J. Biomedical Science and Engineering, 2013, 6, 1040-1049 **JBiSE**
http://dx.doi.org/10.4236/jbise.2013.611130 Published Online November 2013 (http://www.scirp.org/journal/jbise/)

Some pharmacological studies on the methanolic extract of *Inula graveolense* L.

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Received 25 September 2013; revised 21 October 2013; accepted 31 October 2013

ABSTRACT

***Inula graveolens* L. is widely used in Iraq for the treatment of rheumatic fever, infant convulsions, toothache, blood sugar, and also to dissolve internal blood clots, and to aid digestion. However, the efficacy and mechanisms of action of the plant remain unclear. Therefore, the objective of the present study was to investigate the pharmacological effects of the methanolic extract (MEIG) of this plant belonging to compositae family. Anti-diarrheal and antipyretic activities of the extract were examined in rats. Anti-inflammatory and antinociceptive were studied in mice. At the doses of 200 ($P < 0.05$) and 400 mg/kg body weight ($P < 0.01$), the extract displayed remarkable anti-diarrheal activity, evidence by a reduction in the rate of defecation as well as by retardation of intestinal transit of charcoal meal compared to normal saline control group, dose dependently similar to loperamide (5 mg/kg). The methanolic extract (400 mg/kg) showed a significant ($P < 0.01$) dose dependent antipyretic effect in yeast induced elevation of body temperature in experimental rats. The methanolic extract showed significant anti-inflammatory and antinociceptive activity at the dose of 400 mg/kg ($P < 0.01$) as compared to standard drug diclofenac sodium (50 mg/kg). The extract inhibited paw and ear edema in a dose-related manner. A dose-dependent analgesic action was obtained against chemical (writhing test) and thermal (hot-plate test) stimuli indicated that antinociceptive activity may involve inhibition of pain by peripheral and central mechanisms. Again, the methanolic extract (MEIG) was subjected for *in vitro* protein anti-denaturation using Bovine serum albumin and anti-platelet aggregation of human blood activity. It was observed that the extract showed greater percentage of inhibition of BSA ($P < 0.01$) at the highest concentration (400 µg/ml). The extract also showed potential platelet aggregation inhibitory activity in adose-dependent manner. The maximum inhibition was observed at the dose 400 µg/ml ($P < 0.01$) compared to standard drug commercial heparin (20 µg/ml).**

Keywords: Pharmacological Activities; Antidiarrhae; Antipyretic; Anti-Inflammatory; Antinociceptive; Anti-Denaturation; Platelet Aggregation; *Inula graveolens* L.

1. INTRODUCTION

Diarrhea is a gastrointestinal disorder, characterized by an increase in stool frequency and change consistency [1]. From a long time ago, plant kingdom played an important role for discovering new drug source. A number of therapeutic drugs were isolated from plant species. For the treatment of diarrhea, medicinal plants are a potential source of antidiarrheal drugs [2]. Due to poor hygiene practices and malnutrition, children in developing countries frequently suffer from various forms of infections which present as fevers [3]. These fevers are often accompanied by aches and pain which all lead to morbidity and mortality. Herbal medicines are often used as remedies in these conditions since as a result of poverty orthodox medicines may be unaffordable [4]. It is known that a large number of plant species contain various bioactive compounds that may have health-beneficial properties, anti-inflammatory, anti-oxidant and antimicrobial effects, and their preventive and therapeutic use is increasing [5]. Pain is a sensorial modality and primarily protective in nature, but often causes discomfort. It is the most important symptom that brings the patient to physician. Analgesics relieve pain as a symptom, without affecting its cause, currently available analgesic drugs such as opiates and NSAIDS are not useful in all cases due to their adverse effects [6]. Pain and inflammation are associated with many pathophysiologies of various clinical

conditions like arthritis, cancer and vascular diseases. A number of natural products are used in various traditional medical systems to treat relief of symptoms from pain and inflammation [7]. The main cellular components susceptible to damage by free radical and oxidative stress are lipids (peroxidation of unsaturated fatty acids in cell membrane), proteins (denaturation), carbohydrates and nucleic acids [8]. Both inflammation and free radical damage are inter-related aspects that influence each other. As said above, proteins are susceptible to undergo denaturation by formation of free radical and the mechanism of inflammation injury is attributed in part to release of reactive oxygen species from activated neutrophil and macrophages [9]. Plant extracts are an alternative to the currently used anti-platelet agents, because they constitute a rich source of bioactive chemicals [10]. *Inula graveolens* L. is widely distributed in Mediterranean region and Middle East to West Pakistan. In Iraq (Basrah and lower Iraq), this plant is well known in Arabic and English system as "Shuwaser, Suawaid" and "Strong-smellind *Inula*", respectively [11]. Information gathered from some herbalists (in Basra governorate, Iraq) that the plant is useful to reduce rheumatic fever, infant convulsions, toothache, blood sugar, and also to dissolve internal blood clots, and to aid digestion. As there is no scientific report on the biological activity of the plant, the present investigation was done to evaluate the possible anti-diarrheal, antipyretic, anti-inflammatory, antinociceptive, anti-denaturation of protein and anti-platelet aggregation activities of the methanolic extract of *Inula graveolens* (MEIG). The findings from this work may add to the overall value of the medicinal potential of the plant.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), and solvents were from E. Merck (Darmstadt, Germany). All of the reagents were prepared in deionized distilled water to eliminate the contamination of metal ions.

