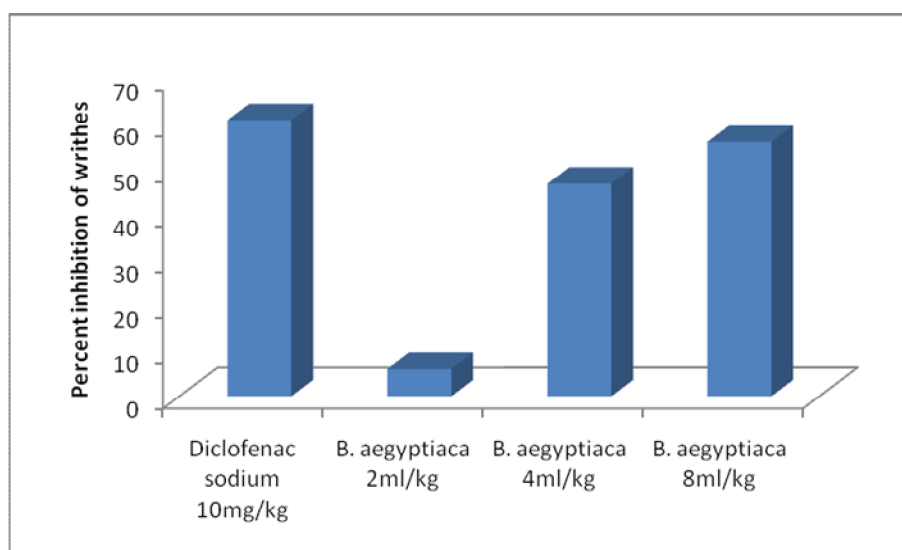
Fig.15. Inhibition of writhes by petroleum ether extract of *B. aegyptiaca* seeds in rats.

3.3 Toxicological studies:

3.3.1 Acute toxicity study:

3.3.1.1 Acute toxicity study of *H. sabdariffa* seeds petroleum ether extract

Acute toxicity study was carried out to confirm the safety of *H. sabdariffa* petroleum extract and to select suitable treatment doses. All the doses (2, 4, 8 and 20 ml/kg) of petroleum ether extract of *H. sabdariffa* seeds employed for acute oral toxicity study were found to be non-toxic. No mortality was observed in the animals that received petroleum ether extract up to 20 ml/kg. Anorexia was observed in the animals receiving the higher doses of extract (8 and 20 ml/kg).

3.3.1.2 Acute toxicity study of *B. aegyptiaca* seeds petroleum ether extract

Oral administration of petroleum ether extract of *B. aegyptiaca* seeds at a dose of 2, 4, 8 and 20 ml/kg was found safe in rats. No mortality or clinical signs were seen in the animals receiving the petroleum ether extract up to 20 ml/kg with the exception of signs of anorexia in animals treated with higher doses (8 and 20 ml/kg).

3.3.2 Sub-chronic toxicity study:

3.3.2.1 Effect of administration of *B. aegyptiaca* seeds oil in rats

3.3.2.1.1 Clinical signs:

No mortality or obvious clinical signs were observed in the control and treated groups during the course of experiment except the signs of anorexia in the animals that received the high dose of oil (8ml/kg).

3.3.2.1.2 Haematological parameters

There were no significant differences in haematological parameters between the control and treated groups at day 0 or day 21. However, when comparing values for each group at day 0 and day 21, there was marked decrease in the mean values of RBC, PCV, Hb and marked ($p < 0.05$) increase in MCHC values at

day 21 in the group receiving 8ml/kg of *B. aegyptiaca* seeds petroleum ether extract. Despite these changes these values are considered within the normal range. The results of administration of *B. aegyptiaca* seeds oil on haematological parameters are presented in Table 12.

3.3.2.1.3 Biochemical parameters

3.3.2.1.3.1 Liver function test

There was no significant change on the levels of AST, ALT, ALP and total proteins between the groups (control and treated) in days 0 or day 21. When comparing the values of each group at day 0 and day 21 the values of AST and ALT were significantly ($p < 0.001$) higher at day 21 in all groups including control. ALP values were also elevated but insignificantly ($p > 0.05$). The results are presented in Table 13.

3.3.2.1.3.2 Kidney function test

The levels of urea and creatinine in control animals were maintained on the normal ranges as well as the treated groups during the period of the experiment. There was no significant ($p > 0.05$) difference between day 0 and day 21 in all groups. The results are shown in Table 14.

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Table 12. The effect of administration of *B. aegyptiaca* seeds oil on haematological parameters.

Groups	$RBC \times 10^6$ µl		Hb (g/dl)		PCV %	
	Day 0	**Day 21**	**Day 0**	**Day 21**	**Day 0**	**Day 21**
Control	7.55±0.88 [a]	7.35±0.18 [a]	13.9±0.64 [a]	14.3±0.21 [a]	44.9±2.35 [a]	45.9±0.92 [a]
***B. aegyptiaca* 4ml/kg**	6.86±0.43 [a]	7.01±0.18 [a]	12.5± 0.71 [a]	13.5±0.33 [a]	40.3±1.75 [a]	44.8±1.28 [a]
***B. aegyptiaca* 8ml/kg**	7.26±0.10 [a]	6.06±0.41 [b]	13.2±0.23 [a]	12.0±0.74 [b]	43.0±1.50 [a]	38.0±2.75 [b]

Groups	MCV (fl)		MCH (pg)		MCHC (g/dl)	
	Day 0	**Day 21**	**Day 0**	**Day 21**	**Day 0**	**Day 21**
Control	59.4±0.71 [a]	62.6±0.62 [a]	18.5±0.14 [a]	19.4±0.25 [a]	30.7±0.43 [a]	30.9±0.47 [a]
***B. aegyptiaca* 4ml/kg**	59.4±2.89 [a]	63.8±0.27 [a]	18.3±0.23 [a]	19.3±0.13 [a]	31.5±0.16 [a]	30.1±0.17 [a]
***B. aegyptiaca* 8ml/kg**	60.6±1.67 [a]	62.6±0.39 [a]	18.22±0.09	19.8±0.19 [a]	30.7±0.88 [a]	31.6±0.46 [b]

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at *P* < 05 (N =5).

Table 13. Effect of *B. aegyptiaca* seeds oil in rats on liver function tests

Groups	ALT (U/I)		AST(U/I)	
	Day 0	**Day 21**	**Day 0**	**Day 21**
Control	35.2±5.55 [a]	63.0±5.84 [a]	102.2±9.48 [a]	160.0±11.13 [a]
***B. aegyptiaca* 4ml/kg**	39.5±7.92 [a]	53.5±3.06 [a]	112.1±12.68 [a]	140.6±6.95 [a]
***B. aegyptiaca* 8ml/kg**	34.2±4.13 [a]	62.3±8.51 [a]	110.8±12.05 [a]	152.4±16.84 [a]

Groups	ALP (U/I)		Total Proteins (g/dl)	
	Day 0	**Day 21**	**Day 0**	**Day 21**
Control	75.5±9.15 [a]	89.4±2.55 [a]	6.45±0.31 [a]	6.93±0.34 [a]
***B. aegyptiaca* 4ml/kg**	85.1±14.25 [a]	100.1±13.54 [a]	6.53±0.71 [a]	6.67±0.27 [a]
***B. aegyptiaca* 8ml/kg**	82.7±6.06 [a]	92.1±2.84 [a]	7.55±0.52 [a]	6.31±0.26 [a]

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).
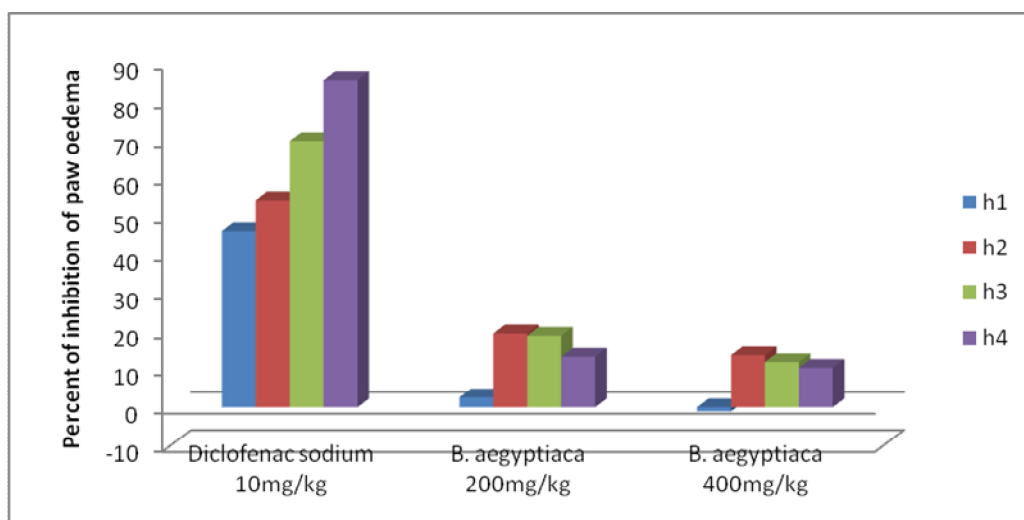
Table 14. Effect of *B. aegyptiaca* seeds oil in rats on kidney function tests

Groups	Urea mg/dl		Creatinine mg/dl	
	Day 0	**Day 21**	**Day 0**	**Day 21**
Control	28.7±2.62[a]	30.0±1.59[a]	0.55±0.07[a]	0.65±0.11[a]
***B. aegyptiaca* 4ml/kg**	35.0±4.38[a]	32.3±2.32[a]	0.55±0.07[a]	0.69±0.05[a]
***B. aegyptiaca* 8ml/kg**	37.3±2.20[a]	30.5±2.32[a]	0.60±0.04[a]	0.64±0.11[a]

Values are expressed as mean ± SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).

3.3.2.1.4 Histopathological findings:

Heart sections of control and treated groups showed no significant pathological changes.

In liver sections hepatocytes appeared normal with normal hepatic cords. No inflammatory, degenerative or necrotic changes were observed in all groups. Some sections in the control and treated animals showed slight dilatation of blood vessels (Fig.16 & 17).

In the kidney sections of the control animals, glomeruli and bowman spaces appeared normal with some lobulation of glomerular tuft and slight widening of cortical tubules (Fig.18).

Kidney sections of rats given 4 ml/kg *B. aegyptiaca* seed oil showed slight dilatation of tubules and lobulated glomerular tufts. Some tubular cells showed detachment from the basement membranes. Generally, cells appeared normal with no evidence of degenerative or necrotic changes (Fig.19).

In kidney sections of rats treated with 8 ml/kg *B. aegyptiaca* seed oil there was slight lobulation in glomerular tuft. In some sections very few areas of degenerative or necrotic changes were noticed (Fig.20).

The white pulps of spleen were distributed normally in the control and treated animals. Very prominent white pulps with dense zone of lymphocytes around central artery surrounded by lighter zone of lymphocytes were seen in some sections in the treated groups. In few sections the white pulps in the treated groups were seen as small collection of condensed lymphocytes around the central artery (Fig.21 & 22).

The pancreatic tissue appeared normal in all groups with no evidence of inflammatory or degenerative changes.

Intestine sections in all groups showed normal appearance with marked presence of goblet cells. Desquamation of villi was seen in some sections (Fig.23 & 24).

There were no significant pathological lesions in glandular and non glandular stomach of the control or treated rats.

Emphysema and congestion of alveolar wall capillaries were observed in the lung sections of all groups. Prebronchial lymphoid hyperplasia was also seen in some sections.
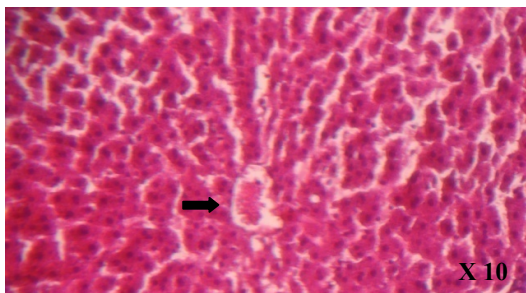
Fig.16. Liver section of rat treated with 4ml/kg *B. aegyptiaca* seed oil, showing normal hepatocytes and congestion of central veins (H&E stain). X10
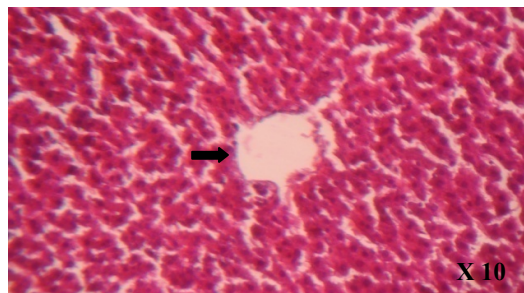
Fig.17. Liver section of rat treated with 8ml/kg *B. aegyptiaca* seed oil, showing normal hepatocytes with dilatation in central vein (H&E stain). X10
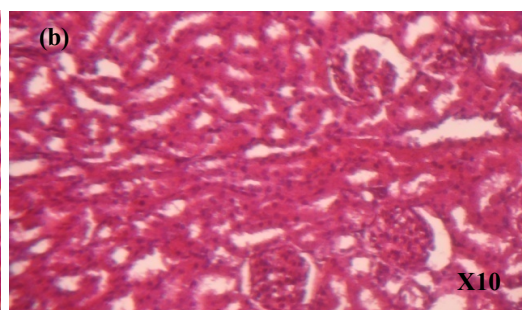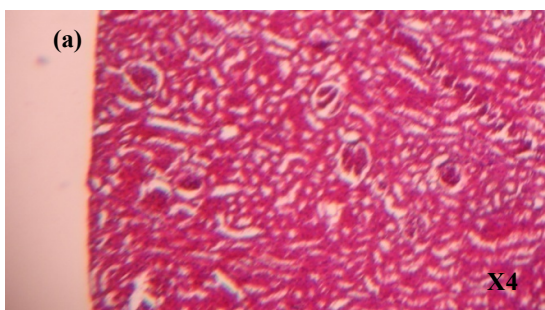
Fig.18. (a) Kidney section of control rat, showing normal appearance with slight lobulation of glomerular tuft (H&E stain). X4. (b) High magnification of (a). X10.
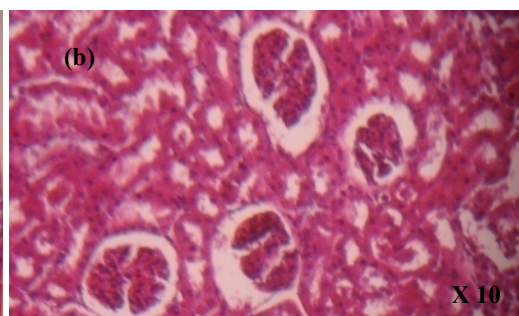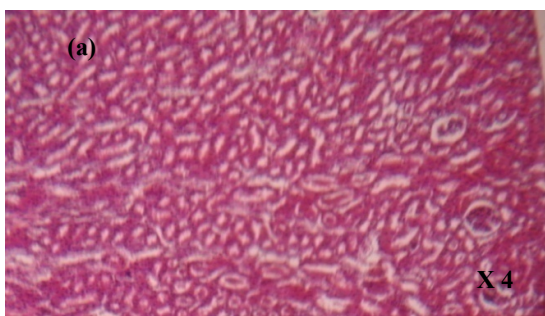
Fig.19. (a) kidney section of treated rat with 4ml/kg *B. aegyptiaca* seed oil, showing normal appearance with slight dilatation of bowman space and lobulation of glomerular tuft (H&E stain). X4. (b) High magnification of (a). X10.
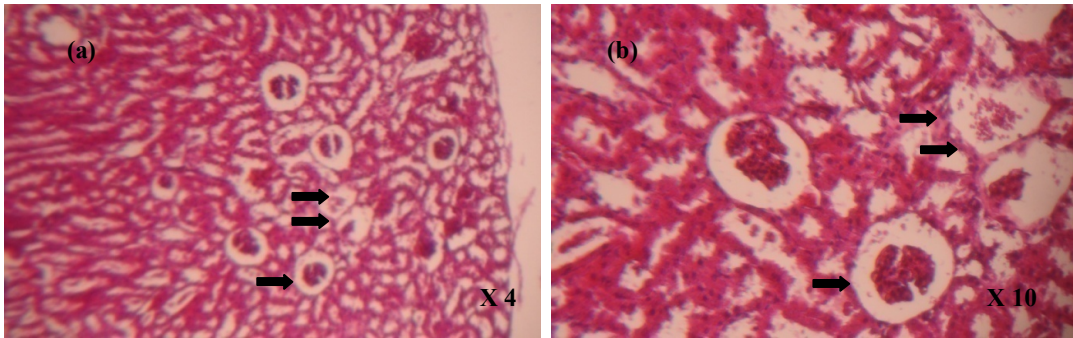
Fig.20. (a) kidney section of treated rat with 8ml/kg *B. aegyptiaca* seed oil, showing normal appearance with dilatation of bowman space and focal area of degenerative or necrotic changes (H&E stain). X4 (b) High magnification of (a). X10.
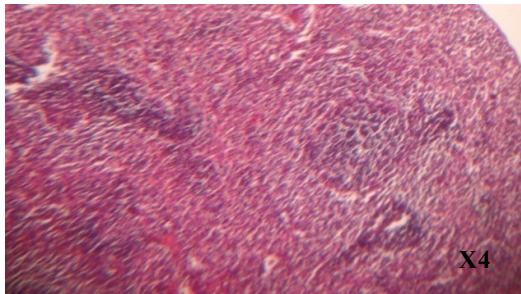
Fig.21. Spleen section of control rat, showing normal appearance (H&E stain). X4.
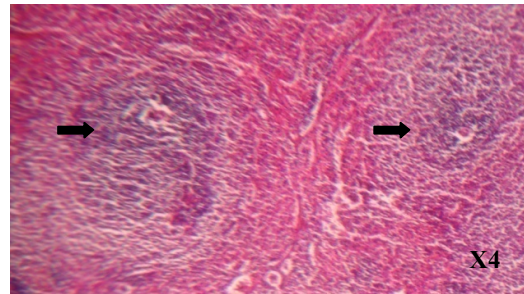
Fig.22. Spleen section of rats treated with 4ml/kg *B. aegyptiaca* seed oil showing normal appearance with dense zone of lymphocytes around central artery (H&E stain). X4. (➡: white pulp).
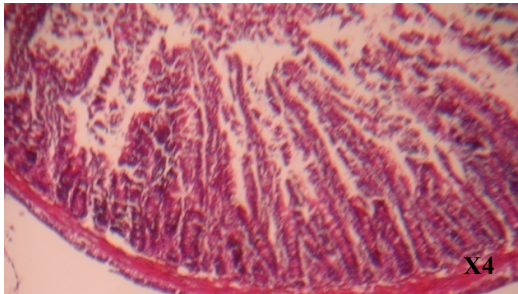
Fig.23. Intestine section of control rat, showing normal appearance of villi, glands and lamina propriea with marked presence of goblet cells and desquamation of villi (H&E stain). X4.
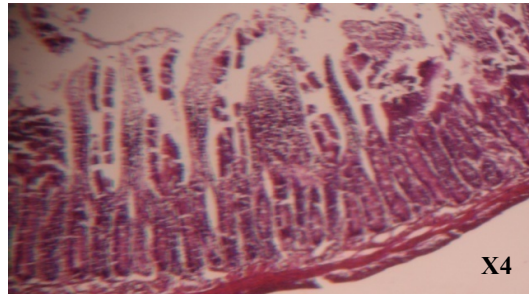
Fig.24. Intestine section of animal treated with 4ml/kg *B.aegyptiaca* seed oil showing desquamation of villi, normal appearance of glands and lamina propriea with marked presence of goblet cells (H&E stain). X4.

