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A PHARMACOLOGICAL STUDY ON THE ANTI-INFLAMMATORY EFFECTS OF CALENDULA *OFFICINALIS* EXTRACT ON EXPERIMENTAL ANIMALS

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ABSTRACT:

Introduction: *Calendula officinalis* is one of those plants which have medicinal importance due to its *flowers* has pharmacological activity the important pharmacological parameters have been less investigated properly and can be scientifically proved by reverse pharmacology. It was found of interest to evaluate these properties of extract of flowers of *Calendula officinalis* scientifically in experimental models.

Methods: Study was conducted in Albino Wistar rats.Fresh *Calendula officinalis* flowers were collected, extraction and drug formulation was made. Anti-inflammatory activity of hydroalcoholic extract of *Calendula officinalis (HECO)* flowers was studied in the Carrageenan induced paw edema model & Cotton pellet induced granuloma models The differences between experimental groups were compared by ANOVA followed by Students "t" unpaired test.

Results: The *Calendula officinalis* extract of flowers at 200 and 400 mg/kg showed significant and highly significant inhibition ($p< 0.05$ and $p<0.001$, respectively) of paw edema after giving the carrageenan in the rat paw in comparison of control. In cotton pellets induced granuloma pouch method also, the Hydrochloric extract of *Calendula officinalis (HECO)* flowers showed very significant reduction of weight of granuloma ($p<0.01$) with 22.80% protection as compared with control.

Conclusion: We conclude that the Anti-inflammatory activity was noted with Hydrochloric extract of *Calendula officinalis* in a dose-dependent manner in acute (carrageenan-induced hind paw edema model) as well as sub-acute (cotton pellet induced granuloma model) inflammatory experimental models. They decrease the chemical mediators of inflammation

Key Words: Calendula Officinalis, anti inflammation, Paw Edema Model, granuloma pouch method , HECO

Introduction

Ayurveda provides the knowledge to use the medicinal plants around us to combat with different diseases. "Herb" is a plant valued since ancient times for its medicinal, savory and aromatic qualities. It is one of the oldest medicinal cares for human since 4000-5000 B.C[1]. It played an important role in improving the quality of human life and maintaining human health for thousands of years. Drugs made from herbs have made their importance felt in the last few decades whose prevalence is continuously increasing in both developing and developed countries due to they are natural in origin and lesser side effects. [2,3] It was also found during the periods of Charaka and Sushruta that many oral formulations of herbal medicines have been recommended for various pathologies and diseases.[4] In medical text of Ayurveda, around 17,000 species are well documented in context of their biological and medicinal properties[5]. Plant extracts are potentially curative and also preventive. It can also augment body's defense against various invading agents and improve immunity[6]. Despite such intensive research on various herbal medicines, many species of plants are still left unexplored. Therefore, plants named *Calendula officinalis* is chosen for the present study. The different parts of *Calendula officinalis* have been found useful in a variety of symptoms and disorders. *Calendula officinalis* is one of those plants which have medicinal importance due to its flowering part has pharmacological activity. The plant is rich in many pharmaceutical active ingredients like carotenoids, auroxanthin, flavoxanthin[7], flavonoids, glycosides, terpenoids esters, steroids and sterols, coumarins, amino acids. It is used as analgesic, antidiabetic, anti-inflammatory, antiseptic, bactericide, in skin problems[8], and as antifungal agent[9]. In the UK, the decoction of the flowers was used as a potent drink for the treatment of smallpox and measles, fresh juice as a remedy for jaundice, costiveness (constipation) and suppression of menstrual flow[10.] Usefulness of *Calendula officinalis* was reported in the form of decoctions, infusions, tinctures in traditional systems of medicines for treating skin diseases like psoriasis, leprosy, etc.[10,11,12,13.] The important pharmacological parameters of *Calendula officinalis* have been less investigated properly and can be scientifically proved by reverse pharmacology. Therefore, it was found of interest to evaluate these properties of extract of flowers of *Calendula officinalis* scientifically in experimental models.

Materials and methods

The present study was conducted in experimental animals i.e. Albino Wistar rats. Animals were procured from Datta Meghe Institute of Medical Sciences, Sawangi (Meghe), Maharashtra after taking permission from Institutional animal ethical committee. The animals were caged in polyvinyl wire mesh cages in the central animal room of the institute approved by CPCSEA. They were maintained under standard laboratory condition (12-hour light and dark cycle and temperature of 22^0 C $\pm$ 3^0 C), humidity (60 ± 10 %) with access to food and water ad libitum according to OECD guidelines, revised draft guidelines 425 and by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India[14]. The animals were allowed to adapt to the new surrounding by giving rest of one week before subjecting them to experimentation.