2.2. Plant Material and Extraction Procedure

Inula graveolens L. plant, used in this study, was collected on october 2012 from Abu-Al-Khaseeb region (Southern of Basra), Iraq. The plant was botanically authenticated and voucher specimens 3897 were deposited in the Herbarium of Basra (Iraq, Basra, College of Science, University of Basra). A quantity (100 g) of powdered plant was extracted in a Soxhelet apparatus with 80% methanol, for 24 h. The methanol extract was filtered and evaporated to dryness under reduced pressure in a rotary evaporator to afford 9.47 g of dry extract.

2.3. Animals

Albino rat (150 - 200 g) of both sex were used to study the anti-diarrhea and antipyretic activity. Healthy albino mice of either sex (20 - 30 g) were used for anti-inflammatory and antinociceptive activity. The animals were housed in polypropylene cages (five in cage) under a 12 h light/12h dark cycle in a controlled temperature room (25 ± 2˚C). All the animals were acclimatized to the laboratory conditions for a week before use. They had free access to food and water. All studies were carried out by using five groups of six animals (3 males and 3 females).

2.4. Antidiarrheal Study

2.4.1. Caster Oil-Induced Diarrhea Test

This test was carried out by using the method of [12]. The animals were fasted for 18 h prior to the test. Test, standard samples and control were administered orally 60 min before received of castor oil at a dose of 1 ml/animal (p.o). Then animals were placed in cages lined with adsorbent papers and observed for 4 h for the presence of diarrhea defined as watery (wet), unformed stool. The control group result was considered as 100%. The anti-diarrhea effect was expressed as the average percent inhibition of defecation, which is calculated by the following equation:

$$\% \text{ Inhibition} = \left[1 - \text{Vt}/\text{Vc}\right] \times 100$$

Where Vt and Vc represent mean number of defecation caused by castor oil in control and standard or test, respectively. Control received normal saline (1 ml/kg, p.o) and loperamide (5 mg/kg, p.o) was used as standard drug.

2.4.2. Gastrointestinal Motility Test

This test was performed according to the method previously described using charcoal as a diet marker [13]. The animals were fasted for 18 h prior to the test. Test, standard samples and control were administered orally 60 min before received of castor oil at a dose of 1 ml/animal (p.o). After 60 min of drug administration, all animals were received 1 ml of charcoal meal (10% charcoal in 5% gum acacia) orally. Sixty minute later, all animals were sacrificed, and the intestine was removed without stretching and placed lengthwise on moist filter paper. The gastrointestinal motility was expressed as the average percent retardation of intestinal transit, which is calculated by the following equation:

$$\% \text{ Retardation} = \left[\text{B}/\text{A}\right] \times 100$$

Where B and A represent the average distances (cm) travelled by the charcoal meal from the pylorus to caecum

and total length (cm) of the small intestine, respectively. Control received normal saline (1 ml/kg, p.o) and loperamide (5 mg/kg, p.o) was used as standard drug.

2.5. Antipyretic Study

The procedure described by [14] was adopted for this study. Fever was induced in the rats by injecting 20% (w/v) suspention of Brewer's yeast (*Saccharomyces cerevisiae*) at a dosage of 1 ml/kg body weight subcutaneously. The rectal temperature of each rat was recorded by clinical thermometer before and after 18 h of yeast adminstration. Rats that did not show a minimum increase of 0.5˚C in temperature 18 h after yeast injection were discarded. Rectal temperature of all the rats was then recorded at the time of 1, 2 and 3 h after oral administration of both test and standard drugs. The control group result was considered as 100%. The antipyretic effect was expressed as the average percent reduction in rectal temperature, which is calculated by the following equation:

$$\%\ \text{Reduction} = \left[B - C_n / B - A\right] \times 100$$

Where B and A represent mean number of rectal temperature after and before 18 h of yeast adminstration respectively, while C_n represent rectal temperature after 1, 2 and 3 h oral administration of drug. Control received normal saline (1 ml/kg, p.o) and paracetamol (33 mg/kg, p.o) was used as standard drug.