CHAPTER FOUR

4. Discussion

The present study was carried out to evaluate the anti-inflammatory and analgesic effects of *H.sabdariffa* and *B. aegyptiaca* seeds petroleum ether and ethanolic extracts in Wistar albino rats using acute and chronic models. The phytochemical screening was performed for active extracts. In addition the toxiclogical status of *B. aegyptiaca* seeds petroleum ether extract was also evaluated in acute and sub-chronic toxicities models in rats.

The anti-inflammatory activity of seeds extracts of both plants was investigated in acute model using carrageenan induced rats' hind paw oedema. Carrageenan-induced inflammation is most commonly used as an experimental model for evaluating the anti-inflammatory potency of compounds or natural products (Chakraborty *et al.*, 2004 and Andrade *et al.*, 2007). The results obtained in this study showed that injection of carrageenan into the rats' hind paw elicited a localized inflammatory response characterized by an increase of paw size (swelling) and pain as a result of increased vascular permeability, cell infiltrations and inflammatory fluids. Control rats injected with carrageenan showed high paw oedema in the first hour and prolonged effect at 2nd, 3rd and 4th hours compared with standard anti-inflammatory drug, indometacin and diclofenac sodium, this is in agreement with the findings of Ravi *et al.* (2009) and Parekar *et al.*, (2012). Those authors suggested that the possible mechanism of action of carrageenan-induced inflammation is bi-phasic, the first phase is attributed to the release of histamine, serotonin and kinins in the first hour, while the second phase is attributed to the release of prostaglandins and lysosome enzymes at 2nd up to 4th hours. The second phase is sensitive to most clinically effective anti-inflammatory drugs (Vasudevan *et al.*, 2006 and Das *et al.*, 2013).

In this study, *H. sabdariffa* seeds petroleum ether extract produced a significant inhibition in the rats' hind paw oedema in the first and second phases of inflammation in a dose dependant manner. The low dose (2ml/kg) failed to reduce paw oedema induced by carrageenan, whereas the medium (4ml/kg) and the high doses (8ml/kg) were found to be more effective in reducing paw oedema at the 3rd hour post carrageenan injection. Indomethacin used as a standard anti-inflammatory drug efficiently decreased the paw oedema from 1st hour to 4th hour. The inhibition by indomethacin was better than that observed by 8ml/kg of *H.sabdariffa* seeds petroleum ether extract. The anti-inflammatory activity of *H.sabdariffa* has been studied by Ali *et al.* (2011). They investigated the anti-inflammatory activity of *H. sabdariffa* calyx ethanolic extract in xylene induced ear oedema model in mice. The results showed a significant inhibition on ear oedema formation in a dose related manner compared with the control. It caused 18.0% and 27.5% inhibition of ear oedema formation at the doses of 250 and 500 mg/kg body weight, respectively. The results obtained from the present study showed similar findings in the inhibition rate of paw oedema. The inhibition was found to be in a dose related manner that is 27.9% and 34.2% by petroleum ether extract at a dose of 4 and 8ml/kg, respectively. Moreover, in one clinical trial involving 50 patients, administration of a decoction of dried fruit (3g/person, three times every day for 7 days to 1 year) was shown to produce anti-inflammatory activity (Ali *et al.*, 2005), which accords well with our results with regard to the anti-inflammatory activity of *H. sabdariffa* seeds petroleum ether extract. Recently, Meaiyebu *et al.* (2013) studied the anti-inflammatory effect of *H. sabdariffa* methanolic extract on carrageenan induced paw oedema in rats. They found that the extract possesses ant-inflammatory activity at high dose (500 mglkg) similar to the action of diclofenac sodium at a dose of 20 mg/kg.

In contrast, the ethanolic extract of *H.sabdariffa* seeds showed insignificant activity in rats' paw oedema at a dose of 100, 200 and 400 mg/kg especially at

the 3rd and 4th hours. These results are supported by the work of Dafallah and Al-Mustafa, (1996), who refuted the anti-inflammatory activity of *H.sbdariffa* aqueous extract, when they adopted the same experimental model.

On the other hand, the petroleum ether extract of *B. aegyptiaca* seeds at a dose of 2, 4 and 8ml/kg, remarkably, inhibited the first phase of inflammation as well as the second phase 1st to 4th hours post carrageenan injection. The inhibition of oedema was comparable to that observed by the standard anti-inflammatory drug, indomethacin. In an independent research work Speroni *et al.* (2005) and Gaur *et al.* (2008) investigated another parts of *B. aegyptiaca* as anti-inflammatory in carrageenan induced paw oedema in rats and their research were in accordance with the present findings.

In this study, the ethanolic extract of *B. aegyptiaca* seeds showed insignificant reduction in carrageenan induced rats' paw oedema at a dose of 200 and 400 mg/kg, while the small dose (10 mg/kg) of the reference drug, diclofenac sodium suppressed significantly the odema indicated the active component(s) to be retained by the non-polar fraction of the extract rather than the polar constituents. These results disagree with the findings of the previous workers (Speroni *et al.*, 2005 and Gaur *et al.*, 2008).

Diclofenac sodium, a standard NSAID, could inhibit transudation by blocking the effects of serotonin, bradykinin, histamine and prostaglandins on vascular membrane and by inhibiting the release of these mediators (Khumpook *et al.*, 2013). It is known that carrageenan-induced paw oedema in rats is sensitive to cyclooxygense inhibitors and anti-inflammatory agents, which are known to inhibit the cyclooxygenase thereby, decrease prostaglandin synthesis (Shaikh., 2011). In this study, the results suggest that the inhibitory effect of petroleum ether extracts of *H.sbdariffa* and *B. aegyptiaca* seeds on carrageenan induced paw oedema may be due to inhibition of the enzyme cyclooxygenase leading to inhibition of prostaglandin synthesis.

Moreover, vascular permeability method was also adopted to confirm the activity of *H.sabdariffa* seeds petroleum ether extract. The vascular permeability test is one of the acute inflammatory models. Acetic acid induced vascular permeability method is widely used to determine the inflammatory response of vascular tissue origin (Shaikh, 2011). In this study, intraperitoneal injection of 0.6% acetic acid caused increased leakage of fluids, including Evans blue across the blood vessel epithelial walls as results of dilatation of blood vessels and increased vascular permeability following stimulation of mast cells which are on line with the finding of the previous studies (Shaikh, 2011 and Anosike *et al.*, 2012).

The present study demonstrated that oral treatment with petroleum ether extract of *H. sabdariffa* seeds although less potent than the standard drug, diclofenac sodium but significantly reduced the intensity of the peritoneal inflammation produced by injection of acetic acid compared with the control, suggesting its ability to inhibit the vascular permeability of small blood vessels during acute inflammation.

 Furthermore, the petroleum ether extracts of both plants were also investigated for the treatment of chronic inflammation using cotton pellet induced granuloma. The cotton pellet granuloma method is widely used to evaluate the transudation and proliferation components of the chronic inflammation (Paschapur *et al.*, 2009 and Patel and Patil, 2012). In the present study subcutaneous implantation of cotton pellet in the control group produced a remarkable formation of granular tissue which was indicated by the noticeable increase of the dried cotton pellets' weight after removal from the incision area. This is in agreement with the results of Chouhan *et al.* (2011) who reported similar findings.

The present study revealed that oral administration of *H. sabdarriffa* and *B. aegyptiaca* seeds petroleum ether extracts for 6 days at a dose of 4 and 8 ml/kg hindered considerably the formation of granular tissue compared with the

control group. In this respect the inhibitory effect of *B. aegyptiaca* seeds petroleum ether extract was shown to be superior to that observed by *H. sabdarriffa* seeds petroleum ether extract. The inhibition of granuloma by *B. aegyptiaca* seeds petroleum ether extract at a dose of 4 and 8 ml/kg was considerably high but inferior to the inhibition observed by diclofenac sodium used as a standard anti-inflammatory drug. This reflects its efficacy to reduce an increase in the amount of fibroblasts and synthesis of collagen with mucopolysaccharide, which are natural proliferative events of granulation tissue formation.

The suppression of granuloma in animals treated with both plants seeds petroleum ether extracts at a dose of 4ml/kg was found to be comparable to that observed in rats treated with 8ml/kg. Diclofenac sodium a standard drug reduces granuloma size by inhibiting the leukocyte infiltration and collagen fiber formation. Suppression of cytokines, such as IL-1 and TNF, as well as growth factors influence fibroblast proliferation and mucopolysaccharides production were suggested as the mechanisms for granuloma inhibition (Khumpook *et al.*, 2013)

The inhibition of granuloma found in the current work may be attributed to the suppression of the proliferation phase of the inflammation of the microphages, neuutrophils, fibroblasts and collagen formation which are basic source for the granuloma formation; therefore, the decrease in granuloma formation indicates the suppression of the proliferation phase as reported by Shaikh, (2011).

The potential mechanism involving anti-granulomatous effect of the extract may contribute to the reduction of some or all key mediating cytokines leading to the inhibition of inflammatory cell recruitment, especially macrophage and mast cells, the key initiators for granulation (Khumpook *et al.*, 2013).

The analgesic effect of *H. sabdarriffa* and *B. aegyptiaca* seeds petroleum ether extracts was also evaluated using acetic acid induced writhing test. The petroleum ether extract of *H. sabdarriffa* demonstrated significant inhibition in

writhes in a dose dependant manner especially at medium and high doses (4 & 8 ml/kg) compared with the control group and the standard analgesic drug diclofenac sodium. The inhibition of writhes by a high dose of (8ml/kg) of *H. sabdarriffa* seeds petroleum ether extract was found to be near to that observed by diclofenac sodium. Ali *et al.* (2011) showed similar results in the study of analgesic activity of the ethanolic calyx extract of *H. sabdarriffa*.

B. aegyptiaca seeds petroleum ether extract at a dose of 4 and 8ml/kg exhibited significant inhibition in abdominal writhes in a dose related manner compared with the standard analgesic drug, diclofenac sodium. The inhibition of writhes by *B. aegyptiaca* seeds petroleum ether extract at a dose of 8ml/kg was comparable to that shown by diclofenac sodium. Low dose (2ml/kg) of *B. aegyptiaca* seeds petroleum ether extract failed to inhibit abdominal writhes compared with the control group. Gaur *et al.* (2008) reported that the ethanol and petroleum ether extracts of aerial parts of *B. aegyptiaca* have significant analgesic activity by using Eddy's hot plate method and tail-flick method in albino rats compared with diclofenac sodium used as a standard analgesic drug. The results showed that the ethanolic extract of *B. aegyptiaca* exhibited more significant activity than petroleum ether as analgesic. Moreover, two saponin fractions isolated from *B. aegyptiaca* showed significant antinociceptive activity in acetic acid-induced writhing test in mice and antioxidant action by using *in vitro* method (Speroni *et al.*, 2005).

Acetic acid is known to induce indirect release of prostaglandin as well as lipooxygenase products into the peritoneum which stimulate the nociceptive neurons sensitive to the non steroidal anti-inflammatory drugs. Therefore, the result of this study strongly suggests that the mechanism of this action may be partly due to inhibition of lipooxygenase and/or cyclooxygenase in the peripheral tissues, thereby reducing prostaglandins synthesis and interfering with the mechanism of transduction in primary afferent nociceptors (Prabhu *et al.*, 2011).

Gas chromatography analysis was achieved to detect the fatty acids composition of *H. sabdariffa* and *B. aegyptiaca* seeds oil. *H. sabdariffa* seeds oil revealed the presence of linolelaidic acid (26.02%), arachidic acid (20.59%) and palmitic acid (16.05%). There are different fatty acids present in *H. sabdariffa* seeds namely palmitic, oleic, linoleic, stearic acids (Ismail *et al.*, 2008). This is in agreement with the current study in the presence of palmitic acid and disagrees with it in the presence of linolelaidic and arachidic acids. It is reported that the seed oil of *H. sabdariffa* is rich in unsaturated fatty acids (70%), of which linoleic acid constituted 44% (Mahadevan *et al.*, 2009).

In the present study, Gas chromatography analysis of *B. aegyptiaca* seeds oil revealed the presence of oleic acid (22.61%) in high levels, palmitic acid (4.90%) and capric acid (1.49%). Chothani and Vaghasiya, (2011) reviewed the phytochemical constituents of *B. aegyptiaca*, they found that the seeds oil contains palmitic, stearic, oleic, and linoleic acids as the main fatty acids, this is in agreement with the present results in the presence of palmitic and oleic acid. However, the current results revealed the presence of capric acid which was not found in the previous research. The difference in fatty acids composition of *B. aegyptiaca* seeds oil may be due to the variation in chemical constituents of seeds at different locations (Elfeel, 2010).

Based on Gas Chromatography analysis, the fatty acids of *H. sabdariffa* and *B. aegyptiaca* petroleum ether extracts could be responsible for their anti-inflammatory activities (Chouhan *et al.*, 2011).

Moreover, the anti-inflammatory effect of the seeds oil extract may be attributed to its antioxidant property (Sokeng *et al.*, 2013). Phytochemical analyses of the *H. Sabdarriffa* seeds showed the presence of flavonoids, steroids and phenolics which are well known for their anti-inflammatory activities (Mungole and Chaturvedi, 2011).

Others have speculated that the anti-inflammatory activity of seeds extract could be strongly due to the presence of steroids which are well known to have strong

anti-inflammatory and analgesic activities (Mungole and Chaturvedi, 2011). Steroids have been isolated from seed oil of *H. sabdarriffa* (Mahadevan *et al.*, 2009). Steroidal saponins were also detected in *B. aegyptiaca* seeds which yield diosgenin, a source of steroidal drugs, such as corticosteriods (Elfeel, 2010).

On the other hand, it has been reported that *H. sabdarriffa* and *B. aegyptiaca* have antioxidant properties (Meda *et al.*, 2010 and Al-Hashimi, 2012). It is well-known that the free radicals are involved in the inflammatory process. Thus the antioxidant property of the plants may have a beneficial role in their anti-inflammatory activity (Sokeng *et al.*, 2013). Antioxidants are documented in several publications to mitigate the inflammatory processes and some of the antioxidant activity of plants have been ascribed to the phenolic compounds present in the plant particularly flavonoids (Mensah *et al.*, 2011).