Rats – Healthy Wistar rats of either sex weighing between180-250 grams (8 to12 weeks old) were used.

Drugs and Chemicals:

- Aspirin or Acetyl salicylic acid (Alkem Ltd.): A 2% suspension in gum acacia was prepared in distilled water.

- Indomethacin (Jagsorpal Pharmaceutical Pvt. Ltd.): A 2% suspension in gum acacia was prepared in distilled water.
- Carrageenan (Analar, BDH): 1% carrageenan was prepared in NS on the day of experiment.
- Ketamine hydrochloride (NEON Laboratories limited thane).

Ethical Clearance

Ethical clearance was taken from Institutional Ethics Committee and Institutional Animal Ethics Committee (IAEC) before commencement of the study.

Collection of plant material

Fresh flowers of Plant *Calendula officinalis* were collected from the nearby area of Wardha and authenticated by a local experienced botanist of Institute - JB COLLEGE OF SCIENCE, (Janki Devi Bajaj College of Science) WARDHA , MAHARASHTRA .The flowers were shade-dried, powdered in a electric mixture and stored in an air tight container for study.

Preparation of extract-

The hydro-alcoholic extracts were obtained by soxhlet extraction at 60˚C using 70 % v/v ethanol as a solvent. Extract obtained by this method was shed dried and yield was measured. Fresh solution was prepared by dissolving extract in distilled water before each experiment.

The yield of flowers extract by soxhlet extraction is 8gm from 40gm of powder.

Preparation of drug formulation-

For oral administration fresh solution was prepared by dissolving the *Calendula officinalis* flowers extract in distilled water before each experiment. Whereas for topical application *Calendula officinalis* flowers *extract* was mixed with simple ointment as mention below.

Preparation of ointment-

Simple ointment (100gm IP):

Soft Paraffin - 85 gm

Hard Paraffin -10

Lanolin/wool fat- 5 gm

5% (w/w) ointment- 10% (w/w) ointment-

Simple ointment- 95gm

Simple ointment- 90gm

CO flowers extract-5 gm

CO flowers extract-10 gm

Assessment of anti-inflammatory activity

Anti-inflammatory activity of hydroalcoholic extract of *Calendula officinalis* flowers was studied in the following two models:

i) Carrageenan induced paw edema model

ii) Cotton pellet induced granuloma models

Carrageenan-Induced Paw Edema Model [15]

The albino Wistar rats of either sex were divided into 4 groups, 6 animals in each group (total 24). Group I served as control and was given only 2% gum acacia (10ml/kg, p.o.).

Group 1- Control- received distilled water (10ml/Kg, p.o)

Group 2- HECO 200mg/kg orally

Group 3- HECO 400mg/kg orally

Group 4- Standard drug aspirin 300mg/kg orally

Wistar rats of either sex weighing 150-250 grams were used for the test. Both hind paws were marked at tibio-talar junction. Right hind paw was kept as control in each animal. Acute inflammation was produced by sub-plantar injection of 0.1 ml of 1% suspension of carrageenan with 2% gum acacia in normal saline, injected in left hind paw (planter aspect) after 60 minutes of oral administration of standard and test drugs. Each paw volume was measured by dipping in Mercury Plethysmometer upto the mark as described

by Chattopadhyay *et al*[16]. Readings were taken at 1, 2 and 3 hour. The difference between the two readings was taken as the volume of edema and the inhibitory percentage of inflammatory reaction was determined for each animal by comparing with control and calculated by the formula described by Sudjarwo Agus[17].

% inhibition = (1- Et/Ec) x 100[17]

Where, Ec = Edema of the control (group I).

Et = Edema of the treated (group II – IV).

Cotton pellet-induced granuloma

The albino Wistar rats of either sex were divided into 4 groups, 6 animals in each group (total 24 animals). Group I served as control and was given only 2% gum acacia (10ml/kg, p.o.).

