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Some Pharmacological & Toxicological Activities of *Calendula officinalis Linn.* Flower 70% Ethanolic Extract

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Abstract: Herbal medicine is the most widely used form of medicine in the world today as it contains curative bioactive ingredients such as alkaloids, coumarins, Saponins and flavonoids. *Calendula officinalis Linn.* (Asteraceae) is used medicinally in Europe, China and India amongst several places in the world. It is also known as "African marigold" and has been a subject of several chemical and pharmacological studies. It is used in traditional medicine, especially for wound healing, jaundice, blood purification, and as an antispasmodic. Therefore, the aim of the present study was to explore phytochemical, toxicological and some pharmacological activities of *Calendula officinalis Linn*. 70% ethanolic extract. The toxicological pattern of the ethaolic extract *Calendula officinalis* flowers was studied by the determination of LD50 in mice by oral administration of upgrading doses 70% ethanolic extract with continuous monitoring.The oral anti-inflammatory (Formalin induced edema), antipyretic (Brewer's yeast induced hyperthermia) and analgesic (writhing test)effects of 70% ethanolic extract of *Calendula officinalis* were carried out on experimental animals. The obtained results revealed that phytochemical screening of 70% ethanolic extract of *Calendula officinalis Linn.* indicates the presence of alkaloid, tannin, Saponin, Glycosides, Resin and flavonoid. The toxicological studies revealed that the minimum lethal dose of *Calendula officinalis Linn*. 70% ethanolic extract was found to be 2000 mg/kg body weight and LD50 of the studied extract was found to be 2450mg/Kg B.Wt. The pharmacological studies performed on *Calendula officinalis* Linn. ethanolic extract exhibited significant antipyretic, analgesic and Anti-inflammatory when given in a dose 250mg/Kg B.Wt.

Keywords: *Calendula officinalis,* Phytochemical, Toxicological, LD50, Antipyretic, Analgesic, Anti-inflammatory

1. Introduction

Medicinal plants are used from ancient times in treatment of various human & animal disorder. Medicinal and aromatic plants are the most widely used form of medicine in the world today where medicinal and aromatic plants contain biologically active chemical substances such as saponins, tannins, essential oils, flavonoids, alkaloids and other chemical compounds [1]. Natural products are gaining a revitalized attention in medical community and their therapeutic uses are gradually increasing. As many synthetic drugs have revealed serious side effects. Therefore, a better strategy is to look for natural substances with strong pharmacological action and less cytotoxicity. In the last few years much attention was directed to the potential health promoting properties of phenolic phytochemicals [2, 3, 4]. Search for safe herbal remedies with potent anti-inflammatory and antipyretic received momentum recently as the available paracetamol and aspirin have toxic effect to various organ of the body [5]. Calendula was cultivated by the Egyptians, Greeks, Hindus and Arabs, Calendula grew in European gardens and has been used medicinally since the 12th century [6]. The name Calendula is from the Middle English calends derived from Latin kalendae, which means the day of the new moon [7]. The flowers are used in diverse preparations, mainly ointments for the treatment of diverse dermatological conditions such as wounds, ulcers, eczema, burns, bruises, eruptions, varicoses and hemorrhoids [8]. Many other properties have been attributed to the flower preparations such as choleretic, anti-inflammatory, analgesic, anti-cancer, bactericidal,

diuretic and tonic actions [9]. For establishing and confirming of therapeutic role of *Calendula officinalis Linn*.in modern medicine, experimental studies are required. The aim of the present study is to Explore and confirm phytochemical, toxicological and some pharmacological activities *Calendula officinalis Linn.* 70% ethanolic extract

2. Materials and Methods

- Plant
- Calendula officinalis Linn. flower:

The flowers were collected & identified in faculty of pharmacy farm – Cairo University, then air dried, pulverized & stored tightly closed in glass container till subjected to phytochemical, toxicological & pharmacological studies

- Preparation of flower extract

In a glass jar dried *Calendula officinalis Linn.* flowers powder were extracted by percolation several times till exhaustion [10] using 70% ethanolic solution then filtered, most of the solvent was removed using Rotatory evaporator apparatus attached with vacuum pump & low temperature 50 c

- Animals
- Mice:

Mature mice of both sexes and weighing (20-25 gm) were used for studying the acute toxicity, LD 50 and analgesic activity.

- Rats*:* Mature albino rats of both sexes and weighing (200-250 gm) were used to reveal antipyretic and anti-inflammatory activity.