A number of publications (Chouhan *et al.*, 2011; Musa *et al.*, 2011 and Madhavi *et al.*, 2012) addressed the anti-inflammatory and analgesic effects of seeds oil. They found that they have promising activities as anti-inflammatory and analgesic agents.

Furthermore, the safety of seeds oil of *H. sabdarriffa* and *B. aegyptiaca* seeds petroleum ether extract was confirmed by acute toxicity study, in accordance to Organization for Economic Co-operation and Development Guidelines (OECD, 2001). No clinical signs or mortality were observed in the animals received petroleum ether extract up to 20 ml/kg for 24 hours, except the symptom of anorexia in rats received higher doses of the oil. This may occur because the stomach is filled with plenty of oil, resulting in anorexia and a decrease in food consumption. Anorexia was also observed in rats given a high dose of oil (8ml/kg) in sub-chronic toxicity.

Moreover, sub-chronic toxicity of *B. aegyptiaca* seeds petroleum ether extract was performed in rats for 21 days at a dose of 4 and 8ml/kg. Haematological studies showed no evidence of significant changes in either control or rats given 4ml/kg petroleum ether extract of *B. aegyptiaca* in the values of WBCs and

RBCs counts, Hb, PCV, MCV, MCH and MCHC during the experimental period. In rats treated with high dose of *B. aegyptiaca* (8ml/kg), results indicated significant decreased in the mean values of RBC, PCV, Hb and increased in MCHC value in day 21, when compared with the control rats and animals treated with *B. aegyptiaca* at a dose of 4ml/kg, these results may strongly be attributed to the reduction of food uptake in this group as a result of administration of a high dose of oil, which led to a decrease in RBC, PCV and Hb values to the minimum normal range and increased MCHC. Despite these findings these values were within the normal ranges.

The present study showed insignificant changes in the activities of serum ALT, AST, ALP and total proteins which proved the safety of the oil in rat livers. These findings are similar to that observed by Ajayi and Folorunso, (2013). The elevation of ALT, AST and ALP levels is taken as markers for hepatocellular injury; these enzymes will be released into the circulation as a result of destruction of hepatocytes (Wolf, 1999). These findings were also confirmed by microscopic examination of liver sections of treated animals which indicated no evidence of any inflammatory, degenerative or necrotic changes in the treated animals.

Urea and creatinine are metabolic waste products that are freely filtered by the glomeruli of the kidneys. The ability of the kidney to excrete creatinine and urea nitrogen decreases with damage, resulting in increased serum creatinine and urea (Bhuvaneswari and Krishnakumari, 2012). In this study, the levels of serum urea and creatinine were not affected significantly; this is in agreement with the results of Obidah *et al.* (2009) and Ajayi and Folorunso, (2013). These levels were maintained within the normal ranges in the treated animals compared with the control group. Serum creatinine and urea levels increase only when significant renal injury has occurred. The insignificant change in serum urea and creatinine is suggestive of normal renal function. Histopathological investigation of kidney sections provided additional support of seeds oil safety. However,

focal degenerative changes were observed in few areas in some kidney sections in rats given the high dose (8ml/kg) of seeds oil without modification in kidney functional mechanism. Additionally, histopathological findings of lung, stomach, intestine, spleen and heart showed normal appearance in rats treated with *B. aegyptiaca* seeds petroleum ether extract at a dose of 4 and 8 ml/kg.

Conclusion

1. It is concluded that petroleum ether extracts of *H. sabdarriffa* and *B. aegyptiaca* seeds have significant anti-inflammatory and analgesic activities in rats. These actions could be due to the inhibition of cyclooxygenase and prostaglandin syntheses.
2. The phytoconstituents, high fatty acid contents and antioxidant properties of the seeds oil extracts of *H. sabdarriffa* and *B. aegyptiaca* may have a valuable role in their anti-inflammatory and analgesic activities.
3. The ethanolic extracts of both plants seeds have no anti-inflammatory activity.
4. The results indicate that the active component(s) of both plant extracts to be attributed to the non-polar fraction of the extract rather than the polar constituents.
5. In acute oral toxicity, the results indicated that the petroleum ether extracts of *H. sabdarriffa* and *B. aegyptiaca* seeds were safe up to 20ml/kg b.wt. in rats.
6. Sub-chronic toxicity of *B. aegyptiaca* seeds petroleum ether extract supported the safety of the extract according to the haematological, biochemical and hitopathological findings.

Recommendations

It is recommended that further studies should be conducted to:

- Isolate, identify and characterize the active ingredient(s) responsible of the anti-inflammatory and analgesic effects of the petroleum ether extract of *H. sabdarriffa* and *B. aegyptiaca* seeds.
- Determine the exact mechanism of actions of the identified ingredients.
- Formulate suitable drug forms in acceptable dosages for both plants extracts and to test them through clinical trials.

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Appendix

Appendix 1

Equipment and materials

   1- Drugs and chemicals

1. Petroleum ether Petroleum ether, E. Merck (India) Limited, Mumbai- 400 018.
2. Chloroform AR Analytical Rasayan.
3. Ethanol.
4. Carrageenan (Vegetable gelatin, Irish moss, chemical company, Mo 63178 U.S.A).
5. NaCl 0.9%
6. Acetic acid.
7. Indomethacin.
8. Diclofenac sodium.
9. Evans blue (KIRAN-LIGHT LABORITERIES, India).
10. Thiopental sodium (NEON LABORATORIES LIMITED, 28. Mahal Ind. Est., M. Caves Red., Andheri (East), Mumbai-400 093).
11. ACCENT-200 diagnostic kits, PZ COMPANY S.A. POLAND.
12. SPINREACT, S.A/S.A.U. Ctra.Sata Coloma, SPAIN, diagnostic kit for total protein.

   2- Apparatus and instruments

1. Soxhlet extractor apparatus, Quick fit, ex5/83. England.
2. Rotary evaporator apparatus, Buchi 011 Switzerland.
3. Sensitive balance HF-300g A&D Company, Limited.
4. Rough balance Sartorius 2351, Germany.
5. Sepectrophotometer, Jenway 6305 UV/VIS., Jenway Ltd, UK.
6. Centrifuge Hettich EBA35.Werk-Nr, Baujahr
7. Mindray, for biochemistry (BS-200 – China).
8. Sysmex – KX-21N, Spain.

9. Digital vernier caliper (P.C.R).

10. Gas chromatography (GC- 2010, SHIMADZU-Japan).

11. Oven.

12. Dissecting set.

13. Capillary tubes heparinized Supe- Rior- Germany

14. Counter ENM- England.

15. Plastic cages.

16. Plastic containers.

17. EDTA tubes.

18. Glass tubes.

19. Appendorf tubes.

20. Petri dishes.

ACCENT 200 II GEN
ACCENT 200

ACCENT-200 ALAT

CE [CORMAY logo]

DIAGNOSTIC KIT
FOR DETERMINATION OF
ALANINE AMINOTRANSFERASE ACTIVITY

INTRODUCTION

Alanine aminotransferase (ALAT, ALT, GPT) is an enzyme participated in amino acids metabolism. ALAT is present in all tissues but the highest level is found in liver and kidney cells. Damage of hepatocytes or kidney cells causes significant release of ALAT into the circulation. Measurement of ALT activity in serum is valuable in the diagnosis of liver diseases: jaundice, mononucleosis or hepatic cirrhosis.

METHOD PRINCIPLE

Optimized, modified method according to International Federation of Clinical Chemistry (IFCC), without pyridoxal phosphate.

$$L\text{-alanine} + 2\text{-oxoglutarate} \xrightarrow{ALAT} \text{pyruvate} + L\text{-glutamate}$$

$$\text{pyruvate} + NADH + H^+ \xrightarrow{LDH} \text{lactate} + NAD^+$$

The rate of absorbance changing at $\lambda=340$ nm is directly proportional to alanine aminotransferase activity.

REAGENTS
Package

1-Reagent	2 x 35 ml
2-Reagent	1 x 17.5 ml

The reagents when stored at 2-8°C are stable up to expiry date printed on the package. Stability on board of the analyser at 2-10°C: ACCENT-200 – 10 weeks, ACCENT-200 II GEN – 10 weeks. Protect from light and avoid contamination!

Concentrations in the test

Tris (pH 7.5)	100 mmol/l
L-alanine	500 mmol/l
LDH	> 36.7 µkat/l
2-oxoglutarate	15 mmol/l
NADH	0.18 mmol/l

Warnings and notes
- Product for in vitro diagnostic use only.
- The reagents contain (< 0.1%) sodium azide as a preservative. Avoid contact with skin and mucous membranes.

SPECIMEN

Serum, heparinized or EDTA plasma free from hemolysis.
Hemolysis should be avoided, since ALAT activity in erythrocytes is 3 to 5 times higher than in normal serum.
Do not freeze the samples. ALAT activity remains stable in specimen up to 3 days at 15-25°C or up to 7 days at 2-8°C.
Nevertheless it is recommended to perform the assay with freshly collected samples!

PROCEDURE

These reagents may be used in automatic analysers ACCENT-200 and ACCENT-200 II GEN.
1-Reagent and 2-Reagent are ready to use.
For reagent blank deionized water is recommended.

APPLICATION for ACCENT-200 and ACCENT-200 II GEN
Parameters

Test Name	ALAT		R1		200	
Test No	15		R2		50	
Full Name	ALAT		Sample Volume		10	
Reference No	15		R1 Blank			
Analy. Type	Kinetic		Mixed Reag. Blank			
Pri. Wave.	340 nm		Concentration		4.5	1000
Secon. Wave.	450 nm		Linearity Limit		0.2	
Trend	Descending		Substrate Limit			
Reac. Time	4	15	Factor			
Incuba. Time	10		☐ Prozone check			
Unit	U/l					
Precision	Integer		q1 ☐ q2 ☐ q3 ☐ q4 ☐			
			PC ☐		Abs ☐	

Calibration Rule

Rule	One-point Linear	
Sensitivity	1	
Replicates	3	
Interval (day)	70	
Difference Limit	0	
SD	0	
Blank Response	0	50000
Error Limit	0	
Coefficient	0	

REFERENCE VALUES [6]

serum / plasma	37°C	
women	up to 31 U/l	up to 0.517 µkat/l
men	up to 41 U/l	up to 0.683 µkat/l

It is recommended for each laboratory to establish its own reference ranges for local population.

QUALITY CONTROL

For internal quality control it is recommended to use the CORMAY SERUM HN (Cat. No 5-172) and CORMAY SERUM HP (Cat. No 5-173) with each batch of samples.
For the calibration of automatic analysers systems the CORMAY MULTICALIBRATOR LEVEL 1 (Cat. No 5-174; 5-176) or LEVEL 2 (Cat. No 5-175; 5-177) is recommended.
The calibration curve should be prepared every 10 weeks (ACCENT-200, ACCENT-200 II GEN), with change of reagent lot number or as required e.g. quality control findings outside the specified range.

PERFORMANCE CHARACTERISTICS

These metrological characteristics have been obtained using automatic analyser ACCENT-200. Results may vary if a different instrument or a manual procedure is used.

- **Sensitivity:** 4.5 U/l (0.075 µkat/l).

- **Linearity:** up to 1000 U/l (16.7 µkat/l).

- **Specificity / Interferences**
  Haemoglobin up to 0.16 g/dl, ascorbate up to 62 mg/l, bilirubin up to 20 mg/dl and triglycerides up to 1000 mg/dl do not interfere with the test.

▪ Precision

Repeatability (run to run) n = 20	Mean [U/l]	SD [U/l]	CV [%]
level 1	29.35	1.42	4.85
level 2	99.75	1.61	1.61

Reproducibility (day to day) n = 80	Mean [U/l]	SD [U/l]	CV [%]
level 1	107.86	1.65	1.53
level 2	208.90	11.68	5.59

▪ Method comparison

A comparison between ALAT values determined at ACCENT-200 (y) and at COBAS INTEGRA 400 (x) using 100 samples gave following results:

$y = 0.9392 x + 2.8336$ U/l;

$R = 0.9966$

(R – correlation coefficient)

WASTE MANAGEMENT
Please refer to local legal requirements.

LITERATURE

1. Wallhofer H., Schmidt E., Schmidt U.F. W.: Synopsis Der Leberkrankheiten. G. Thieme Verlag, Stuttgart (1974).
2. Thefeld W., et al: Dtsch. Med. Wschr. 99, 343 (1974).
3. Bergmeyer H.U., Horder M., Rej R.: J. Clin. Chem. Clin. Biochem. 24, 481-495 (1986).
4. Henry R.J. Cannon D.C. Winkerman J.W.: Clinical Chemistry Principles and Technics, 2nd ed. Hagerstown MD: Harper and Row, 815, 888 (1974).
5. Burtis C.A., Ashwood E.R., ed. Tietz Textbook of Clinical Chemistry, 3rd ed. Philadelphia, PA: Moss D. W., Henderson A. R., 652 (1999).
6. Dembińska-Kieć A., Naskalski J.W.: Diagnostyka laboratoryjna z elementami biochemii klinicznej, Volumed, 776, (1998).

Date of issue: 03. 2012.

ACCENT 200 II GEN
ACCENT 200

CE CORMAY

ACCENT-200 ASAT

DIAGNOSTIC KIT
FOR DETERMINATION OF
ASPARTATE AMINOTRANSFERASE ACTIVITY

INTRODUCTION

Aspartate aminotransferase (ASAT, AST, GOT) is an enzyme participated in amino acids metabolism. ASAT is found in all tissues but particularly high level of ASAT is observed in heart muscle, skeletal muscle, liver and kidney. This is why elevated ASAT serum level is marker of myocardial infarction and kidney, liver or skeletal muscle injury.

METHOD PRINCIPLE

Optimized, modified method according to International Federation of Clinical Chemistry (IFCC), without pyridoxal phosphate.

$$L\text{-aspartate} + 2\text{-oxoglutarate} \xrightarrow{\text{ASAT}} \text{oxalacetate} + L\text{-glutamate}$$

$$\text{oxalacetate} + NADH + H^+ \xrightarrow{\text{MDH}} \text{malate} + NAD^+$$

The rate of absorbance changing at λ=340 nm is directly proportional to aspartate aminotransferase activity.

REAGENTS
Package

1-Reagent	2 x 35 ml
2-Reagent	1 x 17.5 ml

The reagents when stored at 2-8°C are stable up to expiry date printed on the package. Stability on board of the analyser at 2-10°C: ACCENT-200 – 10 weeks, ACCENT-200 II GEN – 10 weeks. Protect from light and avoid contamination!

Concentrations in the test

Tris (pH 7.8)	80 mmol/l
L-aspartate	240 mmol/l
MDH	> 10 µkat/l
LDH	> 20 µkat/l
2-oxoglutarate	15 mmol/l
NADH	0.18 mmol/l
sodium hydroxide	< 1 %

Warnings and notes
- Product for in vitro diagnostic use only.
- The reagents contain (< 0.1%) sodium azide as a preservative. Avoid contact with skin and mucous membranes.
- 1-Reagent is classified as an irritant!

Ingredients: Contains sodium hydroxide.

Xi – Irritating.
R 36/38: Irritating to eyes and skin.
S 26-28-45: In case of contact with eyes, rinse immediately with plenty of water and see medical advice. After contact with skin, wash immediately with plenty of water. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

SPECIMEN

Serum, heparinized or EDTA plasma free from hemolysis.
Do not use heparine ammonium salt.
Hemolysis should be avoided, since ASAT activity in erythrocytes is 10 times higher than in normal serum.
Do not freeze the samples. ASAT activity remains stable in specimen up to 1 day at 15-25°C or up to 4 days at 2-8°C.

ACCENT 200 ASAT

Nevertheless it is recommended to perform the assay with freshly collected samples!

PROCEDURE

These reagents may be used in automatic analysers ACCENT-200 and ACCENT-200 II GEN.
1-Reagent and 2-Reagent are ready to use.
For reagent blank deionized water is recommended.

APPLICATION for ACCENT-200 and ACCENT-200 II GEN
Parameters

Test Name	ASAT	R1	200	
Test No	16	R2	50	
Full Name	ASAT	Sample Volume	10	
Reference No	16	R1 Blank		
Analy. Type	Kinetic	Mixed Reag. Blank		
Pri. Wave.	340 nm	Concentration	5	1000
Secon. Wave.	450 nm	Linearity Limit	0.2	
Trend	Descending	Substrate Limit		
Reac. Time	4 | 15	Factor		
Incuba. Time	10	☐ Prozone check		
Unit	U/l			
Precision	Integer	q1 ☐ q2 ☐ q3 ☐ q4 ☐		
		PC ☐ Abs ☐		

Calibration Rule

Rule	One-point Linear	
Sensitivity	1	
Replicates	3	
Interval (day)	70	
Difference Limit	0	
SD	0	
Blank Response	0	50000
Error Limit	0	
Coefficient	0	

REFERENCE VALUES [6]

serum / plasma	37°C	
female	up to 31 U/l	up to 0.518 µkat/l
male	up to 37 U/l	up to 0.618 µkat/l

It is recommended for each laboratory to establish its own reference ranges for local population.