Group 1 Control- received distilled water (10ml/Kg, p.o)

Group 2- HECO 200mg/kg orally

Group 3- HECO 400mg/kg orally

Group 4- Standard drug indomethacin 10mg/kg orally

Sterile cotton pellet (2 ± 1mg) soaked in 0.2 ml of distilled water containing penicillin (0.1 mg) was implanted subcutaneously on the first day in the inner side of right thigh of each rat with aseptic precautions under ketamine (60mg/kg i.p) anesthesia[18]. Animals were given test and standard drug orally once a daily for 7 days. The pellets were remained in bodies of rats for 7 days. On 8th day the rats were sacrificed and wet cotton pellets with granuloma were dissected out. Weights of these cotton pellets were measured. Then they were dried in hot air oven at 60° C for 24 hours and again the dry weight was determined. Change in weight of granuloma was calculated by subtracting weight of cotton.

Statistical analysis

All the results were expressed as Mean ± Standard Deviation (SD). The differences between experimental groups were compared by one-way Analysis of Variance (ANOVA) followed by Students "t" unpaired test. The results were considered statistically significant when *p < 0.05, **p< 0.01-very significant, ***p< 0.001-Highly significant as compare to control.

Observations and Results

Carrageenan-Induced Paw Edema Model

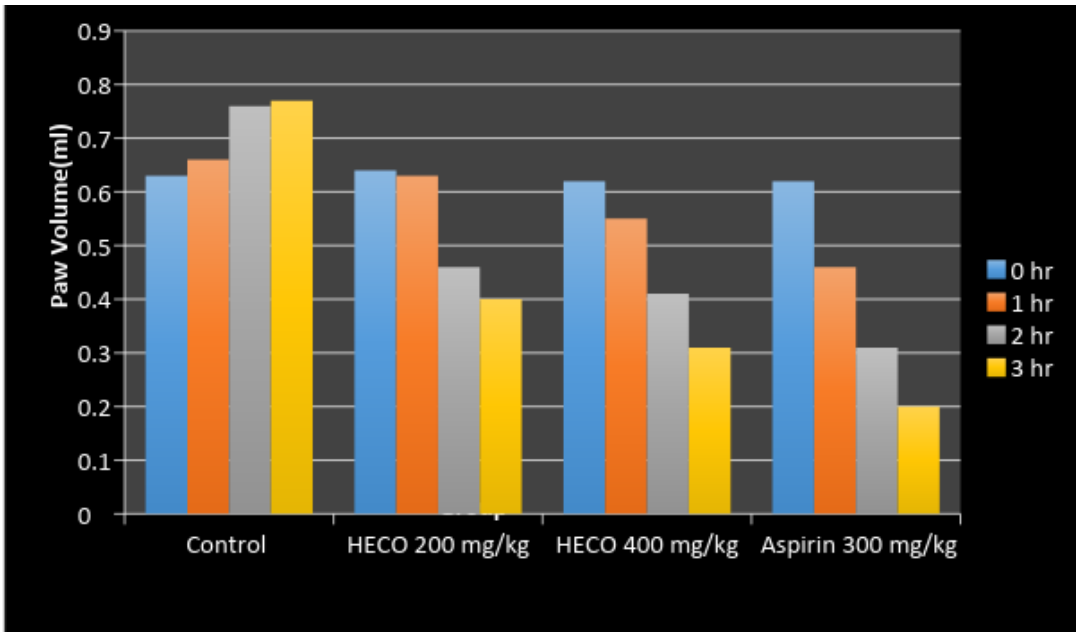
The *Calendula officinalis* extract of flowers at 200 and 400 mg/kg showed significant and highly significant inhibition (p< 0.05 and p<0.001, respectively) of paw edema at end of 1st hour after giving the carrageenan in the rat paw in comparison of control. At the 1st hour, the doses of 200 and 400 mg/kg showed percentage inhibition 4.54%, and 16.67% in comparison of control. (Table 1 & Graph 1)

On the other hand, the *Calendula officinalis* extract of flowers at 200 and 400 mg/kg respectively showed highly significant inhibition (p< 0.001) of paw edema at end of 2nd and 3rd hour after giving the carrageenan in the rat paw in comparison of control. At the 2nd hour, the doses of 200 and 400 mg/kg showed percentage inhibition 39.47% and 46.05% in comparison of control and at the end of 3rd hour, the graded doses of *Calendula officinalis* showed percentage inhibition of 48.05% and 59.74% in comparison with control. Whereas the standard drug aspirin showed highly significant inhibition of paw edema of rat (p<0.001) at 1st, 2nd and 3rd hour with percentage inhibition of 30.30%, 59.21 and 74.02%, respectively in comparison of control. (Table – 1 & Graph 1)

Table 1: Anti-inflammatory activity hydroalcoholic flowers extract of calendula officinalis by Carrageenan induced paw edema method in rats.