Phytochemical studies:

Phytochemical screening of the *Calendula officinalis* flower was carried out to identify the constituents, using standard phytochemical methods as described by [11, 12].The screening involves detection of alkaloids, Saponin, tannins, flavonoids, Carbohydrates /glycosides and resin as shown in Table(1).

Table (1). *Showing Preliminary phytochemical tests on Calendula officinalis Linn. flower.*

Test for	Positive result
1- Alkaloid and /or nitrogenous bases:	
Mayer's test :	
Filtrate was added to Mayer's reagent (Potassium Mercuric iodide)	Yellow cream precipitate
Wagner's test :	
Filtrate was added to Wagner's reagent (Iodine in potassium iodide)	reddish brown precipitate
Dragendorff's test:	
Filtrate was added to Dragendroff's reagent (solution of potassium bismuth iodide)	Orange precipitate
2-Flavonoids:	
Lead acetate test:	
Extract was added to few drops of lead acetate solution	Yellow precipitate
Shinoda test:	
Alcoholic solution of extract was added to few fragments of magnesium ribbon and concentrated hydrochloric acid	Magenta color after few min.
3- Carbohydrates/Glycosides:	
Fehling's test:	
5ml of filtrate was added to equal volume of Fehling solution A & B then heated in water bath	Red precipitate
Benedict's test:	
5ml of filtrate was added to equal volume of Benedict solution then heated in water bath	Red orange precipitate
Molish Test:	n
Filtrate was added to 2 drops of alcoholic α-naphthol solution in a test tube then 2 ml of concentrated sulphuric acid was added carefully along the sides of the test tube	Violet ring at the junctio
4-Saponins:	
Foam test:	
Small amount of extract was shaken with little quantity of water	Persistent foam for ten min.
5- Tannins:	
2 ml of the aqueous extract was stirred with 2 ml of distilled water and few of ferric chloride solution were added	Ink color
6-Resins:	
0.5g extract was diluted with 10ml with water and shaken for 5 minutes	Turbidity formed

Toxicological study:

- Determination of LD50:

Pilot studies was performed to determine minimum lethal dose of *Calendula officinalis Linn.* 70% ethanolic extract. LD50 of *Calendula officinalis Linn*. were determined [13]. For this purpose 5 groups of five mice each weighing 20-25gm injected intraperitoneally in upgrading doses ranging from 1500 to 3500 mg /Kg body weight. Another group was left as control and given diluent only. The toxic symptoms, mortality rate and post-mortem findings in each group was recorded 24 hours post administration. LD50 of tested extract was calculated according to the following formula:

$$LD50 = DM- (\sum AXB)/N$$

Where,
DM: largest dose which kill all animals
A: Mean of dead animals between 2 successive groups
B: Dose difference between 2 successive groups
N: number of animals in each group
∑: summation of multiplying A and B

Pharmacological studies:

- Antipyretic activity:

The method described by [14] was used for studying the antipyretic effect of 70% ethanolic extract of *Calendula officinalis*. Fifteen rats of both sex weighing 200-250 grams, rats were divided into three groups each of five rats. Basal rectal temperature was recorded for each rat, then all rats were made hyperthermic by subcutaneous injection of brewer's yeast in a physiological saline in a dose of 15 g/kg body weight. After 17 hours the body temperature of each rat was measured rectally using a medical thermometer. The first group was kept as control, the second group was given metamezole sodium 50 mg/Kg body weight as a standard antipyretic. The third group was orally administered 250 mg/Kg B.Wt. of *Calendula officinalis Linn*. 70% ethanolic extract. The body temperature of each rat was then recorded every hour for 3 successive hours.

- Analgesic activity (Writhing test):

This experiment was carried out as described by [15]. Fifteen mice of both sex weighing 20-25 g body weight each which previously showed positive writhing (stretch torsion to one side drawing up of hind limb, retraction of abdomen and opisthotonus, so that the belly of the mouse touch the floor) with glacial acetic acid were selected and divided into three groups. Mice of the first group were kept as control non-treated, those of the second group was orally administrated ketoprofen 50 mg/kg body weight as a standard group. Mice of the third group was orally administered *Calendula officinalis Linn.* ethanolic extract 250mg/Kg body weight. After 30 minutes, each mouse was intraperitoneally injected with 0.25 ml of 0.7% glacial acetic acid in distilled water, and the mice were then placed in transparent boxes for observation. The number of writhes were observed after 1, 2, 3, 4 and 5 hours post administration. Number of writhes for each animal in all groups were recorded and the analgesic potency of the tested extract was determined as protection% against writhing according to the following formula:

$$\%\ \text{of protection} = \frac{\text{Control mean} - \text{treated mean}}{\text{Control mean}} \times 100$$