QUALITY CONTROL

For internal quality control it is recommended to use the CORMAY SERUM HN (Cat. No 5-172) and CORMAY SERUM HP (Cat. No 5-173) with each batch of samples.
For the calibration of automatic analysers systems the CORMAY MULTICALIBRATOR LEVEL 1 (Cat. No 5-174; 5-176) or LEVEL 2 (Cat. No 5-175; 5-177) is recommended.
The calibration curve should be prepared every 10 weeks (ACCENT-200, ACCENT-200 II GEN), with change of reagent lot number or as required e.g. quality control findings outside the specified range.

PERFORMANCE CHARACTERISTICS

These metrological characteristics have been obtained using automatic analyser ACCENT-200. Results may vary if a different instrument or a manual procedure is used.

- **Sensitivity:** 5 U/l (0.084 µkat/l).

- **Linearity:** up to 1000 U/l (16.7 µkat/l).

■ **Specificity / Interferences**

Haemoglobin up to 0.16 g/dl, ascorbate up to 62 mg/l, bilirubin up to 20 mg/dl and triglycerides up to 1000 mg/dl do not interfere with the test.

■ **Precision**

Repeatability (run to run) n = 20	Mean [U/l]	SD [U/l]	CV [%]
level 1	40.40	3.48	8.62
level 2	188.00	5.50	2.93

Reproducibility (day to day) n = 80	Mean [U/l]	SD [U/l]	CV [%]
level 1	119.96	2.66	2.22
level 2	278.59	4.50	1.62

■ **Method comparison**

A comparison between ASAT values determined at ACCENT-200 (y) and at COBAS INTEGRA 400 (x) using 100 samples gave following results:

$y = 1.0601 x - 1.1263$ U/l;

$R = 0.9979$ \qquad\qquad (R – correlation coefficient)

WASTE MANAGEMENT

Please refer to local legal requirements.

LITERATURE

1. Clin. Chem. Acta 105, 147-172 (1980).
2. Wallhofer H., Schmidt E., Schmidt U.F.W.: Synopsis Der Leberkrankheiten. G. Thieme Verlag, Stuttgart (1974).
3. Thefeld W. et al: Dtsch. Med. Wschr. 99, 343 (1974).
4. Bergmeyer H.U., Horder M., Rej R.: J. Clin. Chem. Clin. Biochem. 24, 497 (1986).
5. Tietz N.W., ed. Clinical Guide to Laboratory Tests, 3rd ed. Philadelphia, PA: W.B. Saunders, 76 (1995).
6. Dembińska-Kieć A., Naskalski J.W.: Diagnostyka laboratoryjna z elementami biochemii klinicznej, Volumed, 777, (1998).

Date of issue: 05. 2012.

Cat.No 7-212 *II GEN*

ACCENT 200
ACCENT 200

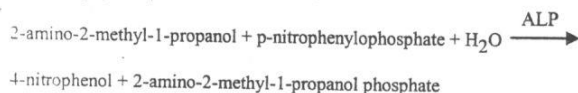CE CORMAY

ACCENT-200 ALP

DIAGNOSTIC KIT
FOR DETERMINATION OF
ALKALINE PHOSPHATASE
ACTIVITY

INTRODUCTION
Alkaline phosphatase (ALP) is actually a group of isoenzymes that hydrolyse monophosphate esters in alkaline medium. Optimum pH for these ALP isoforms activities is about 9-10. Alkaline phosphatase level is the highest in liver, bone, intestine, kidney and placenta. Measurement of ALP isoenzymes is useful in diagnosis of these organs diseases.

METHOD PRINCIPLE
Kinetic method recommended by International Federation of Clinical Chemistry (IFCC).

$$\text{2-amino-2-methyl-1-propanol} + \text{p-nitrophenylophosphate} + H_2O \xrightarrow{ALP}$$

4-nitrophenol + 2-amino-2-methyl-1-propanol phosphate

The rate of 4-nitrophenol formation is directly proportional to the ALP activity.

REAGENTS
Package

1-Reagent	1 x 30 ml
2-Reagent	1 x 8 ml

The reagents when stored at 2-8°C are stable up to expiry date printed on the package. Stability on board of the analyser at 2-10°C: ACCENT-200 – 6 weeks, ACCENT-200 II GEN – 6 weeks. Protect from light and avoid contamination!

Concentrations in the test

2-amino-2-methyl-1-propanol (AMP)	350 mmol/l
Mg^{2+}	2.0 mmol/l
Zn^{2+}	1.0 mmol/l
HEDTA	2.0 mmol/l
p-nitrophenylphosphate	16.0 mmol/l

Warnings and notes
- Product for in vitro diagnostic use only.
- The reagents contain sodium azide (< 0.1%) as a preservative. Avoid contact with skin and mucous membranes.
- During the reaction p-nitrophenol is produced. Do not swallow or inhale, avoid contact with skin.

SPECIMEN
Serum, heparinized plasma free from hemolysis.
Do not use EDTA, citrate and oxalate as anticoagulants because of ALP activity inhibition!
ALP activity remains stable in specimen up to 4 hours at 15-25°C. Freezing of sample causes a loss of enzyme activity. Frozen specimens should be thawed and kept at room temperature for 18 to 24 hours before measurement to achieve full enzyme reactivation. Nevertheless it is recommended to perform the assay with freshly collected samples!

PROCEDURE
These reagents may be used in automatic analysers ACCENT-200 and ACCENT-200 II GEN.
1-Reagent and 2-Reagent are ready to use.
For reagent blank deionized water is recommended.

APPLICATION for ACCENT-200 and ACCENT-200 II GEN
Parameters

Test Name	ALP	R1	200	
Test No	18	R2	50	
Full Name	Alkaline phosphatase	Sample Volume	5	
Reference No	18	R1 Blank		
Analy. Type	Kinetic	Mixed Reag. Blank		
Pri. Wave.	405 nm	Concentration	28	700
Secon. Wave.	670 nm	Linearity Limit	0.2	
Trend	Ascending	Substrate Limit		
Reac. Time	2 13	Factor		
Incuba. Time	10	Prozone check		
Unit	U/l			
Precision	Integer	q1 ☐ q2 ☐ q3 ☐ q4 ☐		
		PC ☐ Abs ☐		

Calibration Rule

Rule	One Point Linear	
Sensitivity	1	
Replicates	3	
Interval (day)	10	
Difference Limit	0	
SD	0	
Blank Response	0	50000
Error Limit	0	
Coefficient	0	

REFERENCE VALUES [9, 10]

gender	age	U/l (37°C)	µkat/l (37°C)
female	1 – 30 days	48 – 406	0.80 – 6.77
	31 days – 1 year	124 – 341	2.07 – 5.68
	1 year – 3 years	108 – 317	1.80 – 5.28
	4 – 15 years	54 – 369	0.91 – 6.23
	20 – 50 years	42 – 98	0.71 – 1.67
	≥ 60 years	53 – 141	0.90 – 2.40
male	1 – 30 days	75 – 316	1.25 – 5.27
	31 days – 1 year	82 – 383	1.37 – 6.38
	1 year – 3 years	104 – 345	1.73 – 5.75
	4 – 15 years	54 – 369	0.91 – 6.23
	20 – 50 years	53 – 128	0.90 – 2.18
	≥ 60 years	56 – 119	0.95 – 2.02

It is recommended for each laboratory to establish its own reference ranges for local population.

QUALITY CONTROL
For internal quality control it is recommended to use the CORMAY SERUM HN (Cat. No 5-172) and CORMAY SERUM HP (Cat. No 5-173) with each batch of samples.
For the calibration of automatic analysers systems the CORMAY MULTICALIBRATOR LEVEL 1 (Cat. No 5-174; 5-176) or LEVEL 2 (Cat. No 5-175; 5-177) is recommended.
The calibration curve should be prepared every 10 days (ACCENT-200, ACCENT-200 II GEN), with change of reagent lot number or as required e.g. quality control findings outside the specified range.

Total protein
Biuret. Colorimetric

Quantitative determination of total protein
IVD

Store at 2-8ºC

PRINCIPLE OF THE METHOD
Proteins give an intensive violet-blue complex with copper salts in an alkaline medium. Iodide is included as an antioxidant.
The intensity of the color formed is proportional to the total protein concentration in the sample[1,4].

CLINICAL SIGNIFICANCE
The proteins are macromolecular organic compounds, widely distributed in the organism. They act like structural and transport elements. The proteins of the serum are divide in two fractions, albumin and globulins
The determination of total proteins is useful in the detection of:
- High protein levels caused by hemoconcentration like in the dehydrations or increase in the concentration of specific proteins.
- Low protein level caused by hemodilution by an impared synthesis or loss (as by hemorrhage) or excessive protein catabolism[4,5].
Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

REAGENTS

R Biuret	Sodium potassium tartrate	15 mmol/L
	Sodium iodide	100 mmol/L
	Potassium iodide	5 mmol/L
	Copper (II) sulphate	19 mmol/L
T PROTEIN CAL	Bovine albumin primary standard 7 g/dL	

PRECAUTIONS
Corrosive (C):R35:Causes severe burns.
Copper (II) sulphate: Environmentally dangerous (N): R50/53: Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
S22: Do not breathe dust. S60: This material and its container must be disposed of as hazardous waste. S61: Avoid release to the environment. Refer to special instructions/safety data sheets.

PREPARATION
The reagents are ready to use.

STORAGE AND STABILITY
All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8ºC protected from light and contaminations prevented during their use.
Do not use reagents over the expiration date.
Signs of reagent deterioration:
- Presence of particles and turbidity.
- Blank absorbance (A) at 540 nm $\geq$ 0.22.

ADDITIONAL EQUIPMENT
- Spectrophotometer or colorimeter measuring at 540 nm.
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment.

SAMPLES
Serum or heparinized plasma[1]:
Stability of the sample: 1 month at refrigerator (2-8ºC).

PROCEDURE
1. Assay conditions:
   Wavelength: . . . . 540 (530-550) nm
   Cuvette: . . . . 1 cm. light path
   Temperature . . . . .37ºC / 15-25ºC
2. Adjust the instrument to zero with distilled water.
3. Pipette into a cuvette:

	Blank	Standard	Sample
R (mL)	1.0	1.0	1.0
Standard[Note 1-2] (μL)	–	25	–
Sample (μL)	–	–	25

4. Mix and incubate 5 min at 37ºC or 10 min at room temperature.
5. Read the absorbance (A) of the samples and Standard, against the Blank. The colour is stable for at least 30 minutes.

CALCULATIONS
$$\frac{(A)Sample}{(A)Standard} \times 7 \ (Standard\ conc.) = g/dL\ of\ total\ protein\ in\ the\ sample$$

QUALITY CONTROL
Control sera are recommended to monitor the performance of assay procedures: SPINTROL H Normal and Pathologic (Ref. 1002120 and 1002210).
If control values are found outside the defined range, check the instrument, reagents and calibrator for problems.
Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

REFERENCE VALUES[1]
Adults: 6.6 – 8.3 g/dL
Newborn: 5.2 – 9.1 g/dL
These values are for orientation purpose; each laboratory should establish its own reference range.

PERFORMANCE CHARACTERISTICS
Measuring range: From detection limit of 0,20 g/dL to linearity limit of 15 g/dL.
If the results obtained were greater than linearity limit, dilute the sample 1/2 with NaCl 9 g/L and multiply the result by 2.
Precision:

	Intra-assay (n=20)		Inter-assay (n=20)	
Mean (g/dL)	5.07	9.64	5.15	9.74
SD	0.04	0.08	0.06	0.14
CV (%)	0.88	0.90	1.23	1.43

Sensitivity: 1 g/dL = 0,07 A.
Accuracy: Results obtained using SPINREACT reagents (y) did not show systematic differences when compared with other commercial reagents (x).
The results obtained using 50 samples were the following:
Correlation coefficient (r): 0.9918.
Regression equation: y= 1.0164x – 0.1264.
The results of the performance characteristics depend on the analyzer used.

INTERFERENCES
Hemoglobin and lipemia[1,4].
A list of drugs and other interfering substances with total protein determination has been reported by Young et. al[2,3].

NOTES
1. T PROTEIN CAL: Proceed carefully with this product because due its nature it can get contamined easily.
2. Calibration with the aqueous standard may cause a systematic error in automatic procedures. In these cases, it is recommended to use a serum Calibrator.
3. Use clean disposable pipette tips for its dispensation.
4. **SPINREACT has instruction sheets for several automatic analyzers. Instructions for many of them are available on request.**

BIBLIOGRAPHY
1. Koller A. Total serum protein. Kaplan A et al. Clin Chem The C.V. Mosby Co. St Louis. Toronto. Princeton 1984; 1316-1324 and 418.
2. Young DS. Effects of drugs on Clinical Lab. Tests, 4th ed AACC Press, 1995.
3. Young DS. Effects of disease on Clinical Lab. Tests, 4th ed AACC 2001.
4. Burtis A et al. Tietz Textbook of Clinical Chemistry, 3rd ed AACC 1999.
5. Tietz N W et al. Clinical Guide to Laboratory Tests, 3rd ed AACC 1995.

PACKAGING

Ref: 1001290	Cont.	R:2 x 50 mL, CAL: 1 x 2 mL
Ref: 1001291		R:2 x 250 mL, CAL: 1 x 5 mL
Ref: 1001292		R:1 x 1000 mL,CAL: 1 x 5 mL

Cat. No 7-206

ACCENT 200 *II GEN*
ACCENT 200

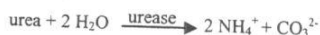CE [CORMAY]

ACCENT-200 UREA

DIAGNOSTIC KIT
FOR DETERMINATION OF
UREA CONCENTRATION

INTRODUCTION

Urea is a product of amino acids catabolism. It is produced in liver and excreted in urine. Urea in the blood is reported as the blood urea nitrogen (BUN). Increased urea concentration in the serum, called uremia, is observed due to dehydration, renal failure, high-protein diet, increased protein catabolism caused by tissue injury or massive bleeding into the alimentary tract. The reason of reduced urea level could be overhydration, low-protein diet or starvation and severe liver disease.

METHOD PRINCIPLE

Kinetic, enzymatic method with urease and glutamate dehydrogenase.

$$urea + 2 H_2O \xrightarrow{urease} 2 NH_4^+ + CO_3^{2-}$$

$$NH_4^+ + 2\text{-oxoglutarate} + NADH \xrightarrow{GLDH} L\text{-glutamate} + NAD^+ + H_2O$$

The rate of absorbance changing at $\lambda=340$ nm is proportional to the urea concentration.

REAGENTS
Package

1-Reagent	2 x 30 ml
2-Reagent	1 x 15 ml

The reagents when stored at 2-8°C are stable up to expiry date printed on the package. Stability on board of the analyser at 2-10°C: ACCENT-200 – 10 weeks, ACCENT-200 II GEN – 10 weeks. Protect from light and avoid contamination!

Concentrations in the test

Tris (pH 7.8)	96 mmol/l
ADP	0.6 mmol/l
urease	266.7 µkat/l
GLDH	16 µkat/l
NADH	0.25 mmol/l
2-oxoglutarate	9 mmol/l

Warnings and notes

- Product for in vitro diagnostic use only.
- The reagents contain sodium azide (< 0.1%) as a preservative. Avoid contact with skin and mucous membranes.

SPECIMEN

Serum, EDTA or heparinized plasma free from hemolysis, 24-hours urine.
Do not use heparine ammonium salt and fluoride as anticoagulants.
Urine preparation: before analysis urine sample should be diluted 100-fold with 0.9% NaCl, and the results multiplied by 100. Mix well probes before measurement. 24-hours urine samples should be kept at 2-8°C preserved by maintenance of pH less than 4.
Specimen can be stored up to 7 days at 2-8°C.
Nevertheless it is recommended to perform the assay with freshly collected samples!

PROCEDURE

These reagents may be used in automatic analysers ACCENT-200 and ACCENT-200 II GEN.
1-Reagent and 2-Reagent are ready to use.
For reagent blank deionized water is recommended.

APPLICATION for ACCENT-200 and ACCENT-200 II GEN
Parameters

Test Name	UREA		R1		200	
Test No	10		R2		50	
Full Name	Urea		Sample Volume		3	
Reference No	10		R1 Blank			
Analy. Type	Kinetic		Mixed Reag. Blank			
Pri. Wave.	340 nm		Concentration		3	260
Secon. Wave.	450 nm		Linearity Limit		0.2	
Trend	Descending		Substrate Limit			
Reac. Time	3	10	Factor			
Incuba. Time	10		☐ Prozone check			
Unit	mg/dl					
Precision	Integer		q1 ☐ q2 ☐ q3 ☐ q4 ☐			
			PC ☐ Abs ☐			

Calibration Rule

Rule	One-point Linear	
Sensitivity	1	
Replicates	3	
Interval (day)	70	
Difference Limit	0	
SD	0	
Blank Response	0	50000
Error Limit	0	
Coefficient	0	

REFERENCE VALUES [8]

serum / plasma	mg/dl	mmol/l
	< 50	< 8.3
24-hours urine	g/24h	mmol/24h
	20 – 35	300 – 550

1 mg of urea corresponds to 0.467 mg of urea nitrogen.