2.6. Anti-Inflammtory Study

2.6.1. Carrageenan-Induced Paw Edema Test

The test was determined according to the technique of [15]. After 0.5 h, 0.1 ml of 1% (w/v) carrageenan suspension was injected subcutaneously to the plantar surface of the left hind paw. The paw volume was measured using a plethysmometer (model 7140, Ugo Basile, Italy), immediately and 0.5, 1, 1.5 and 2 h after drug treatment. The anti-inflammatory effect is expressed as the average percent inhibition of edema, which is calculated by the following equation:

$$\%\ \text{Inhibition} = \left[1 - \text{Vt}/\text{Vc}\right] \times 100$$

Where Vt and Vc represent the increase in paw volumes of mice treated with drug and control, respectively. Control received normal saline (1 ml/kg, p.o) and diclofenac sodium (10 mg/kg, p.o) was used as standard drug.

2.6.2. Xylene-Induced Ear Edema Test

A published method by [16] was adopted. After 0.5 h, 0.03 ml xylene was applied to the anterior and posterior surfaces of the right ear. The left ear was considered as control. Two hours after xylene application, mice were killed and both ears were removed. Circular sections were taken, using a cork borer with a diameter of 7 mm, and weighed. The increase in weight caused by the irritant was measured subtracting the weight of the untreated left ear section from that of the treated right ear sections. The anti-inflammatory effect is expressed as the average percent inhibition of writhes, which is calculated by the following equation:

$$\%\ \text{Inhibition} = \left[1 - \text{Vt}/\text{Vc}\right] \times 100$$

Where Vt and Vc represent the average writhes in the drug and control groups, respectively. Control received normal saline (1 ml/kg, p.o) and diclofenac sodium (10 mg/kg, p.o) was used as standard drug.

2.7. Antinociceptive Study

2.7.1. Hot-Plate Test

The hot plate test was assessed according to the method described by [16], with minor modification. The temperature of a metal surface was maintained at 55˚C ± 0.5˚C. Latency to a discomfort reaction (jumping, withdrawal or licking of the paws) was determined before and after drug administration. A cut-off time was 15 sec, to avoid damage of the paw. Reaction time and the type of response were noted using a stopwatch. The latency was recorded before and 0.5, 1, 1.5 and 2 h after oral administration of both test and standard drugs. Average reaction times were then calculated and the percentage variation calculated using following relation:

$$\%\ \text{Inhibition} = \left[\left(\text{Before treatment}/\text{after treatment}\right) - 1\right] \times 100$$

Control received normal saline (1 ml/kg, p.o) and diclofenac sodium (10 mg/kg, p.o) was used as standard drug.

2.7.2. Writhing Test

The test was performed as described by [7]. Test, standard samples and control were administered orally 30 min before intraperitoneal administration of 0.7% (v/v) acetic acid (volume of injection 0.1 ml/10g body weight). The mice were placed individually into glass beakers and 5 min were allowed to elapse. The number of stretching or writhing was recorded for the next 10 min. A percentage reduction in the writhing number was considered evidence for analgesia, which is calculated by the following equation:

$$\%\ \text{Inhibition} = \left[1 - \text{Vt}/\text{Vc}\right] \times 100$$

Where Vt and Vc represent the average number of writhes in the drug and control groups, respectively. Control received normal saline (1 ml/kg, p.o) and di-

clofenac sodium (10 mg/kg, p.o) was used as standard drug.

2.8. Anti-Denaturation Study

This test was carried out by using the method of [17] with slight modification. Briefly, 0.2% w/v bovine serum albumin (BSA) was prepared in Tris-buffer saline. pH was adjusted at 6.8 using glacial acetic acid. Stock solution of 10,000 µg/ml of methanolic extract (MEIG) was prepared by using methanol as a solvent. From this stock solution 3 different concentrations of 100, 200 and 400 µg/ml were prepared by using methanol as a solvent. In Eppendorf tube, 50 µL of the extract and 5 mL of 0.2% (w/v) BSA solution and 50 µL of methanol were added. Test tubes containing the sample mixture were heated at 72˚C for 5 minutes. After cooling for 10 min, the absorbance at 660 nm was measured. The experiment was performed in triplicate. The inhibition percentage of precipitation (denaturation of the protein) was determined on % basis relative to the control using the following formula:

$$\% \text{ Inhibition} = \left[1 - \text{Vt}/\text{Vc}\right] \times 100$$

Where Vt and Vc represent the average denaturation of the drug and control groups, respectively. Control was 50 µL of methanol and Ibuprofen (100 µg/ml) was used as standard drug.