- Anti-inflammatory effect:

The method described by [16] was used. Fifteen rats of both sex weighing 200-250 grams body weight were used. Edema or inflammation was induced in the right hand paw of all rats by subcutaneous injection of 0.1 ml formalin 6% solution in normal saline. After four hours, the thickness of each rat paw was measured in mm using Vernier caliber to detect the inflammatory process achieved by the formalin solution. Rats were then divided into three equal groups of five rats each, Rats of the first group were left as control with induced inflammation only, those of the second group was orally administered diclofenac sodium (Voltarin®) in a dose of 30 mg /kg body weight as a standard. The third group was orally administrated ethanolic extract of *Calendula officinalis Linn.* at a dose 250 mg/kg body weight. Thirty minutes after drug or test compound administration, 0.1ml of 6% formalin solution in normal saline was injected subcutaneously in the right hind paws of all groups for induction of edema. The thickness of each rat paw was measured in mm by Vernier caliber after 1, 2, 3, 4, 5, and 6 hours post administration.

Statistical analysis:

SPSS 16.0 software package was used for statistical analysis. Differences among means were determined using one. Differences were considered significant at $p \leq 0.05$ and highly significant at $p \leq 0.01$

3. Results and Discussion

3.1. Phytochemical Screening

Preliminary phytochemical tests for detection of active principles in the *Calendula officinalis Linn.* flower were carried out & recorded in Table (2). The obtained results revealed the presence of tannin, Saponin, Glycosides, Resin and flavonoid and absence of alkaloids.

Table (2). *Showing results of Preliminary phytochemical on Calendula officinalis Linn. flower.*

Test for	*Calendula officinalis Linn.*
1-Alkaloid and /or nitrogenous bases	-ve
2-Flavonoids	+ve
3- Carbohydrates/Glycosides	+ve
4-Saponins	+ve
5- Tannins	+ve
6-Resins	+ve

These obtained finding correlates with that obtained by [17, 18, 19] who reported that ethanolic extract of *Calendula officinalis Linn.* flower contains flavonoids.[18,19] specified that flavonoids present in *Calendula officinalis* flower are isorhamnetin-3-One-O- hesperidoside, quercetin. [19] suggested that these ingredients are responsible for *Calendula officinalis* antioxidant activities and [20] added that flavonoids has also antinociceptive and anti-inflammatory effect. [18] added that *Calendula officinalis* flower ethanolic extract contains other flavonoids as isoquercetin, isorhamnetin-3-O-D-glycoside, narcissin, calendoflaside, calendoflavoside, calendoflavobioside, rutin, isoquercitrin, neohesperidoside, isorhamnetin-3-O-2 Grhamnosyl rutinoside, isorhamnetin-3-Orutinoside, quercetin-3-O-glucoside and quercetin-3-O-rutinoside. *Calendula officinalis* methanolic extract contains also some of these flavonoids as Quercetin-3-o-glucoside, Rutin, and Isohamnetin-3-o-glucoside and also gallic acid as mentioned by [21, 22] who added that these ingredients are present also in aqueous extract. [17] stated that *Calendula officinalis* ethanolic, aqueous and methanolic extract contains also Glycosides and Saponin. [6, 23] confirmed presence of these chemical compounds in methanolic extract. [24] reported that phytochemical analysis of *Calendula officinalis Linn.* aerial parts aqueous extract contains also flavonoids and tannins, but he diversely mentioned that it doesn't contain Saponin and contains alkaloids

3.2. Toxicological Study

Acute toxicity studies on the tested extract of *Calendula officinalis Linn.* was carried out in mice via intraperitoneal injection. The obtained data showed that minimum lethal dose of *Calendula officinalis Linn.* 70% ethanolic extract was found to be 2000mg/kg body weight. Symptoms of Toxicity observed following *Calendula officinalis Linn.* injection was characterized by tremors, convulsions, arched back, sweating rapid respiration, coma followed by death. Post mortem Examination revealed the presence of some petechial hemorrhages on liver and congestion in lungs, heart & Kidneys. LD50 of the studied extract was found to be 2450mg/Kg body weight.