It is recommended for each laboratory to establish its own reference ranges for local population.

QUALITY CONTROL

For internal quality control it is recommended to use the CORMAY SERUM HN (Cat. No 5-172) and CORMAY SERUM HP (Cat. No 5-173) for determination in serum or CORMAY URINE CONTROL LEVEL 1 (Cat. No 5-161) or LEVEL 2 (Cat. No 5-162) for determination in urine with each batch of samples.
For the calibration of automatic analysers systems the CORMAY MULTICALIBRATOR LEVEL 1 (Cat. No 5-174; 5-176) or LEVEL 2 (Cat. No 5-175; 5-177) is recommended.
The calibration curve should be prepared every 10 weeks (ACCENT-200, ACCENT-200 II GEN), with change of reagent lot number or as required e.g. quality control findings outside the specified range.

PERFORMANCE CHARACTERISTICS

These metrological characteristics have been obtained using automatic analyser ACCENT-200. Results may vary if a different instrument or a manual procedure is used.

- **Sensitivity:** 3 mg/dl (0.5 mmol/l).

- **Linearity:** up to 260 mg/dl (43.29 mmol/l).

- **Specificity / Interferences**
  Haemoglobin up to 5 g/dl, ascorbate up to 62 mg/l, bilirubin up to 20 mg/dl and triglycerides up to 1000 mg/dl do not interfere with the test.

- **Precision**

Repeatability (run to run) n = 20	Mean [mg/dl]	SD [mg/dl]	CV [%]
level 1	34.62	0.31	0.90
level 2	98.05	1.16	1.18

Reproducibility (day to day) n = 80	Mean [mg/dl]	SD [mg/dl]	CV [%]
level 1	33.96	0.71	2.08
level 2	96.69	2.58	2.67

- **Method comparison**
  A comparison between urea values determined at ACCENT-200 (y) and at COBAS INTEGRA 400 (x) using 51 samples gave following results:
  $y = 0.9113 x + 1.6867$ mg/dl;
  $R = 0.9985$ (R – correlation coefficient)

WASTE MANAGEMENT
Please refer to local legal requirements.

LITERATURE
1. Kassirer J.P.: New Eng. J. Med. 285, 385 (1971).
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II GEN

ACCENT 200
ACCENT 200

CE | CORMAY

ACCENT-200 CREATININE

DIAGNOSTIC KIT
FOR DETERMINATION OF
CREATININE CONCENTRATION

INTRODUCTION

Creatinine is a product of creatine nonenzymatic dehydration in skeletal muscle. The amount of creatinine generated and excreted by kidney is proportional to muscle mass and usually is higher in men than women. Daily creatinine generation remains fairly constant, with the exception of crushing injury or degenerative diseases that cause massive damage to muscle. Creatinine blood and urine level depends on glomelural filtration so creatinine clearance is excellent index of renal function.

METHOD PRINCIPLE

Modificated Jaffe's method, without deproteinization. In alkaline solution picrate reacts with creatinine to form a yellow-red 2,4,6-trinitrocyclohexadienate. The colour intensity is proportional to the creatinine concentration.

REAGENTS
Package
1-Reagent	4 x 15 ml
2-Reagent	2 x 7.5 ml

The reagents when stored at 15-25°C are stable up to expiry date printed on the package. Stability on board of the analyser at 2-10°C: ACCENT-200 – 4 weeks, ACCENT-200 II GEN – 4 weeks. Protect from light and avoid contamination!

Concentrations in the test
sodium hydroxide	300 mmol/l
carbonate buffer	100 mmol/l
picric acid	6.5 mmol/l

Warnings and notes
- Product for in vitro diagnostic use only.
- 1-Reagent is classified as an irritant!

Ingredients: sodium hydroxide;
Xi – Irritant.
R 36/38: Irritating to eyes and skin.
S 26-28-37/39-45: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. After contact with skin, wash immediately with plenty of water. Wear suitable gloves and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

SPECIMEN

Serum, EDTA or heparinized plasma free from hemolysis, 24-hours urine.

Urine preparation: Before analysis urine sample should be diluted 100-fold with 0.9% NaCl and the results multiplied by 100. Mix well probes before measurement.

Specimen can be stored up to 7 days at 2-8°C. For longer storage samples should be frozen at -20°C.

Nevertheless it is recommended to perform the assay with freshly collected samples!

PROCEDURE

These reagents may be used in automatic analyser ACCENT-200 and ACCENT-200 II GEN.
1-Reagent and 2-Reagent are ready to use.
For reagent blank deionized water is recommended.

APPLICATION for ACCENT-200
Parameters

Test Name	CREA	R1	200
Test No	8	R2	50
Full Name	Creatinine	Sample Volume	25
Reference No	8	R1 Blank	
Analy. Type	Fixed Time	Mixed Reag. Blank	
Pri. Wave.	510 nm	Concentration	0.32	20
Secon. Wave.	670 nm	Linearity Limit	0.2
Trend	Ascending	Substrate Limit	
Reac. Time	1 4	Factor	
Incuba. Time	3	☐ Prozone check	
Unit	mg/dl		
Precision	0.01	q1 ☐ q2 ☐ q3 ☐ q4 ☐

PC ☐   Abs ☐

Calibration Rule

Rule	Two-Point Linear
Sensitivity	1
Replicates	3
Interval (day)	7
Difference Limit	0
SD	0
Blank Response	0	50000
Error Limit	0
Coefficient	0

APPLICATION for ACCENT-200 II GEN
Parameters

Test Name	CREA	R1	200
Test No	8	R2	50
Full Name	Creatinine	Sample Volume	25
Reference No	8	R1 Blank	
Analy. Type	Fixed Time	Mixed Reag. Blank	
Pri. Wave.	510 nm	Concentration	0.32	20
Secon. Wave.	670 nm	Linearity Limit	0.2
Trend	Ascending	Substrate Limit	
Reac. Time	1 4	Factor	
Incuba. Time	4	☐ Prozone check	
Unit	mg/dl		
Precision	0.01	q1 ☐ q2 ☐ q3 ☐ q4 ☐

PC ☐   Abs ☐

Calibration Rule

Rule	Two-Point Linear
Sensitivity	1
Replicates	3
Interval (day)	7
Difference Limit	0
SD	0
Blank Response	0	50000
Error Limit	0
Coefficient	0

REFERENCE VALUES [7]

serum / plasma	mg/dl	µmol/l
female	0.6 – 1.1	53 – 97
male	0.7 – 1.3	62 – 115
24-hours urine	mg/kg/24h	µmol/kg/24h
female	11 – 20	97 – 177
male	14 – 26	124 – 230

It is recommended for each laboratory to establish its own reference ranges for local population.

QUALITY CONTROL

For internal quality control it is recommended to use the CORMAY SERUM HN (Cat. No 5-172) and CORMAY SERUM HP (Cat. No 5-173) for determination in serum or CORMAY URINE CONTROL LEVEL 1 (Cat. No 5-161) or LEVEL 2 (Cat. No 5-162) for determination in urine with each batch of samples.

For the calibration of automatic analysers systems the CORMAY MULTICALIBRATOR LEVEL 1 (Cat. No 5-174; 5-176) and LEVEL 2 (Cat. No 5-175; 5-177) is recommended.

The calibration curve should be prepared every week (ACCENT-200, ACCENT-200 II GEN), with change of reagent lot number or as required e.g. quality control findings outside the specified range.

PERFORMANCE CHARACTERISTICS

These metrological characteristics have been obtained using automatic analyser ACCENT-200. Results may vary if a different instrument or a manual procedure is used.

- **Sensitivity:** 0.32 mg/dl (28.3 µmol/l).

- **Linearity:** up to 20 mg/dl (1768 µmol/l).
  For higher concentration dilute the sample with 0.9% NaCl and repeat the assay. Multiply the result by dilution factor.

- **Specificity / Interferences**
  Haemoglobin up to 2.5 g/dl, triglycerides up to 500 mg/dl, ascorbate up to 62 mg/l and bilirubin up to 20 mg/dl do not interfere with the test.

- **Precision**

Repeatability (run to run) n = 20	Mean [mg/dl]	SD [mg/dl]	CV [%]
level 1	0.88	0.06	6.65
level 2	3.48	0.08	2.33

Reproducibility (day to day) n = 80	Mean [mg/dl]	SD [mg/dl]	CV [%]
level 1	1.44	0.07	4.86
level 2	3.92	0.16	4.00

- **Method comparison**

A comparison between creatinine values determined at ACCENT-200 (y) and at COBAS INTEGRA 400 (x) using 63 samples gave following results:

$y = 1.1032 x - 0.0409$ mg/dl;

$R = 0.9985$ (R – correlation coefficient)

WASTE MANAGEMENT

Please refer to local legal requirements.

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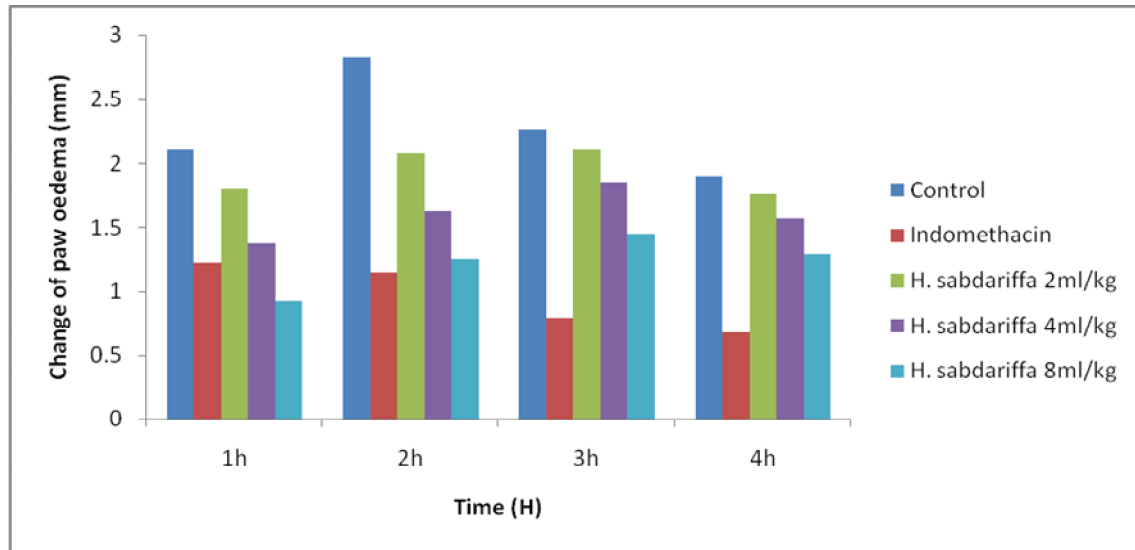
Appendix 2

Fig. The effect of *H. sabdariffa* petroleum ether extract and indomethacin on rats hind paw oedema induced by carrageenan.

Appendix 3
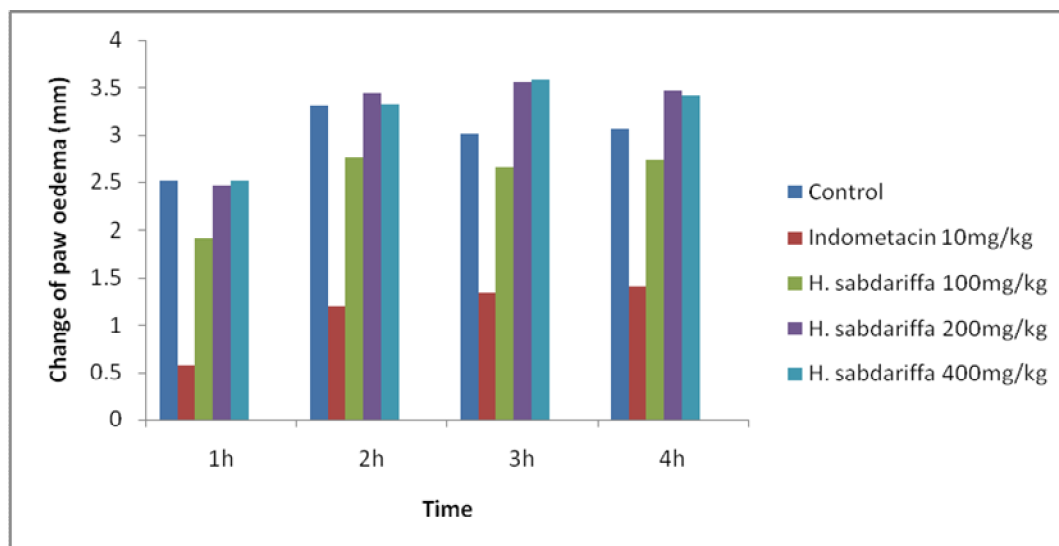
Fig. The effect of *H. sabdariffa* ethanolic extract and indomethacin on rats hind paw oedema induced by carrageenan.

Appendix 4
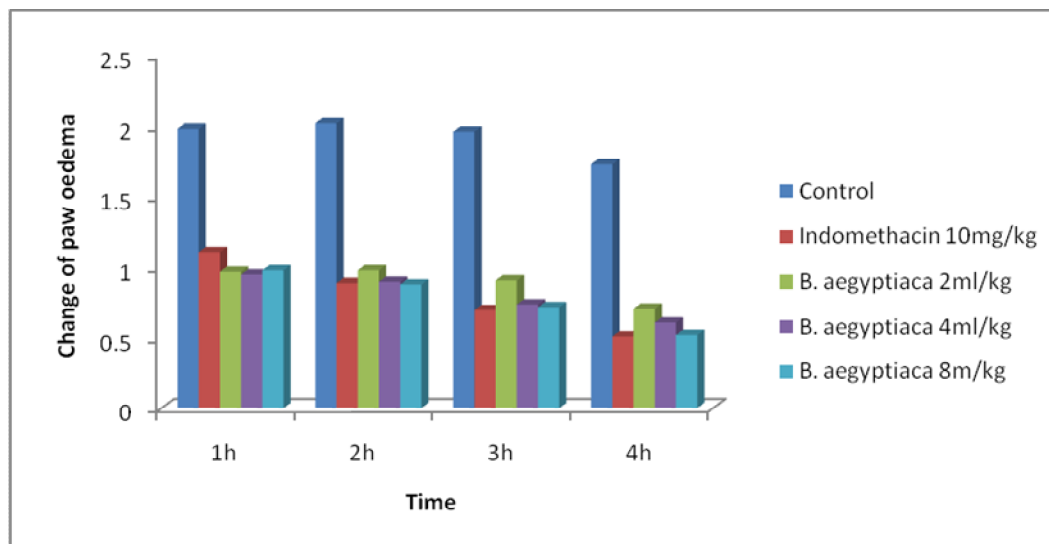
Fig. Effect of petroleum ether extract of *B. aegyptiaca* seeds on carrageenan induced rat paw oedema.

Appendix 5
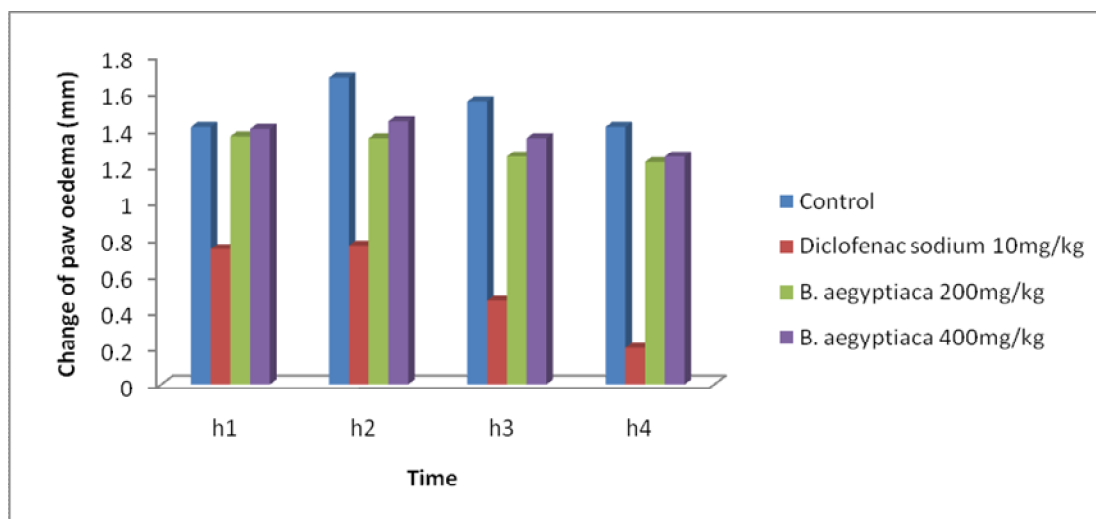
Fig. Effect of ethanolic extract of *B. aegyptiaca* seeds on carrageenan induced rat paw oedema.