2.9. Anti-Platelet Aggregation Study

A published method by [18] was adopted. Blood was taken collected from healthy human volunteers who have not taken any medication two weeks prior to participation in the study. The blood was mixed with 3.8% (w/v) sodium citrate solution in a ratio of 9:1 and centrifuged at 260 × g for 15 min at 20˚C in order to obtain the supernatant which contained platelets (*i.e*. platelet rich plasma (PRP)). The remaining blood was centrifuged at 1200 × g for 10 min to obtain another supernatant which did not contained platelets (*i.e*. platelet poor plasma (PPP)). The cuvettes were incubated at 37˚C for 5 min. The platelet aggregation was initiated by adding 20 µl of 10 µM adenosine di-phosphate (ADP) to 1 ml of PRP. The platelet aggregation was recorded for 5 min at 600 nm. The effect of different concentration (100, 200 and 400 µg/ml) of methanolic extract (MEIG) was studied by incubation with PRP at 37˚C for 5 min before the addition of ADP. The inhibition percentage of maximal aggregation was calculated using the following equation:

$$\% \text{ Inhibition} = \left[1 - \text{Vt}/\text{Vc}\right] \times 100$$

Where Vt and Vc represent the maximal aggregation of the drug and control groups, respectively. Control was the platelet poor plasma (PPP) and commercial heparin (20 µg/ml) was used as standard drug.

2.10. Statistical Analysis

The data were expressed as mean values ± SEM and tested with analysis of variance followed by Dunnett's t-test. P-values < 0.05, 0.01 were considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Antidiarrheal Study

The results of this study are presented in **Tables 1** and **2**, respectively. The results revealed that the extract at the doses of 100, 200 and 400 mg/kg, produced a dose dependent decrease in the number of faecal matters and decreased propulsion of charcoal meal of the gastrointestinal tract passed by the animals in castor oil-induced

Table 1. Effect of the methanolic extract of *Inula graveolens* (MEIG) on castor oil-induced diarrhea in rats.

Groups	Dose mg/kg	No. of faecal droppings in 4 h	% Inhibition
Group I	Control	19.2 ± 1.21	-
Group II	Standard	5.7 ± 0.64**	70.31
Group III	100	13.6 ± 0.73	29.16
Group IV	200	9.4 ± 0.45*	51.04
Group V	400	6.1 ± 0.87**	68.22

N = 6, values are mean ± SEM, *P < 0.05, **P < 0.01, dunnet test as compared to control.

Table 2. Effect of the methanolic extract of *Inula graveolens* (MEIG) on charcoal meal-stimulated gastrointestinal transit.

Groups	Dose mg/kg	Intestinal length (cm)	Distance traveled by charcoal (cm)	% Retardation
Group I	Control	89.34 ± 1.31	76.61 ± 1.2	85.75
Group II	Standard	84.25 ± 1.12	36.13 ± 1.08**	42.88
Group III	100	84.20 ± 1.26	52.31 ± 1.11	62.12
Group IV	200	86.18 ± 1.72	47.22 ± 1.49*	54.79
Group V	400	84.33 ± 1.34	39.15 ± 1.72**	46.42

N = 6, values are mean ± SEM, *P < 0.05, **P < 0.01, dunnet test as compared to control.

diarrheal model, respectively. At doses (200 and 400 mg/kg) of the extract, a significant ($P < 0.05$ and $P < 0.01$) inhibitions (51.04% and 68.22%, respectively) of characteristic diarrheal feces were observed. The effect of the highest dose of the extract was similar to that of the standard drug, loperamide (5 mg/kg). Diarrhea results from an imbalance between the absorptive and secretory mechanisms in the intestinal tract accompanied by hurry resulting in an excess loss of fluid in the faeces. In some diarrhea the secretory component predominates while other diarrhea is characterized by hypermotility [19]. Castor oil is a triglyceride characterized by a high content of the hydroxylated unsaturated fatty acid ricinoleic acid [20]. After oral ingestion of castor oil, ricinoleic acid is released by lipases in the lumen, and considerable amount of ricinoleic acid are absorbed in the intestine [21]. Presence of ricinoleate in small intestine, the peristaltic activity of small intestine increases as a result of permeability of Na^+ and Cl^- changed in the intestinal mucosa. Secretion of endogenous prostaglandin is also stimulated by ricinoleate [22]. Prostaglandins of the E series are considered to be good diarrheogenic agents in experimental animals as well as in human beings. The inhibitors of prostaglandins biosynthesis are therefore considered to delay castor oil-induced diarrhea. Prostaglandins are associated with changes in the bowel that stimulate diarrhea. Recent study shows that the laxative effect of ricinoleic acid present in castor oil is due to the induction of contraction of intestinal smooth muscle which is mediated by activation of EP3 receptors on intestinal smooth muscle [23]. Many anti-diarrheal agents act by reducing the gastrointestinal motility and/or the secretion. Inhibitors of prostaglandin biosynthesis delay castor oil induced diarrhea [12]. Methanolic extract of *Inula graveolens* (MEIG) exhibit significant anti-diarrheal activity. Earlier reports suggest that anti-diarrheal properties of medicinal plants might be ascribed to tannins, alkaloids, flavonoids, saponin and steroids [24]. The methanolic extract of *Inula graveolens* (MEIG) contain higher amount of both phenolic and flavonoid compounds which may be responsible for its effect [25]. Flavonoids, present in plant extract, are reported to inhibit release of autacoids and prostaglandins, thereby may inhibit motility and secretion induced by castor oil [26]. However, the components responsible for the anti-diarrheal activity of *Inula graveolens* L. are currently unclear. Therefore, further work must be carried out to isolate and identify these components.