Group	Dose mg/kg PO	Paw Volume(ml) (Percentage Inhibition)			
		0 Hr	1 Hr	2 Hr	3. Hr
1-Control Distilled Water	10 ml/kg	0.63±0.01	0.66±0.01	0.76±0.008	0.77±0.01
2-HECO	200	0.64±0.010 (1.58)	0.63±0.001** (4.54)	0.46±0.011*** (39.47)	0.40±0.01*** (48.05)
3-HECO	400	0.62±0.01 (1.58)	0.55±0.01*** (16.67)	0.41±0.01*** (46.05)	0.31±0.007*** (59.74)
4-Standard Aspirin	300	0.62±0.005 (1.59)	0.46±0.008*** (30.30)	0.31±0.02*** (59.21)	0.20±0.01*** (74.02)

Note: Number of animals n=6. One-way ANOVA followed by Student's "t" unpaired test. Results are expressed in Mean±SEM: *p<0.05- significant, **p<0.01- very significant and ***p<0.001- highly significant as compared to control. HECO–Hydroalcoholic extract of *Calendula officinalis* flowers.



Graph 1: Anti-inflammatory activity of hydroalcoholicflowers extract of *Calendula officinalis* by Carrageenan induced paw edema method in rats.

Cotton pellet induced granuloma pouch model

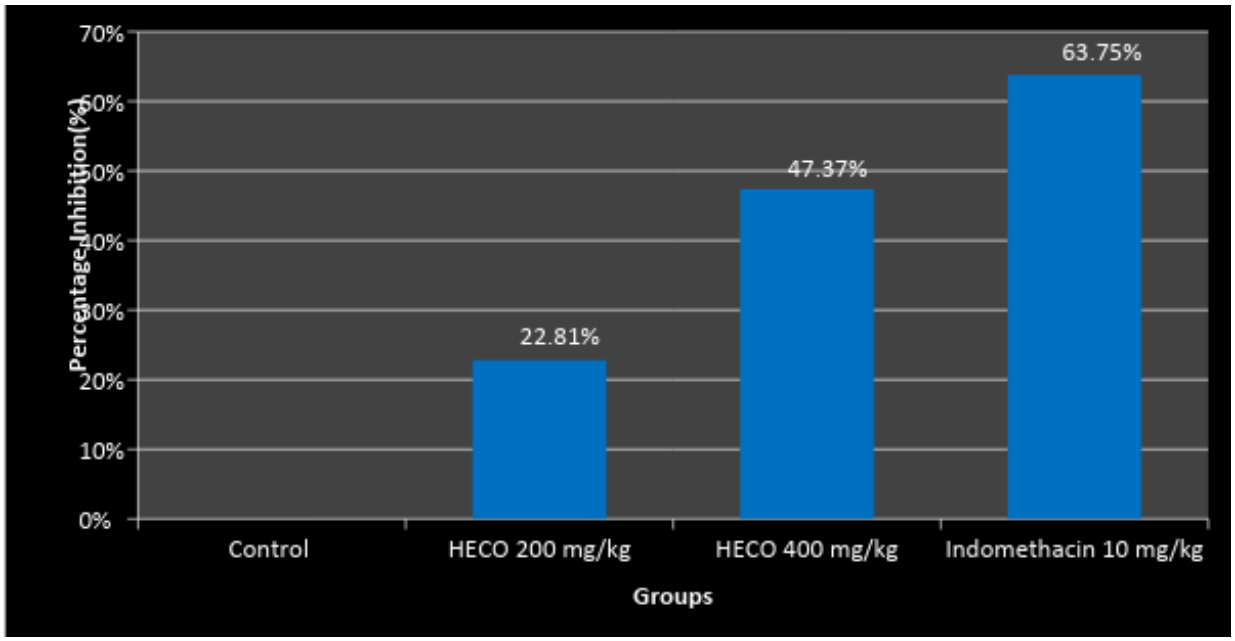
In cotton pellets induced granuloma pouch method, the hydroalcoholic extract of *Calendula officinalis* flowers at 200 mg/kg showed very significant reduction of weight of granuloma (p<0.01) with 22.80% protection as compared with control. On the other hand, 400 mg/kg showed highly significant reduction (p<0.001) in the weights of granuloma with 41.64% protection in comparison with control. The standard, indomethacin 20mg/kg showed a protection of 63.75% with highly significant reduction (p<0.001) in the weight of granuloma as compared with control. (Table 2 & Graph 2)