Appendix 6

Paper 1

Ali, S. A., Mohamed, A. H and Mohammed .G. E. (2014). Fatty acid composition, anti-inflammatory and analgesic activities of *Hibiscus sabdariffa* Linn. Seeds. *Journal of Advanced Veterinary and Animal Research.,* 1(2): 50-57.

RESEARCH ARTICLE

Fatty acid composition, anti-inflammatory and analgesic activities of *Hibiscus sabdariffa* Linn. seeds

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ABSTRACT

Hibiscus (H.) sabdariffa (commonly known as "*Karkadeh*" in Arabic) is widely used in various pharmacological applications in Sudan. The present study was carried out to investigate the anti-inflammatory and analgesic activities of *H. sabdariffa* seed extracts using rat models. In acute anti-inflammatory models, oral administration of petroleum ether extract of *H. sabdariffa* seeds inhibited the hind paw edema ($p<0.01$) which was induced by carrageenan. The petroleum ether extract exhibited significant ($p<0.01$) inhibition of vascular permeability in rats induced by intraperitoneal injection of acetic acid (0.6%). In cotton pellet granuloma method, the petroleum ether extract of *H. sabdariffa* seed showed significant inhibition of granuloma. The extract reduced ($p<0.001$) abdominal constrictions which was induced by injection of acetic acid (0.7%). Analysis of seed oil of *H. sabdariffa* using Gas Chromatography revealed the presence of three fatty acids; these were linolelaidic acid, arachidic acid, and palmitic acid. In conclusion, *H. sabdariffa* seeds possess anti-inflammatory and analgesic activities in rat model.

Keywords:

INTRODUCTION

Inflammation is a protective reaction by the body in response to physical or chemical injury; acute inflammatory response begins immediately after cellular injury (Sivaraman et al., 2010). At present, several drugs are used for relieving pain, and in management of inflammatory conditions. These drugs include narcotics (e.g., opioids), non-narcotics (e.g., salicylates), and corticosteroids (e.g., hydrocortisone). However, it is reported that these drugs may exert several adverse effects on health (Ahmed et al., 1992).

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used as analgesic and anti-inflammatory agents. However, as side effects, NSAIDs may cause ulceration and bleeding in the gastrointestinal tract (Schoenfeld et al., 1999). Recently, many medicines of plant origin have been used taking advantages of their safety in use. Medicinal plants are considered as major sources of novel chemical compounds. Therefore, the medicinal plants attracted researchers for the development of novel, cheap, and non-toxic drugs (Ahmed et al., 1992).

Hibiscus (H.) sabdariffa Linn., belonging to the family Malvaceae, is commonly termed as "*Karkadeh*" in Arabic, and "*Roselle*" or "*Red sorrel*" in English. This plant is widely grown in Central and West Africa, South-East Asia, and in parts of West India, Jamaica and Central America (Ali et al., 2005). It has been traditionally used as antiseptic, astringent, cholagogue, aphrodisiac, demulcent, diuretic, emollient, purgative,

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digestive stomachic, sedative and tonic. The plant was also reported to be used for high blood pressure, liver diseases, fever, ulcers, abscesses and anemia (Mahadevan et al., 2009). There are many reports on the pharmacological effects of the extracts prepared from different parts of *H. sabdariffa*; the pharmacological effects include hepatoprotective (Dahiru et al., 2003), antihypertensive (Onyenekwe et al., 1999; Adegunloye et al., 1996), antimutagenic (Chewonarim et al., 1999), antispasmodic (Ali et al., 1991), antioxidant (Bako et al., 2009), anticancer (Tseng et al., 1998) cytotoxicity and antibacterial (Olaleye, 2007), immunomodulatory (Fakeye, 2008), antinociceptive, anti-inflammatory, and antidiarrheal activities (Ali et al., 2011). The plant has been vigorously used in phytochemical studies, through which a number of active ingredients have been isolated. The petroleum ether extract of *H. sabdariffa* seed contains flavonoids, carotenoid, steroids, fatty acids, and phenolic compounds, whereas alcoholic extract of the seed contains alkaloids and tannins (Mungole and Chaturvedi, 2011).

The seed oil of *H. sabdariffa* revealed the presence of cholesterol, campasterol, stigmasterol, ß-sitosterol, α-spinasterol, and ergosterol (Ali et al., 2005). Besides, the seed oil contains steroids, tocopherols, unsaturated fatty acids (70%) such as linoleic acid, cellulose, pentosans, and starch (Mahadevan et al., 2009). However, reports on analgesic and anti-inflammatory activities of the seed extracts of *H. sabdariffa* are very few. Therefore, the present study was designed to investigate the anti-inflammatory and analgesic activities of petroleum ether and ethanolic extracts of *H. sabdariffa* seeds using rat model.

MATERIALS AND METHODS

Plant material: The *H. sabdariffa* seeds were purchased from local market in Omdurman, Sudan. The seeds were then identified and authenticated by expert botanists in Medicinal and Aromatic Plants Research Institute (MAPRI), National Centre of Research (NCR), Khartoum, Sudan.

Preparation of the extracts: The powdered seeds of *H. sabdariffa* were successively extracted with petroleum ether at 40-60°C, and/or ethanol (98%) using Soxhlet extractor apparatus. The extraction was carried out for about 4 h for petroleum ether and 8 h for ethanol. The solvents were then collected and evaporated under vacuum pressure using rotary evaporator apparatus. The percentages of the residues were calculated. The ethanolic extract was stored in glass bottle that was protected from light and humidity. On the other hand,

the oil extract obtained from petroleum ether was stored in dark bottles (Harborne, 1984).

Gas chromatography: Fatty acid composition of *H. sabdariffa* was identified by Gas Chromatography (GC-2010, SHIMADZU, Japan) according to the procedure described by Christie (1989). In brief, methyl ester of *H. sabdariffa* seed oil was prepared prior to inject into GC. One mL of seed oil was taken into a screw capped tube, in which about 6 mL of NaOH (0.5 M) and 6 mL of 1% H_2SO_4 dissolved in methanol were added. After a vigorous homogenization, the tube was kept overnight to allow the reaction to proceed. Then, 2 mL of n-hexane was added and mixed well. Then, saturated NaCl was added and mixed. The supernatant was recovered after filtering with anhydrous Na_2SO_4, and finally injected in GC. The GC was equipped with flame ionization detector (FID) and capillary column (BD-1, Japan; 30m × 0.25 mm × 0.25 mm). The detector temperature was programed at 300°C with a flow rate of 1 mL/min. The injector temperature was adjusted at 250°C. Nitrogen was used as the carrier gas. The identification of fatty acids of *H. sabdariffa* seed oil corresponding to the peaks was performed by comparison with the retention times of standard fatty acids analyzed under the same conditions.

Experimental animals: Adult Wistar albino rats of either sex were purchased from Medicinal and Aromatic Plants Research Institute (MAPRI), National Centre for Research (NCR), Sudan. The animals were kept in polypropylene cages in the Laboratory Animal House of the College of Veterinary Medicine, Sudan University of Science and Technology (SUST). The animals were acclimatized for 7 days under standard environmental conditions (*i.e.*, relative humidity: 40-60%, temperature: 24±2°C, and 12 h light-dark cycle), and fed with mash feed consisting of flour, meat, edible oil, sodium chloride, vitamins, minerals and tap water *ad libitum*. Supply of food was withdrawn 12 h prior to the commencement of the experiment; however, the rats were allowed for free access to water always. All the experiments were carried out by using five animals in each group. This study was approved by the Scientific Research Committee of the College of Veterinary Medicine, SUST in accordance with good clinical practice and international guidelines for animal use in experimentations.

Acute toxicity study: The acute toxicity test was performed according to the guidelines of the Organization for Economic Cooperation and Development (OECD), which determined the use of a test dose limit of maximum 20 mL/kg b.wt. (OECD,

2001). Twenty adult Wistar albino rats of both sexes weighing between 100-136 gm were used for this study. The rats were fasted for 12 h with free access to water only. *H. sabdariffa* seeds petroleum ether extract was administered orally dosed at 2, 4, 8, 20 mL/kg b.wt., and mortality of the rats was observed for 24 h.

Anti-inflammatory activity evaluation

Carrageenan induced paw edema (for acute inflammation): Anti-inflammatory activities of *H. sabdariffa* seed extracts were determined in albino rats of both sexes weighing between 86-128 gm according to the procedure described by Ramprasath et al. (2004). Two different experiments were performed for this study. Five groups (5 rats in each) were allotted to different treatments as follows; Group 1 (control): the rats were kept untreated in petroleum ether extract experiment, and administered distilled water (10 mL/kg) only in ethanolic extract experiment, Group 2: the rats were given standard drug (indomethacin) at 10 mg/kg b.wt., Group 3: the rats were treated with petroleum ether extract through oral route dosed at 2 mL/kg b.wt., or ethanolic extract dosed at 100 mg/kg b.wt., Group 4: the rats were administered petroleum ether extract orally dosed at 4 mL/kg b.wt., or ethanolic extract dosed at 200 mg/kg b.wt., Group 5: the rats were received petroleum ether extract dosed at 8 mL/kg b.wt., or ethanolic extract dosed at 400 mg/kg b.wt. Thirty minutes after the administration of the treatments, edema was induced with the injection of carrageenan (0.1 mL, 1% w/v in saline) into the sub planter tissue of the right hind paw. The paw volume, up to the tibiotarsal articulation, was measured using a digital vernier calliper. The measures were determined at 0 h (before carrageenan injection) and 1, 2, 3 and 4 h after carrageenan injection. The percent paw volume inhibition was measured using the following formula:

$$\% \ inhibition = \frac{(V_f - V_0)control - (V_f - V_0)treated}{(V_f - V_0)control} \times 100$$

Where; V_0 = paw volume before administration of carrageenan (i.e., initial paw volume), and V_f = paw volume after administration of carrageenan.

Acetic acid-induced vascular permeability: Twenty albino rats of either sex weighing between 108-180 gm were taken and divided randomly into 4 equal groups. Group 1 (control): rats were given distilled water at 10 mL/kg b.wt., Group 2: the rats were given standard drug (i.e., diclofenac sodium) orally dosed at 10 mg/kg b.wt., Group 3: the rats received 4 mL/kg b.wt. petroleum ether extract of *H. sabdariffa* seeds orally,

Group 4: the animals were treated with 8 mL/kg b.wt. petroleum ether extract of *H. sabdariffa* seeds orally. After 1 h of administration of petroleum ether extract and diclofenac sodium, the rats were injected with 0.25 mL of 0.6% (v/v) acetic acid solution intraperitoneally (IP). Immediately after the acetic acid injection, Evan's blue was injected intravenously (dosed at 10 mg/kg b.wt.) via tail vein. After 30 min of Evan's blue injection, the animals were anesthetized and sacrificed, and the peritoneum was washed with 10 mL of normal saline; the washing was collected in tubes, which was centrifuged at 3,000 rpm for 15 min. The absorbance of the supernatant was measured at 620 nm using spectrophotometer. The vascular permeability effect was expressed as the absorbance (A), which represented the total amount of dye leaked into the intraperitoneal cavity (Shaikh, 2011; Anosike et al., 2012).

Cotton pellet granuloma (for chronic inflammation): Twenty rats of either sex of 85–155 gm were divided into 4 equal groups. The animals were anesthetized with thiopental sodium (40 mg/kg b.wt., IP). The subcutaneous implantations of sterile cotton pellets (20 mg) were done in lumbar region to induce chronic inflammation (Lalitha and Sethuraman, 2010). The test extract (4 and 8 mL/kg b.wt. of petroleum ether of *H. sabdariffa* seeds) and standard drug (diclofenac sodium; dosed at 10 mg/kg b.wt.) were administered orally for 6 consecutive days from the day of cotton pellet implantation. On the 7th day, the animals were sacrificed by an over dose of chloroform. The cotton pellets were removed surgically, dried at 60°C for 24 h until a constant weight was obtained. The increment in dry weight of the pellets over 20 mg were taken as an index of granuloma formation.

Analgesic study

Acetic acid induced writhing test: The peripheral analgesic activity of petroleum ether extract was measured by acetic acid induced writhing test (Nwafor and Okwuasaba, 2003). Twenty five animals of male and female rats weighing 102–146 gm were divided into equal 5 groups. Group 1 (Control): Animals received distilled water only (dosed at 1 mL/100g b.wt.), Group 2: rats were administered orally with diclofenac sodium dosed at 10 mg/kg b.wt, Group 3: rats were administered orally petroleum ether extract of *H. sabdariffa* seeds dosed at 2 mL/kg b.wt., Group 4: rats were administered orally petroleum ether extract of *H. sabdariffa* seeds at a dose of 4 mL/kg b.wt., and Group 5: rats were administered orally petroleum ether extract of *H. sabdariffa* seeds dosed at 8 mL/kg b.wt.

After 1 h following the administration of the treatments, acetic acid (0.7%) dosed at 0.1 mL/10g b.wt. was injected (IP) to initiate pain sensation. The number of writhing was calculated immediately after the application of acetic acid for 20 min. The inhibition of writhing produced by the petroleum ether extract and standard analgesic drug (diclofenac sodium) was measured by comparing with the inhibition produced by the control group.

Statistical analysis: Data were expressed as the mean±SEM. Differences between experimental groups were compared by one way analysis of variance (ANOVA) followed by Duncan test (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The analysis of seed oil of *H. sabdariffa* petroleum ether extract using Gas Chromatography (GC) revealed the presence of linolelaidic acid (26.02%), arachidic acid (20.59%) and palmitic acid (16.05%) (Figure 1). These fatty acids could be responsible for its anti-inflammatory effect (Chouhan et al., 2011). Moreover, anti-inflammatory effect of the plant might be attributed to its antioxidant properties (Sokeng et al., 2013).

In acute toxicity study, all the doses (2, 4, 8 and 20 mL/kg b.wt.) of petroleum ether extract of *H. sabdariffa* seeds were found to be non-toxic. No animal mortality was observed after receiving petroleum ether extract up to the dose of 20 mL/kg b.wt.

The oral administration of petroleum ether extract of *H. sabdariffa* seeds reduced the paw edema significantly that was induced by carrageenan in dose dependent manner. After 3 h of the treatment dosed at 4 and 8 mL/kg b.wt., paw edema was reduced by 27.9% ($p<0.05$) and 34.2% ($p<0.01$), respectively. Besides, indomethacin was used as standard drug, which decreased paw edema by 57.1% ($p<0.001$) after 3 h (Table 1). In contrast, the ethanolic extract of *H. sabdariffa* seeds did not show significant reduction in paw edema (inhibition 0%) even at the test doses of maximum 400 mg/kg b.wt.

The vascular permeability test is considered as one of the acute inflammatory models. Oral administration of diclofenac sodium dosed at 10 mg/kg b.wt., and petroleum ether extract of *H. sabdariffa* seeds (dosed at 4 and 8 mL/kg b.wt.) significantly ($p<0.01$) inhibited the dye leakage induced by acetic acid as compared to control (Table 2).

The petroleum ether extract was also assessed for reduction of cotton pellet induced granuloma in rats (Table 3). Granuloma formation was inhibited significantly after administration of petroleum ether extract of *H. sabdariffa* seeds for 6 consecutive days as compared to control group. The test dose (4 and 8 mL/kg b.wt.) showed 30.3% and 27.2% of inhibition ($p<0.01$) respectively as compared to the control group; however, diclofenac sodium exhibited the highest inhibition rate (52.7%; $p<0.001$) as compared to control. A significant ($p<0.05$) difference was found between the inhibition rates of standard drug and test groups.

The animals treated with *H. sabdariffa* seed petroleum ether extract exhibited a significant level of inhibition in abdominal writhes produced by acetic acid especially when the dose was higher (8 mL/kg b.wt.; 45.0%, $p<0.001$) as compared to control group. However, maximum writhing inhibition (52.6%) was recorded in the cases of using diclofenac sodium (Table 4) as standard.

The most effective and widely used model for evaluating anti-inflammatory drugs is carrageenan-induced paw edema. Carrageenan induced edema is a biphasic response. The first phase is mediated through the release of histamine, serotonin and kinins during first hour. The second phase is related to the release of prostaglandins (Vasudevan et al., 2006; Andrade et al., 2007), which peaks after 3 h (Chakraborty et al., 2004). The carrageenan-induced paw edema in rats is known to be sensitive to cyclooxygenase inhibitors and anti-inflammatory agents causing the inhibition of cyclooxygenase activity; thus, the level of prostaglandin synthesis is decreased (Shaikh, 2011).