This indicates for a great extent that this flower is nontoxic as LD50 value is very high. In this respect [25] mentioned that plants with LD50 less than 10 mg /Kg body weight are considered highly toxic and others with LD50 greater than 50 mg/Kg body weight are considered nontoxic.

3.3. Pharmacological Studies

Antipyretic effect:

The antipyretic effect of 70% ethanolic extract of *Calendula officinalis Linn.* was studied using brewer's yeast to induce hyperthermia in rats Table (3).

Table (3). *Showing the antipyretic effect of 70% ethanolic extract of Calendula officinalis Linn.in Hyperthermic rats (Mean ± SE, N=5).*

Treatment	Dose in mg/Kg B.Wt.	Mean Rectal Temperature in °C			
		Before treatment	1 hour	2 hours	3 hours
Control –ve (Non treated)	0	38.86±0.024$^{(a)}$	39.06±0.06$^{(a)}$	39.06± 0.06$^{(a)}$	39.06±0.06$^{(a)}$
Metamezole sodium	50	39.02±0.091$^{(a)}$	36.88±0.08 $^{(b)}$	36.48±0.08$^{(b)}$	36.48±0.08$^{(b)}$
Calendula officinalis Linn.	250	39±0.063$^{(a)}$	38±0.16$^{(c)}$	37.1±0.29$^{(b)}$	36.76±0.11$^{(c)}$

Data were expressed as mean ±SE, means with different superscript letters (a, b, c) are significant different at P≤ 0.05

The subcutaneous injection of brewer's yeast suspension markedly elevated the rectal temperature after 17hr of administration. It was noticed that oral administration of both *Calendula officinalis* ethanolic extract (250 mg / kg) body weight & the standard metamezole sodium (50 mg/Kg) body weight induce marked antipyretic activity as they significantly decrease rectal temperature after 3 hours post oral administration as compared with control non treated group. Significance was indicated by lowering the body temperature after their administration at 2 hours (37.1±0.29 and 36.48±0.08) of 250mg/Kg of tested extract and 50mg /kg of standard metamezole respectively and at 3 hours (36.76±0.11and 36.48±0.08) of 250mg/Kg of tested extract and 50mg /kg of standard metamezole respectively.

Hyperthermia due to the infected or damaged tissue promotes the formation of pro-inflammatory mediators (cytokines like interleukin 1β, α, β and TNF-α) which increase the synthesis of PGE2 near pre-optic hypothalamus area thereby triggering the hypothalamus to elevate the body temperature [26]. Its effect may be due to antagonizing the prostaglandins effect on hypothalamus.

Analgesic effect:

The peripheral anti-nociceptive activity of *Calendula officinalis Linn.* ethanolic extract studied using writhing technique in mice. The obtained results were registered in Table (4).

Table (4). *Showing the analgesic activity of Calendula officinalis Linn. 70% ethanolic extract in mice using writhing test (n=5).*

Groups	Dose mg/Kg B.Wt	Protection percentage against writhing after 5 hours				
		1 hr	2hr	3hr	4hr	5hr
Control	0	0	0	0	0	0
Standard Ketoprofen	50	100	100	100	100	100
Calendula officinalis Linn.	250	20	20	20	20	20

Standard group orally administered Ketoprofen (50 mg/Kg b.wt) showed 100% protection against writhing induced by glacial acetic acid for 5 hours. Oral administration of *Calendula officinalis* ethanolic extract in a dose of 250 mg/Kg body weight exhibited analgesic activity with 20% protection percentage against writhing for 5 hours. These results correlates with that obtained by [27] who reported that *Calendula officinalis Linn.* flower ethanolic extract has anti-nociceptive effects.Prostaglandins and sympathomimetic system mediators such as PGE2 and PGF2a were released by intraperitoneal administration of acetic acids. Also, levels of these mediators were increased in the peritoneal fluid of the acetic acid induced mice [28]. Thus, the anti-nociceptive effect of the hydroalcoholic extract could be mediated by peripheral effects, including the prostaglandin synthesis inhibition [20].

[29] reported that plants showing the antipyretic effect also possess analgesic activity. Obtained results matches with those obtained by [30] who reported that crude extract of *Calendula officinalis Linn.* exhibited significant antipyretic (74.95% inhibition) effects at a dose of 300 mg/Kg and analgesic (27.42% inhibition) effects.

Anti-inflammatory effect:

The anti-inflammatory effect of 70% ethanolic extract of *Calendula officinalis Linn.* was studied using formalin 6% solution induced inflammation in rats paw and the data was compared with that of control in Table (5).

