

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

Sumaia Awad Elkariem Ali^{1*}, Abdelwahab Hassan Mohamed² and Galal Eldin Elazhari Mohammed¹

¹College of Veterinary Medicine, Sudan University of Science and Technology, P.O. Box 204, Hilat Kuku, Khartoum North, Sudan;

²Faculty of Pharmacy, National Ribat University, Khartoum, Sudan.

*Corresponding author's e-mail: somiahadloul@yahoo.com, sumaiaawadelkareim@sustech.edu

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 linoleic acid, arachidic acid, and palmitic acid. In conclusion, *H. sabdariffa* seeds possess anti-inflammatory and analgesic activities in rat model.

Keywords:

Anti-inflammatory, Analgesic, Fatty acid, *Hibiscus sabdariffa* seed, Rat model

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, campesterol, stigmasterol, β -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% H₂SO₄ 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 Na₂SO₄, 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 programmed 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:

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

Where; V_o = 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 (Sokenget 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 |
| <i>H. sabdariffa</i> | 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 |
| <i>H. sabdariffa</i> | 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 |
| <i>H. sabdariffa</i> | 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 |
| <i>H. sabdariffa</i> | 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).

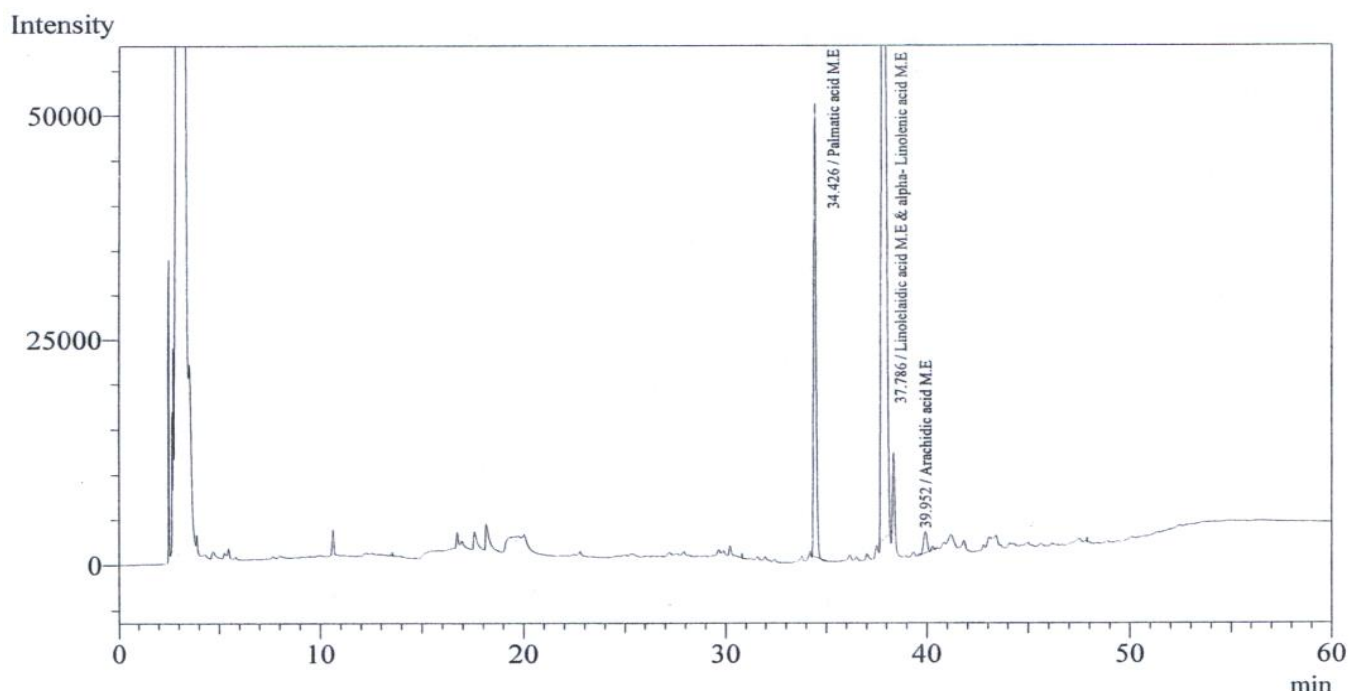


Figure 1. Chromatogram of Gas Chromatography (GC) for *H. sabdariffa* seed oil. Three distinct peaks corresponding to the authentic fatty acids were seen.

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 macrophages, 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. sabdariffa* 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 lipoxygenase 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 lipoxygenases 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. sabdariffa* 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. sabdariffa* 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. sabdariffa* 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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