In the present study, *H. sabdariffa* seeds petroleum ether extract significantly inhibited the first and second phases of inflammation. These findings suggested that the inhibitory effect of this extract on carrageenan-induced paw edema could be attributed to inhibition of cyclooxygenase, leading to inhibition of prostaglandin synthesis. However, the inhibition level was less than that of standard drug, indomethacin. Anti-inflammatory activity of *H. sabdariffa* calyx ethanolic extract was studied by Ali et al. (2011), in xylene induced ear edema model in mice; where significant inhibition was occurred on ear edema formation. Another study by Dafallah and Al-Mustafa (1996) showed different results in evaluating anti-inflammatory activity of *H. sabdariffa* aqueous extract.

Table 1: Effect of petroleum ether extract of *H. sabdariffa* seeds on carrageenan induced rats' paw edema.

Group	Dose	Increase in paw volume (mm)				Inhibition %			
		1h	2h	3h	4h	1h	2h	3h	4h
Control	-	2.11±0.13	2.82±0.24	2.27±0.20	1.90±0.09	-	-	-	-
Indomethacin	10mg/kg	1.22±0.09***	1.15±0.23***	0.97±0.23***	0.68±0.12***	42.1	59.9	57.1	64.9
H. sabdariffa	2 mL/kg	1.81±0.12	2.08±0.14*	2.11±0.05	1.76±0.15	16.3	22.8	7.8	7.5
	4 mL/kg	1.37±0.21**	1.63±0.13**	1.85±0.12*	1.57±0.08	32.0	38.5	27.9	15.8
	8 mL/kg	0.92±0.11***	1.25±0.20***	1.45±0.22**	1.29±0.27*	55.3	55.7	34.2	32.9

The values are expressed as mean±SEM *p<0.05, **p<0.01, ***p<0.001 (n=5).

Table 2: Effect of petroleum ether extract of *H. sabdariffa* seeds on acetic acid-induced vascular permeability.

Treatment	Dose	Absorbance ±SE	Inhibition %
Control	-	0.140±0.03	-
Diclofenac sodium (standard)	10mg/kg	0.026±0.00**	76.7
H. sabdariffa	4 mL/kg	0.072±0.02*	48.5
	8 mL/kg	0.047±0.01**	56.5

The values are expressed as mean±SEM *p<0.05, **p<0.01 (n=5).

Table 3: Effect of petroleum ether extract of *H. sabdariffa* seeds on cotton pellet granuloma in rats.

Groups	Dose	Granuloma dry weight (mg)	Inhibition %
Control	-	44.3±3.24	-
Diclofenac sodium (standard)	10 mg/kg	20.4±1.90***	52.7
H. sabdariffa	4 mL/kg	30.2±0.81***	30.3
	8 mL/kg	31.5±0.59***	27.2

The values are expressed as mean±SEM, ***p<0.001 (n=5).

Table 4: Analgesic effect of petroleum ether extract of *H. sabdariffa* seeds in rats writhing model.

Groups	Dose	Number of writhes	Inhibition rate %
Control	-	77.8±1.80	-
Diclofenac sodium (standard)	10 mg/kg	36.8±1.74***	52.6
H. sabdariffa	2 mL/kg	66.6±3.04**	14.5
	4 mL/kg	61.6± 2.23***	20.7
	8 mL/kg	42.8±1.62***	45.0

The values are expressed as mean±SEM, **p<0.01, ***p<0.001 (n=5).
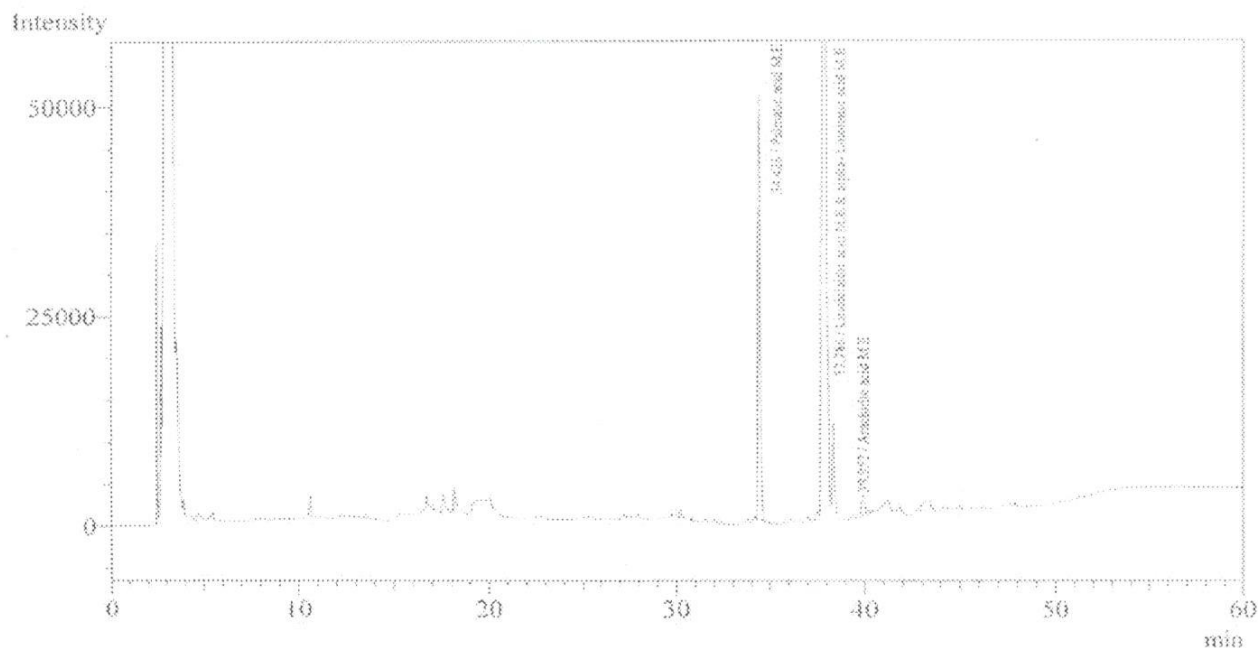
Figure 1. Chromatogram of Gas Chromatography (GC) for *H. sabdariffa* seed oil. Three distinct peaks corresponding to the authentic fatty acids were seen.

Vascular permeability induced by acetic acid is widely used to determine the inflammatory response of vascular tissue. In this study, IP injection of 0.6% acetic acid caused dilatation of blood vessels, and vascular permeability was increased. These effects were mediated by different inflammatory mediators (e.g., histamine, prostaglandins, leukotrienes etc.) and increased leakage of fluids including Evan's blue located at the blood vessel epithelial walls (Shaikh, 2011; Anosike et al., 2012). The present study demonstrated that oral treatment with petroleum ether extract of *H. sabdariffa* seeds (dosed at 4 and 8 mL/kg b.wt.) reduced peritoneal inflammation, suggesting its ability to inhibit the permeability of small blood vessels during acute inflammation.

The cotton pellet granuloma method is widely used to evaluate the transudation and proliferation components in the chronic inflammation (Paschapur et al., 2009; Patel and Patil, 2012). Subcutaneous implantation of cotton pellet in rat induced the formation of granular tissue. The amount of this tissue formation was measured by weighing the dried pellet. Cotton pellet granuloma involved the proliferation of microphages, neutrophils, fibroblasts and collagen formation, which were basic source for the granuloma formation; therefore, decrease in granuloma formation indicated the suppression of the proliferation phase (Shaikh, 2011). Administration of *H. sabdarriffa* seeds petroleum ether extract significantly ($p<0.001$) suppressed the formation of granular tissue. The inhibition was found to be similar in rats dosed at 4 and 8 mL/kg b.wt., but not as strongly as diclofenac sodium which was used as a standard drug.

Acetic acid is well known to induce indirect release of prostaglandins as well as lipooxygenase products into the peritoneum which stimulate the nociceptive neurons sensitive to the non-steroidal anti-inflammatory drugs. The results of this study strongly suggested that the mechanism of action of the petroleum ether extracts might be involved in part to inhibit the lipooxygenases and/or cyclooxygenases. It indicated that prostaglandins synthesis was reduced, which interfered the mechanism of transduction in primary afferent nociceptors (Prabhu et al., 2011). Ali et al. (2011) reported that the ethanolic calyx extract of *H. sabdarriffa* showed dose-dependent writhing inhibition significantly as compared to the control group. Maximum writhing inhibition was recorded as 66.85% when the dose was 500 mg/kg b.wt., which was comparable to the effect of diclofenac sodium. Phytochemical analysis of the *H. sabdarriffa* seeds showed the presence of flavonoids, steroids and phenolics; these are well known for their anti-inflammatory activities (Mungole and Chaturvedi, 2011).

CONCLUSION

Petroleum ether extract of *H. sabdarriffa* seeds have significant anti-inflammatory and analgesic activities in acute and chronic anti-inflammatory models. These actions could be due to inhibition of cyclooxygenase and prostaglandin synthesis.

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Appendix 7

Paper 2

Ali, S. A., Mohamed, A. H and Mohammed .G. E. (2014). Fatty Acid Composition, Anti-inflammatory and Analgesic Activities of *Balanites aegyptiaca* Seeds in Rats. *Journal of Agricultural and Veterinary Science.,* 15(1): 16-26.

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Fatty acid Composition, Anti-inflammatory and Analgesic Activities of *Balanites aegyptiaca* Seeds in Rats

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Abstract

The anti-inflammatory and analgesic effects of *B. aegyptiaca* seeds petroleum ether extract was investigated in Wistar albino rats. The anti-inflammatory effect was assessed on acute and chronic models by using carrageenan induced paw oedema and cotton pellet induced granuloma respectively. The analgesic activity of the petroleum ether extract of seeds was evaluated by acetic acid induced writhes. Oral administration of *B. aegyptiaca* seeds petroleum ether extract at dose rate of 2, 4 and 8ml/kg significantly ($P < 0.05$) inhibited carrageenan induced paw oedema in rats. The inhibition was comparable to that observed by indomethacin used as a standard anti-inflammatory drug. In cotton pellet granuloma method, the formation of granuloma significantly ($P < 0.05$) hindered by oral administration of the extract at dose rate of 4 and 8ml/kg compared to the control and standard drug diclofenac sodium. The petroleum ether extract also significantly ($P < 0.05$) reduced the writhes produced by acetic acid compared to control and standard analgesic drug diclofenac sodium. Moreover, the acute toxicity study indicated that oral administration of seeds oil in rats did not have any side or toxic effects up to 20 ml/kg except of anorexia in rats received higher doses of the oil. Gas Chromatography analysis of seed oil revealed the presence of capric acid, palmitic acid and oleic acid. This study confirmed the traditional uses of *B. aegyptiaca* seeds as anti-inflammatory and as an analgesic agent which may be attributed to its fatty acids content.

Keywords: *Balanites aegyptiaca, carrageenan, acetic acid, oedema.*

Introduction

Medicinal plants are considered to be an important source of new chemical substances with potential therapeutic effects (Gupta *et al.*, 2006). The use of the medicinal herbs for curing diseases has been documented in history of all civilizations. They contain active constituents that are used in the

treatment of many human diseases including inflammation (Das *et al.*, 2013).

Inflammatory diseases are currently treated with steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). Despite their widespread use, NSAIDs are often associated with severe adverse effects; the most common being gastro-intestinal ulcers, bleeding and renal disorders (Nonato *et al.*, 2009 and Sivaraman *et al.*, 2010). Due of the deleterious side effects attributed to the prolonged use of NSAIDs and their ineffectiveness in some cases, the control of inflammation is still a major challenge (Nonato *et al.*, 2009).

Balanites aegyptiaca L. (Zygophyllaceae), is locally known *Hegleig* tree and its fruits called *lal'loub*. It is also known as Desert date' in English. *B. aegyptiaca* is a small to medium-sized semidecidous tree which attains a height of about 6 m. The tree is found in most and to sub humid tropical savannas of African, all over the Sahel and on many sites of the Sudan savanna, extending from the Atlantic coastline of Senegal to the red sea and Indian Ocean and the Arabian Peninsula (Maydell, 1986).

Hegleig is an extremely useful tree which has been utilized over thousands of years. All parts of the tree have medicinal uses including fruits, seeds, barks and roots (Elfeel, 2010; Maydell, 1986). The bark and roots are used as laxatives or tranquilizers (for colic). The bark is used against stomachaches, sterility, mental diseases, epilepsy, yellow fever, syphilis and as a vermifuge. Fruits and leaves, and especially the kernel-oil are applied for rheumatism and bark extracts for toothaches (Maydell, 1986). The oil exhibited anticancer activity, antimutagenic activity against *Fasciola gigantica* -induced mutagenicity, anthelmintic activity against hepatic worms (*Schistosoma mansoni* and *Fasciola gigantica*), antiviral activity against Herpes simplex virus and antimicrobial activity against selected strains of Gram-positive bacteria, Gram-negative bacteria and *Candida* (Al Ashaal *et al.*, 2010). It is also reported that *B. aegyptiaca* have potent wound-healing, antioxidant (Annan and Dickson, 2008), hepatoprotective (Jaiprakash *et al.*, 2003), anti-inflammatory, analgesic (Gaur *et al.*, 2008) and antidiabetic activities (Mansour and Newairy, 2000).

On the other hand researchers have been isolated huge numbers of active constituents from different parts of *B. aegyptiaca* as reviewed by Chothani and Vaghasiya, (2011). The most important constituent is found to be steroidal saponins (Elfeel, 2010). The kernels contained 45.0 to 46.1% oil and protein (32.4%), oil contains mainly palmitic, steric, oleic and linoleic acids which were the main fatty acids (Chothani and Vaghasiya, 2011). However, kernel contains a xylopyranosyl derivative of saponin present in mesocarp (Staerk *et al.*, 2007). Nine saponin have been also isolated from kernel cake of *B. aegyptiaca* (Chothani and Vaghasiya, 2011)

Scientific researches in the anti-inflammatory and analgesic activities of *B. aegyptiac*a especially on seeds oil extract have not yet been performed. The present study was designed to evaluate the anti-inflammatory and analgesic effects of *B. aegyptiaca* seeds petroleum ether extract using different models in rats and to determine the fatty acids composition of the seeds oil using Gas chromatography.

Materials and Methods
Plant materials:

Fruits of *B. eagyptiaca* were purchased from local market in Omdurman, Sudan. The fruits were then identified and authenticated by the botanists in Medicinal and Aromatic Plants Research Institute (MAPRI), National Centre for Research (NCR), Khartoum, Sudan.

Preparation of the extract:

The fruits of *B. aegyptiaca* were opened and the kernels were separated and powdered using a blender. 200 gm of *B. aegyptiaca* seeds weighed and packed in soxhlet apparatus. The powdered seeds were extracted with petroleum ether (40 – 60) °C to extract oil. The solvent was then collected and evaporated under reduced pressure using rotary evaporator apparatus. The petroleum ether extract yielded pale yellow colour oil which was stored in dark bottles in room temperature till use (Harborne, 1984).

Fatty acid composition of seeds oil of *B. eagyptiaca*:

Methyl ester of seeds oil of *B. aegyptiaca* was prepared according to the procedure of Christie, (1989); Christie, (1972) to determine fatty acids composition using Gas chromatography (GC- 2010, SHIMADZU- Japan).

Experimental animals:

Wistar albino rats were obtained from Medicinal and Aromatic Plants Research Institute (MAPRI), National Centre for Research (NCR), and kept in plastic cages in the laboratory animal house in the College of Veterinary Medicine, Sudan University of Science and Technology. They were maintained under standard environmental conditions and provided with standard feed and water *ad libitum*. The animals were fasted overnight before the commencement of the experiment but were allowed with free access to water. All experiments were carried out using five animals in each group.

Acute toxicity study:

According to Organization for Economic Co-operation and Development (OECD/OCDE) guidelines (OECD, 2001) the acute toxicity study was performed in Wistar albino rats of either sex. Twenty rats (100 – 120 g) were divided randomly to 4 groups of 5 rats each. *B. aegyptiaca* seeds petroleum ether extract was administered orally to treated groups at a dose of 2, 4, 8, 20 ml/kg respectively and mortality was observed for 24 hours.

Anti-inflammatory activity evaluation: Carrageenan induced paw oedema (for acute inflammation)

Anti-inflammatory activity was performed in albino rats of either sex (80 – 122 g) according to the method of Ramprasath *et al.*, (2004). Five groups of 5 rats per each were subjected to different treatments as follows:

Group 1: Control: Animals were kept untreated.

Group 2: Standard anti-inflammatory drug: Rats were administered orally with indomethacin at a dose of 10 mg/kg.

Group 3: Low dose: Rats were treated with petroleum ether extract of *B. aegyptiaca* seeds at a dose of 2 ml/kg orally.

Group 4: Medium dose: Animals were administered with petroleum ether extract of *B. eagyptiaca* seeds at a dose of 4 ml/kg orally.

Group 5: High dose: Rats were given petroleum ether extract of *B. eagyptiaca* seeds at a dose of 8 ml/kg orally.