Table (5). *Showing the anti-inflammatory effect of 70% ethanolic extract of Calendula officinalis Linn. in rats.*

Treatment	Dose mg / Kg B.Wt.	Mean of right paw thickness in mm						
		Pre- treat	1hr. post-treat	2hr. post- treat	3hr. post-treat	4hr. post-treat	5hr.post-treat	6hr. post-treat
C -ve	0	7.75± 0.12$^{(a)}$	7.87± 0.15$^{(a)}$	7.87± 0.15$^{(a)}$	7.87±0.15$^{(a)}$	7.87± 0.15$^{(a)}$	7.87± 0.15$^{(a)}$	7.87± 0.15$^{(a)}$
Diclo-fenac sodium	30	7.75± 0.12$^{(a)}$	6.16±0.39$^{(b)}$	6.16± 0.39$^{(b)}$	5.74± 0.15$^{(b)}$	5.49± 0 $^{(b)}$	4.74± 0.32 $^{(b)}$	4.74± 0.32$^{(b)}$
C. officinalis Linn.	250	7.142±0.44$^{(a)}$	5.87±0.15$^{(b)}$	5.87± 0.15$^{(b)}$	5.49± 0 $^{(b)}$	4.61±0.22$^{(c)}$	4.412±0.02$^{(b)}$	4.4± 0.22$^{(b)}$

Data were expressed as mean ±SE, means with different superscript letters (a, b, c) are significant different at $P \leq 0.01$

Formalin injected subcutaneously in the planter surface of rat's paw in all groups rapidly induced a significant increase in the paw thickness.

It was noticed that oral administration of ethanolic extract of *Calendula officinalis* in a dose of (250 mg / kg) body weight and Diclofenac sodium in a dose of (30 mg /Kg) body weight induced a significant decrease in inflamed rat paw thickness when compared with control non treated group for 6 hours Induction of edema in rat's paw by formalin is a biphasic response, in which the first phase is mediated by histamine, serotonin and kinins whereas the second phase is mediated by prostaglandins (cyclooxygenase product of arachidonic acid metabolism) and production of reactive oxygen species [31,32]. The present findings show that anti-inflammatory actions associated with ethanol extract results from inhibition of one or more signaling intracellular pathways which are involved with effects from these mediators. *Calendula officinalis* extract potent anti- inflammatory response may be mediated by the inhibition of pro-inflammatory cytokines and Cox-2 and subsequent prostaglandin synthesis [33]. The ethanolic extract of *Calendula officinalis* ethanolic extract at both 250 mg/kg b. wt. possessed significant reduction in paw edema at 1and 3 hours when its potency is compared to standard diclofenac sodium. The effectiveness of the ethanolic extract to reduce edema at 1and 6 hours may attributed its antagonist effect to first phase products (histamine, serotonin, and kinins) or its antagonist effect to second phase products (prostaglandins)or its synthesis by inhibition of cyclooxygenase enzyme leading to subsequent reduction in prostaglandins production and may attributed to inhibit liberation of the reactive oxygen species (second phase mediator) from phagocytes invading the site of inflammation and leading to tissue damage [34, 35, 36]. These Results matches also with that obtained by [18, 37, 19] who reported that Ethyl acetate soluble fraction of the methanol extract of *Calendula officinalis* flowers exhibited the most potent inhibition (84%) of 12-o tetradecanoylphorbol-13-acetate(TPA)-induced inflammation and with [38] who reported that the ethanolic extract of *Calendula officinalis Linn.* possessed significant anti-inflammatory activity.

Also, the link between both anti-nociceptive activity and moderate anti-inflammatory effect observed with the extract has been indicated in non-steroidal anti-inflammatory drugs (NSAIDs). It is a well-established fact that NSAIDs exert their analgesic and anti-inflammatory activity by the inhibition of cyclooxygenase activity [39]. Based on the pharmacological tests results, the *Calendula officinalis* hydroalcoholic extract has antinociceptive and anti-inflammatory activities. [20] who reported also anti-nociceptive effect and anti-inflammatory effects of hydroalcoholic extract of *Calendula officinalis Linn.* aerial part in both chemical pain and anti-inflammatory tests.

4. Conclusion

Calendula officinalis Linn. Flower is a safe flower as it's LD50 is 2000 mg/Kg body weight with many pharmacological activities as it is proved to have antipyretic, analgesic and anti-inflammatory actions that may be a result of its active biochemical ingredients Flavonoids, glycosides, Saponin, Tannin and resin.

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