3.2. Antipyretic Study

The effect of methanolic extract of *Inula graveolens* L. is illustrated in **Table 3**. It provoked dose dependent reduction in body temperature when studied at 100, 200 and 400 mg/kg, p.o. during various assessment times (1 - 3 h). Maximum protection (82.14%) was observed after 3 h of drug administration at 400 mg/kg ($P < 0.01$). Standard drug, paracetamol produced 88.23% protection after 3 h drug treatment 33 mg/kg (p.o). Fever has been recognized as a major sign of diseased condition right from the very beginning of human civilization. The febrile response is coordinated by the central nervous system through endocrine, neurological, immunological and behavioral mechanisms [27]. The initiation, manifestations and regulation of the febrile response are dependent on the pyrogenic and anti-pyrogenic properties of various exogenous and endogenous substances. There is a general consensus that fever is caused by a regular rise in body temperature above normal daily, fluctuations originating in conjucation with an elevated thermoregulatory set point [28,29]. The neurons of thermoregulatory system center are sensitive not only to changes in blood temperature but to cold and warm receptors located in the skin and muscle and thus maintain an appropriate balance between the heat production and loss [30]. In routine practice, non-steroidal anti-inflammatory drugs (NSAID) are commonly prescribed for the treatment of different conditions. It has been suggested that prostaglandins inhibition are involved in the antipyretic mechanisms of NSAID through cyclooxygenase pathway [31]. As a result, it could be speculated that the pharmacologically active components from this extract (MEIG) might contain active principle(s) that demonstrated inhibitory action on cyclooxygenase and thus, they produced antipyretic activity by preventing the formation of prostaglandins or by increasing the concentration of the body's

Table 3. Effect of the methanolic extract of *Inula graveolens* (MEIG) on yeast-induced pyrexia in rats.

Groups	Dose mg/kg	Rectal temperature (˚C)		Rectal temperature after administration of drug (˚C)			% Reduction		
		Normal (A)	18 h after yeast administration (B)	1 h (C_1)	2 h (C_2)	3 h (C_3)	1 h (C_1)	2 h (C_2)	3 h (C_3)
Group I	Control	37.87 ± 0.14	38.60 ± 0.12	38.57 ± 0.15	38.58 ± 0.11	38.55 ± 0.12	-	-	-
Group II	Standard	37.62 ± 0.16	38.30 ± 0.17	37.87 ± 0.14**	37.80 ± 0.12**	37.70 ± 0.16**	63.23	73.52	88.23
Group III	100	37.67 ± 0.19	38.22 ± 0.18	38.12 ± 0.18	38.02 ± 0.17	38.00 ± 0.13	18.18	36.36	40.00
Group IV	200	37.37 ± 0.13	38.35 ± 0.15	38.01 ± 0.12	37.85 ± 0.16*	37.70 ± 0.20*	34.69	51.02	66.32
Group V	400	37.17 ± 0.20	38.01 ± 0.18	37.62 ± 0.15**	37.47 ± 0.16**	37.32 ± 0.14**	46.42	64.28	82.14

N = 6, values are mean ± SEM, *P < 0.05, **P < 0.01, dunnet test as compared to control.

own antipyretic component [28,30]. Flavonoids are known to target prostaglandins which are involved in the pyrexia [32]. Hence the presence of flavonoids in the methanolic extract of *Inula graveolens* (MEIG) may be contributory to its antipyretic activity.