Carrageenan was injected to all groups after one hour of the administration of the treatments into the sub planter tissue of the right hind paw at a dose of 0.1 ml (1% w/v in saline) to induce oedema. The paw volume was determined using a digital vernier calliper. The measurements were recorded at 0 h (before carrageenan injection) and 1, 2, 3 and 4 hours after carrageenan injection the % paw volume inhibition was measured using the following formula:

$$\% \text{ inhibition} = (Pv_t - Pv_O)_{control} - (Pv_t - Pv_O)_{treated} / (Pv_t - Pv_O)_{control} \times 100$$

Where Pv_O = paw volume before administration of carrageenan and Pv_t = is the paw volume after administration of carrageenan.

Cotton pellet granuloma (for chronic inflammation)

Twenty rats weighing between 80 – 166 g were allotted randomly into 4 groups of 5 rats each. The sterile cotton pellets (20 mg) were implanted under anaesthesia in shaved lumbar region of rats using small incision to induce chronic inflammation (Chouhan et al., 2011).

Group 1 served as control, group 2 served as standard anti-inflammatory drug diclofenac sodium administered at a dose 10 mg/kg.

Group 3 and 4 served as test groups and received medium dose (4ml/kg) and high dose (8ml/kg) of the petroleum ether extract of B. aegyptiaca seeds respectively. All treatments were administered orally for 6 consecutive days from the day of cotton pellet implantation. On the 7th day, animals were killed by an over dose of anaesthesia. The cotton pellets were removed surgically, dried at 60 °C for 24h and weighed. The increase of pellets weight over 20 mg after dryness was taken as an index of granuloma formation.

Analgesic study
Acetic acid induced writhing test:

Acetic acid induced writhing test was used to determine the peripheral analgesic effect of B. aegyptiaca seeds petroleum ether extract (Nwafor and Okwuasaba, 2003). Twenty five animals (90 – 156 g) were divided randomly into 5 groups of 5 rats per each.

Group1: Control: Animals received distilled water only (1 ml/100g).

Group 2: Standard analgesic drug: Rats were administered orally with diclofenac sodium at a dose of 10 mg/kg.

Group 3: Low dose: Rats were administered orally with petroleum ether extract of B. aegyptiaca seeds at a dose of 2 ml/kg.

Group 4: Medium dose: Animals were given petroleum ether extract of B. eagyptiaca seeds at a dose of 4 ml/kg orally.

Group 5: High dose: Rats were administered orally petroleum ether extract of B. eagyptiaca seeds at a dose of 8 ml/kg.

After one hour following the administration of the treatments, acetic acid (0.7%) at a dose of 0.1 ml/10g of body weight was injected intra-peritoneal to produce pain sensation. The number of writhing was calculated immediately after the injection of acetic acid for 20 min. The inhibition of writhing produced by the plant extract and standard drug was measured by comparing with the inhibition produced by the control group.

Statistical analysis:

Data were expressed as the mean ± SEM. Differences between experimental groups were compared by one way analysis of variance (ANOVA) followed by Duncan test. The results were considered statistically significant when $P < 0.05$ (Gomez and Gomez, 1984).

Results

Gas chromatography analysis of seed oil obtained from B. aegyptiaca petroleum ether extract revealed the presence oleic acid (22.61%), palmitic acid (4.90%) and capric acid (1.49%). The fatty acids composition of B. aegyptiaca seed oil is presented in Figure (1).
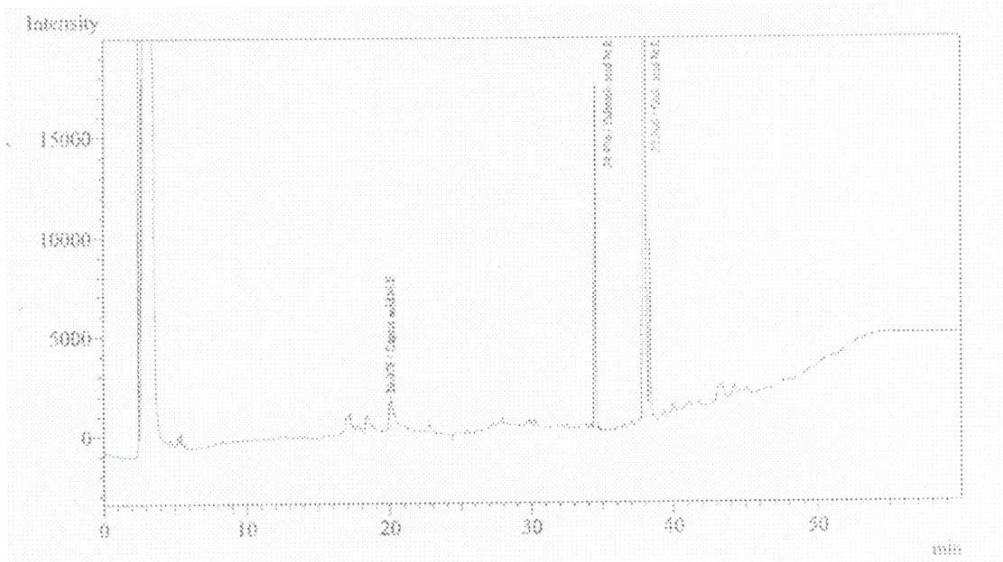
Figure 1: Chromatogram-Gas Chromatography for *B. aegyptiaca* seeds oil

In acute oral toxicity study, *B. aegyptiaca* seeds petroleum ether extract (2, 4, 8 and 20 ml/kg) was found to be safe. No mortality was observed in the animals received petroleum ether extract up to 20 ml/kg, except the observation of anorexia in rats received higher doses of the oil.

In this study, *B. aegyptiaca* seeds petroleum ether extract administered orally at 2, 4 and 8 ml/kg in rats significantly ($P < 0.05$) reduced the paw oedema induced by carrageenan at 1st, 2nd, 3rd and 4th hours post carrageenan injection. The inhibition of rats treated with *B. aegyptiaca* seeds petroleum ether extract was comparable to indomethacin used as a standard anti-inflammatory drug (Table 1).

Table 1: Effect of petroleum ether extract of *B. aegyptiaca* seeds on carrageenan induced rat paw oedema

Groups	Dose	Increase in paw volume (mm)				Inhibition%			
		1h	2h	3h	4h	1h	2h	3h	4h
Control	-	1.98 ± 0.07^a	2.02 ± 0.04^a	1.96 ± 0.06^a	1.73 ± 0.03^a	-	-	-	
Indomethacin	10mg/kg	1.11 ± 0.07^b	0.89 ± 0.11^b	0.70 ± 0.09^b	0.51 ± 0.05^b	43.5	55.9	63.5	70.4
Low dose	2ml/kg	0.97 ± 0.06^b	0.98 ± 0.02^b	0.91 ± 0.08^b	0.71 ± 0.06^b	51.0	51.2	53.4	59.0
Medium dose	4ml/kg	0.95 ± 0.05^b	0.90 ± 0.04^b	0.74 ± 0.03^b	0.61 ± 0.03^b	51.9	55.5	62.3	64.8
High dose	8ml/kg	0.98 ± 0.02^b	0.88 ± 0.02^b	0.72 ± 0.05^b	0.52 ± 0.01^b	50.1	56.5	62.8	69.8

Values are expressed as mean $\pm$ SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).

The petroleum ether extract of *B. aegyptiaca* seeds was also examined for cotton pellet induced granuloma in rats (Table 2). Granuloma formation was inhibited significantly ($P < 0.05$) in rats treated with standard drug, diclofenac sodium and petroleum ether extract of *B. aegyptiaca* seeds for 6 consecutive days compared to the control group. The animals treated with petroleum ether extract of *B. aegyptiaca* seeds at a dose of 4 and 8 ml/kg showed 42.7% and 45.4% of inhibition respectively as compared to the control group, whereas

diclofenac sodium exhibited the highest inhibition rate (59.9%).

Table 2: Effect of petroleum ether extract of *B. aegyptiaca* seeds on cotton pellet granuloma in rats

Groups	Dose	Granuloma dry weight (mg)	Inhibition %
Control	-	38.8 ± 2.3^a	-
Diclofenac sodium	10 mg/kg	15.4 ± 0.9^c	59.9
Medium dose	4 ml/kg	22.0 ± 2.1^b	42.7
High dose	8 ml/kg	20.9 ± 0.9^b	45.4

Values are expressed as mean $\pm$ SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).

The treatment of animals with *B. aegyptiaca* seeds petroleum ether extract produced a significant inhibition ($P < 0.05$) in abdominal writhes induced by acetic acid especially at a dose of 4 (46.8 %) and 8 ml/kg (56.0%) compared with the control group. Maximum writhing inhibition was 60.8% in diclofenac sodium group used as standard drug (Table 3).

Table 3: Effect of petroleum ether extract of *B. aegyptiaca* seeds on acetic acid induced writhes in rats.

Groups	Dose	Number of writhes	Inhibition rate %
Control	-	100.2 ± 2.52^a	-
Diclofenac sodium	10 mg/kg	37.6 ± 1.9^c	60.8
Low dose	2 ml/kg	94.2 ± 2.42^a	6.0
Medium dose	4 ml/kg	53.2 ± 2.53^b	46.8
High dose	8 ml/kg	44.0 ± 0.89^{bc}	56.0

Values are expressed as mean $\pm$ SEM, means within the same column with different superscripts are significantly different at $P < 05$ (N =5).

Discussion

Acute toxicity study showed that the petroleum ether extract of *B. aegyptiaca* seeds safe in rats when administered orally up to 20 ml/kg (OECD, 2001).

In the present study, the anti-inflammatory and analgesic activities of *B. aegyptiaca* seeds petroleum ether extract were evaluated using different models in rats. Carrageenan-induced inflammation is most commonly used as an experimental model for evaluating the anti-inflammatory potency of compounds or natural products (Parekar *et al.*, 2012). The results obtained in this study showed that injection of carrageenan into the rats' hind paw elicited a localized inflammatory response characterized by an increase of paw size (swelling) and pain as a result of increased vascular permeability, cell infiltrations and inflammatory fluids. Control rats injected with carrageenan showed high paw oedema in the first hour and prolonged effect after 2 – 4 hours compared with standard anti-inflammatory drug, indometacin; this is in agreement with the finding of (Ravi *et al.*, 2009 and Parekar *et al.*, 2012). The probable mechanism of action of carrageenan-induced inflammation is bi-phasic; the first phase is attributed to the release of histamine, serotonin and kinins in the first hour, while the second phase is attributed to the release of prostaglandins and lysosome enzymes in 2 to 4 hours. The second phase is sensitive to most clinically effective anti-inflammatory drugs (Das *et al.*, 2013).

The petroleum ether extract of *B. aegyptiaca* seeds at a dose of 2, 4 and 8ml/kg remarkably

inhibited the first phase of inflammation as well as the second phase. The inhibition of oedema in rats treated *B. aegyptiaca* seeds petroleum ether extract was comparable to that observed by the standard anti-inflammatory drug, indomethacin. In agreement with our findings Gaur *et al.*, (2008) reported that the ethanol and petroleum ether extracts of aerial parts of *B. aegyptiaca* have significant anti-inflammatory activity on carrageenan-induced hind paw oedema in rats compared with the standard drug, indomethacin. Another study by Speroni *et al.*, (2005) showed similar finding in the investigation of the anti-inflammatory activity of two new saponins isolated from *B. aegyptiaca* in the carrageenin-induced oedema in the rats. The results indicated that the inhibitory effect of the extract on carrageenan induced paw oedema might be due to inhibition of the enzyme cyclooxygenase leading to inhibition of prostaglandins synthesis.

In cotton pellet induced granuloma, oral administration of *B. aegyptiaca* seeds petroleum ether extract for 6 days at a dose of 4 and 8 ml/kg hindered significantly (P < 0.05) the formation of granular tissue compared with the control. The inhibition of granuloma by *B. aegyptiaca* seeds petroleum ether extract at a dose of 4 and 8 ml/kg was considerably high but inferior to the inhibition observed by diclofenac sodium used as a standard anti-inflammatory drug. This reflects its efficacy in reducing an increase in the amount of fibroblasts and synthesis of collagen with mucopolysaccharide, which are natural proliferative events of granulation tissue formation. The potential mechanism involving anti-granulomatous effect of the extract could be due to the reduction of some or all key mediating cytokines leading to the inhibition of inflammatory cell recruitment, especially macrophage and mast cells, the

key initiators for granulation (Khumpook *et al.*, 2013).

In this study, the analgesic activity of *B. aegyptiaca* seeds petroleum ether extract was also investigated using acetic acid induced writhing test in rats. The extract exhibited significant inhibition of abdominal writhes (P < 0.05) especially at the medium and high doses (4 & 8 ml/kg) compared to the control and standard analgesic drug, diclofenac sodium. The low dose (2ml/kg) was found to be ineffective in reducing abdominal writhes compared with the control. Acetic acid is well-known to induce indirect release of prostaglandin as well as lipooxygenase products into the peritoneum which stimulate the nociceptive neurons sensitive to the non steroidal anti-inflammatory drugs. For that reason, the results of this study suggest that the mechanism of this action might be partially due to inhibition of lipooxygenase and/or cyclooxygenase in the peripheral tissues, thereby reducing the production of prostaglandins and interfering with the mechanism of transduction in primary afferent nociceptors (Prabhu *et al.*, 2011).

In this study, Gas Chromatograohic analysis of *B. aegyptiaca* seeds oil revealed the presence of oleic, palmitic and capric acids. Researchers reported that the oil of *B. aegyptiaca* seeds contains palmitic, stearic, oleic, and linoleic acids as the main fatty acids (Chothani and Vaghasiya, 2011), this is in agreement with our results in the presence of oleic and palmitic acid. The higher content of fatty acids could be responsible for the anti-inflammatory effect of *B. aegyptiaca* petroleum ether extract (Chouhan *et al.*, 2011).

Others have speculated that the anti-inflammatory of seeds extract could be strongly due to the presence of steroids (Elfeel, 2010) which are well known to have potent anti-inflammatory and analgesic activities (Mungole and Chaturvedi, 2011).

Meda *et al.,* (2010) reported that *B. aegyptiaca* has antioxidant properties, which may be responsible for its anti-inflammatory activity (Sokeng *et al.,* 2013).

In conclusion, *B. aegyptiaca* seeds petroleum ether extract possess potent anti-inflammatory and analgesic effects in different models used during this study. The inhibitory effect of this extract could be attributed to inhibition of the enzyme cyclooxygenase leading to inhibition of prostaglandins synthesis. Moreover, the phytoconstituent, high fatty acids content and antioxidant property of the plant may have a valuable role in its anti-inflammatory and analgesic activities.

Acknowledgements

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محتوى الاحماض الدهنية و الاثر المضاد للالتهاب و الفعالية كمسكن للالم
لمستخلص بذور الهجليج في الفئران

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فى هذه الدراسة تم التقصي عن محتوى الاحماض الدهنية و الاثار المضادة للالتهاب والمسكنة للألم لمستخلص البتروليوم ايثر لبذور الهجليج فى الجرذان. الاثر المضاد للالتهاب تم تقويمه فى نموذج للالتهاب الحاد و نموذج للالتهاب المزمن باستخدام الكراجنان لاحداث الوذمة وكرات القطن لاحداث الورم الحبيبى على التوالي. الاثر المسكن للالم تم تقويمه بواسطة حمض الخليك لاحداث الانقباضات. الإعطاء الفموي لمستخلص البتروليوم ايثر لبذور الهجليج بجرعة 2، 4 و 8 مل/كجم ثبط معنوياً الوذمة المحدثة بالكراجنان فى الجرذان. وجد ان التثبيط مشابه لذلك الذي لوحظ بواسطة عقار الاندوميثازين المستخدم كعقار معياري مضاد للالتهاب. فى طريقة الورم الحبيبي بكرات القطن، أعيق معنوياً تكوين الورم الحبيبي بالاعطاء الفموي للمستخلص بجرعة 4 و 8 مل/كجم مقارنة بالمجموعة الضابطة والعقار المعياري ديكلوفيناك صوديوم. مستخلص البتروليوم ايثر ايضا قلل معنوياً الانقباضات المحدثة بحمض الخليك مقارنة بالمجموعة الضابطة و العقار المعياري المسكن للالم، ديكلوفيناك صوديوم. ايضاً الدراسة السمية الحادة اوضحت ان الاعطاء الفموي لزيت البذور فى الجرذان لايسبب اي اثار جانبية او سامة حتى 20مل/كجم باستثناء قلة الشهية فى الجرذان المعطاة الجرعات العالية من الزيت. تحليل الغاز اللوني لزيت بذور الهجليج اظهر وجود حامض الكابريك ، حامض البالمتيك و حامض الاوليك. هذه الدراسة أكدت صحة الاستخدام التقليدي لبذور الهجليج كمضاد للالتهاب ومسكن للألم والذى يمكن ان يعزى لمحتواه من الاحماض الدهنية.