3.3. Anti-Inflammtory Study

The results of this study for carrageenan and xylene tests are presented in **Tables 4** and **5**, respectively. The extract (400 mg/kg) prevented the formation of edema induced by carrageenan and thus showed significant anti-inflammatory activity ($P < 0.01$). The methanolic extract (MEIG) reduced the edema induced by carrageenan by 29.46% after 3 h injection of noxious agent as compared to the control group that received normal saline (1 ml/kg). On the other hand, applied of xylene to the anterior and posterior surfaces of the right ear in control animals, produced a local edema that increased progressively to reach a maximal intensity 2 h after the applied of the phlogistic agent. The extract showed a significant ($P < 0.05$ and $P < 0.01$) dose dependent reduction at the doses of 200 and 400 mg/kg, respectively as compared to the control group that received normal saline (1 ml/kg). Diclofenac sodium (10 mg/kg) also caused a significant inhibition of paw and ear edema volumes, respectively. Inflammation is a normal protective response to tissue injury and it involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair [33]. According to our findings, the methanolic extract (MEIG) produced potential anti-inflammatory effect when assessed in both carrageenan and xylene tests in experimental mice, as shown in **Tables 4** and **5**, respectively. The most widely used primary test to screen new anti-inflammatory agents measure the ability of acompound to reduce local edema induced in mice paw by injection of an irritant agent [15]. Carrageenan induced inflammation is a useful model to detect oral action of anti-inflammatory agents [34]. The development of oedema in the paw of the mice after the injection of carrageenan is due to release of histamine, serotonin and prostaglandin like substances [35]. The early phase (0.5 - 1 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandin in the damaged tissue surroundings. The late phase is sustained prostaglandin release and mediated by bradykinin, leukotrienes, polymorph nuclear cells and prostaglandins produced by tissue macrophages [36]. A number of natural products are used in various traditional medical systems to treat relief of symptoms from pain and inflammation. The significant ameliorative activity of the methanolic extract (MEIG) and standard drug observed in the present study may be due to inhibition of the mediators of inflammation such as histamine, serotonin and prostaglandin. The carrageenan assay is a good method for the comparative bioassay of anti-inflammatory. The xylene-induced ear edema method [16], has been widely employed to assess the transudative, oxidative and proliferative components of chronic inflammation.

3.4. Antinociceptive Study

The results of this study for acetic acid induced writhing and hot plate tests are presented in **Tables 6** and **7**, respectively. The methanolic extract (MEIG) at the doses of 100, 200 and 400 mg/kg caused an inhibition on the writhing response induced by acetic acid. The maximal inhibition (43.75%) of the nociceptive response was achieved at a dose of 400 mg/kg ($P < 0.01$). The extract

Table 4. Effect of the methanol extract of *Inula graveolens* (MEIG) on carrageenan-induced paw edema in mice.

Groups	Dose mg/kg	Oedema diameter (cm)					% Inhibition			
		0 h	0.5 h	1 h	1.5 h	2 h	0.5 h	1 h	1.5 h	2 h
Group I	Control	0.82 ± 0.01	0.88 ± 0.03	0.9 ± 0.03	0.96 ± 0.02	1.01 ± 0.01	-	-	-	-
Group II	Standard	0.68 ± 0.03	0.65 ± 0.02	0.64 ± 0.02	0.61 ± 0.05**	0.59 ± 0.02**	41.59	36.46	28.89	26.32
Group III	100	0.80 ± 0.08	0.76 ± 0.01	0.74 ± 0.06	0.71 ± 0.04	0.70 ± 0.03*	30.7	26.05	17.78	13.64
Group IV	200	0.75 ± 0.07	0.70 ± 0.03	0.69 ± 0.05	0.66 ± 0.01*	0.62 ± 0.04*	38.62	31.25	23.34	20.46
Group V	400	0.64 ± 0.02	0.62 ± 0.04	0.59 ± 0.07	0.57 ± 0.02**	0.54 ± 0.03**	46.54	40.63	34.45	29.46

N = 6, values are mean ± SEM, *P < 0.05, **P < 0.01, dunnet test as compared to control.

Table 5. Effect of the methanolic extract of *Inula graveolens* (MEIG) on xylene-induced ear swelling in mice.

Groups	Dose mg/kg	Ear swelling (mg)	% Protection
Group I	Control	6.7 ± 0.51	-
Group II	Standard	3.6 ± 0.62**	46.26
Group III	100	5.4 ± 0.45	19.40
Group IV	200	4.8 ± 0.52*	28.35
Group V	400	4.0 ± 0.6**	40.29

N = 6, values are mean ± SEM, *P < 0.05, **P < 0.01, dunnet test as compared to control.

Table 6. Effect of the methanolic extract of *Inula graveolens* (MEIG) on latency to hot-plate test.

Groups	Dose mg/kg	Mean latency(s) before and after drug administration					% Inhibition			
		0 h	0.5 h	1 h	1.5 h	2 h	0.5 h	1 h	1.5 h	2 h
Group I	Control	1.78 ± 0.17	1.56 ± 0.26	1.45 ± 0.19	1.26 ± 0.22	1.07 ± 0.21	-	-	-	-
Group II	Standard	4.12 ± 0.33**	5.49 ± 0.1**	4.63 ± 0.64**	3.68 ± 0.62**	1.76 ± 0.08**	56.79	71.58	68.68	65.76
Group III	100	2.32 ± 0.62	2.61 ± 0.38	1.78 ± 0.72	1.43 ± 0.25	1.21 ± 0.07	23.27	40.22	18.53	11.88
Group IV	200	2.45 ± 0.51	1.97 ± 0.46	2.27 ± 0.54	1.86 ± 0.81	1.32 ± 0.20	27.34	20.81	36.12	32.25
Group V	400	2.91 ± 0.47	3.17 ± 0.43**	4.35 ± 0.32**	2.24 ± 0.73**	1.97 ± 0.24**	38.83	50.78	66.66	43.75

N = 6, values are mean ± SEM, *P < 0.05, **P < 0.01, dunnet test as compared to control.

Table 7. Effect of the methanolic extract of *Inula graveolens* (MEIG) on acetic acid-induced writhing in mice.

Groups	Dose mg/kg	No. of writhing	% Protection
Group I	Control	36.2 ± 0.44	-
Group II	Standard	6.4 ± 0.39**	82.32
Group III	100	26.7 ± 0.33*	26.24
Group IV	200	19.5 ± 1.2**	46.13
Group V	400	15.8 ± 1.02**	56.35

N = 6, values are mean ± SEM, *P<0.05, **P < 0.01, dunnet test as compared to control.

showed a significant dose-dependent reduction in the number of writhing with approximately 26.24%, 46.13% and 56.35% of inhibition respectively. The oral dose of methanolic extract at 400 mg/kg ($P < 0.01$) elicited a significant analgesic activity as evidenced by increase in latency time on comparison with negative control at the end of 0.5, 1, 1.5 and 2 h. The increase in latency time was found in a dose dependent manner. Acetic acid causes an increase in the peritoneal fluid level of prostaglandins (PGE2 & PGF2a) as well as lipooxygenase products, involving in part peritoneal receptors and inflammatory pain by inducing capillary [37]. Collier *et al*. [38] postulated that acetic acid acts indirectly by inducing the release of endogenous mediators, which stimulate the nociceptive neurons. The most important transmission pathways for inflammatory pain are that comprising peripheral polymodal nociceptors sensitive to protons, such as acid sensitive ion channels and to algogen substances, such as bradykinin and cytokines. Although the writhing test has poor specificity (e.g., anticholinergic, tricyclic antidepressants and antihistaminic and other agents show activity in this test), it is a very sensitive method of screening the antinociceptive of compounds [39]. The hot-plate test is commonly used to assess narcotic analgesia. Although the central and peripheral analgesics respond by inhibiting the number of contractions provoked by chemical pain stimuli, only the central analgesics increase the time of response in the hot plate test [40]. These observations tend to suggest that the methanolic extract of *Inula graveolens* (MEIG) may possess centrally- and peripherally-mediated antinociceptive properties. The peripheral antinociceptive effect of the extract may be mediated via inhibition of cyclooxygenases and/or lipooxygenases (and other inflammatory mediators), which its central antinociceptive action may be due its possible action as partial agonist of adrenergic, serotoninergic, cholinergic and dopaminergic recptors [41].

3.5. Anti-Denaturation Study

In the present investigation, the effect of methanolic extract (MEIG) was evaluated against heat induced denaturation of Bovine serum albumin (BSA). The results are summarized in **Table 8**. The present findings exhibited a concentration dependent inhibition of BSA denaturation by the test extract throughout the concentration of 100, 200 and 400 µg/ml. The extract has shown a significant ($P < 0.01$) anti-denaturation activity (62.16% and 75.67%) on BSA at 200 and 400 µg/ml, respectively. Ibuprofen a standard drug showed the maximum inhibition 86.48% at the concentration 100 µg/ml. Protein denaturation is a process in which proteins loss their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Denaturation of tissue proteins is one of the well documented causes of inflammatory and rheumatoid arthritis. Production of auto-antigens in certain arthritic diseases may be due to denaturation of proteins *in vivo* [42]. Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development. The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding [43]. Several anti-inflammatory drugs have been reported to show dose dependent ability to inhibit thermally induced protein denaturation [44]. Bovine serum albumin (BSA) on denaturation expresses antigens associated to Type III hypersensitive reaction, related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus [45]. From the result of the present study, it can be stated that the methanolic extract (MEIG) is capable of controlling the production of auto antigen and thereby it inhibit the denaturation of proteins and its effect was compared with the standard drug Ibuprofen. The plant extract revealed to contain phenolic compounds, known to produce inhibitory effect on protein denaturetion [44].

Table 8. Effect of the methanolic extract of *Inula graveolens* (MEIG) on anti-denaturation of bovine serum albumin.

Groups	Dose µg/ml	Absorbance at 660 nm	% Inhibition
Group I	Control	0.37 ± 0.04	-
Group II	Standard	0.05 ± 0.02**	86.48
Group III	100	0.23 ± 0.04	37.83
Group IV	200	0.14 ± 0.01**	62.16
Group V	400	0.09 ± 0.06**	75.67

N = 6, values are mean ± SEM, *P < 0.05, **P < 0.01, dunnet test as compared to control.

Literature survey also suggests that, the antidenaturation property of BSA was due to the presence of two interesting binding sites in the aromatic tyrosine rich and aliphatic threonine and lysine residue regions of the BSA [17]. They have also reported that therapeutic molecules could be activating the tyrosine motif rich receptor dually with threonine that regulates signal transduction biological pathways for their overall biological action [46]. Further studies are needed to elucidate other mechanisms of the anti-denaturation activity of the methanolic extract (MEIG) and to identify the active constituents responsible for the anti-denaturation effect.

3.6. Anti-Platelet Aggregation Study

Platelets are essential for normal haemostasis. Activation of the clotting cascade by trauma, results in platelet activation, which is followed by aggregation. Results of platelet aggregation were expressed as a percent of aggregation at a given time interval (5 min) from reagent addition (**Table 9**). The percentage aggregation inhibition of the methanolic extract (MEIG) at 400 µg/ml and standard, commercial heparin at 20 µg/ml ($P < 0.01$) were 70.58% and 82.35% respectively. Platelets play an important role in the process of a thrombosis by adhering to the damaged regions (caused by reactive oxygen species) of the endothelial surface. The activated platelets to platelets bond, binds also to leucocytes bringing them in to a complex process of plague formation and growth. The anti-platelet therapy constitutes the best available tool for ameliorating the mechanism related to atherogenesis and have interestingly inhibited platelet aggregation. Platelets stick to the damaged vessel wall, they stick to each other (aggregate) and release ADP, thromboxane A_2 (TXA_2) which promotes further aggregation, and thus a platelet plug is formed [47]. In the veins, due to sluggish blood flow, the fibrinous tail is formed which traps RBC's the red tail. In arteries platelet mass are the main constituents of the thrombus. Anti-platelet drugs are more useful in arterial thrombosis, while anti-coagulant are more effective in venous thrombosis [48]. The methanolic extract (MEIG) showed significant anti-platelet aggregation at 400 µg/ml. The mechanism behind this effect is yet not clear but it might be said that the compound(s) responsible for this effect are methanol-soluble, heat-resistant plant botanicals, which might be different from chemical anti-coagulating agents (salicylates). Natural compounds were the first historical source of antithrombotic compounds (heparin, vitamin K, antagonists, streptokinase and urokinase). Recently several natural anti-platelet agents from natural products including polyphenols [49] and flavonoids [50] have been reported. Polyphenols may inhibit platelet aggregation through a number of different mechanisms, including inhibition of cyclooxygenaes, lipoxygenase and phosphodiesterase activities [51]. Flavonoids anti-aggregation effects may be attributed to inhibition of thromboxane formation, thromboxane receptor antagonism, blunting hydrogen peroxide production, or inhibition of phospholipase C [50,52].

Table 9. Effect of the methanolic extract of *Inula graveolens* (MEIG) on platelet aggregation.

Groups	Dose µg/ml	Absorbance at 660 nm	% Inhibition
Group I	Control	0.34 ± 0.03	-
Group II	Standard	0.06 ± 0.01**	82.35
Group III	100	0.24 ± 0.06	29.41
Group IV	200	0.19 ± 0.01**	44.11
Group V	400	0.10 ± 0.04**	70.58

N = 6, values are mean ± SEM, *P < 0.05, **P < 0.01, dunnet test as compared to control.

4. CONCLUSION

From the results obtained in the present study, it may be concluded that the methanolic extract of *Inula graveolens* L. possessed good pharmacological activities, which might be helpful in preventing or slowing the progress of various inflammatory, nociceptive, albumin denaturation and plaetelet aggregation-related diseases and it showed dose dependent activities. Further investgation on isoltion and identification of the active component(s) in the plant may lead to chemical entities with potential for clinical use.

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