Table 2: Anti-inflammatory activity of hydroalcoholic flowers extract of calendula officinalis by cotton pellet induced granuloma method in rats

Group	Drug Treatment mg/kg orally	Initial dry wt of CP(mg)	Final dry wt of CP(mg)	Wt of granulomatous tissue	% Inhibition
1-Control Distilled Water	10 ml/kg	2	30.50±1.87	28.50±1.87	
2-HECO	200	2	24±1.26	22±1.26***	22.81%
3-HECO	400	2	17.00±2.09	15.00±2.09***	47.37%
4-Standard Indomethacin	10	2	12.33±1.50	10.33±1.50***	63.75%

a. t cannot be computed because the standard deviations of both groups are 0

Note: Number of animals n=6. One-way ANOVA followed by Student's "t" unpaired test. Results are expressed in Mean±SEM: *p<0.05- significant, **p<0.01- very significant and ***p<0.001- highly significant as compared to control. HECO–Hydroalcoholic extract of *Calendula officinalis* flowers.



Graph 2: Anti-inflammatory activity of hydroalcoholic flowers extract of C*alendula officinalis* on rats by cotton pellet induced granuloma showing percentage inhibition.

Discussion

Carrageenan-induced paw edema[19,20,21,22]

An anti-inflammatory drug of herbal origin is suitable for screening of anti-inflammatory properties in the acute as well as sub-acute model of inflammation[19]. Carrageenan model is commonly used as it is highly reproducible with apparent no systemic side effects.[20]

Carrageenan develops edema in the paw of rat in a biphasic event.[21] The initial phase of inflammation produced by carrageenan causes release of histamine, kinin and serotonin in the first hour. While the second phase is related to the release of prostaglandin like substances, protease and lysosome in 2-3 hour and these mediators produce edema in rat paw.[21,22] These mediators leads to a dilation of vessels with extravasations of fluid and plasma proteins with formation of edema. These mediators as the metabolites of arachidonic acid (AA) and its COX pathway are capable of

producing the characteristic signs of inflammation: vasodilatation, hyperemia, pain, edema, and cellular filtration.

Oral administration of HECO extract at the dose 200 mg/kg and 400mg /kg showed significant ($p<0.001$) percent inhibition of edema at the end of 2 and 3 hours as compare to control. (Table 1, graph 1) This action may be due to inhibition of histamine, serotonin, kinin and prostaglandin by HECO. The carotenoids present in *Calendula officinalis* flowers like lycopene has been reported to consistently reduce transcript levels of proinflammatory cytokines (Herzog et al,2005). Moreover, the expression of cyclooxygenase-2, a key enzyme involved in inflammatory process was found to be initiated by the treatment of extract. Thus, the *Calendula officinalis* flower extract may be contributes to its anti-inflammatory activity through the inhibition of mediators of inflammation especially proinflammatory cytokines as well as by the inhibiting the Cox-2 and thereby prostaglandins which has major role in inflammatory process.

The cotton pellet-induced granuloma

It is widely used to assess the exudative and proliferative component of inflammation. Cotton pellets by absorbing fluid greatly influence the weight of granuloma and dry weight. It is well corrected with the amount of granulomatous tissue[23]. The size of granuloma is inhibited by NSAIDs by suppressing the generation of collagen fiber and mucopolysaccharides.[24] HECO at the dose of 200 and 400 mg/kg showed significant ($p <0.01$ and $p<0.001$ respectively) inhibition of granuloma as compare to control (Table 2). The percentage inhibition of granuloma at dose of 400 mg/kg of HECO is almost comparable to the standard, Aspirin. With increasing doses of HECO, there is a gradual increase in the percentage inhibition of granuloma, indicating its anti-inflammatory activity.*Calendula officinalis flowers* extract contain flavonoids and genins[25] as constituents which may be responsible for decreasing both final dry weight of cotton pellet and weight of granuloma which can attribute to their anti-inflammatory activity.

Conclusions

We conclude that the Anti-inflammatory activity was noted with HECO in a dose-dependent manner in acute (carrageenan-induced hind paw edema model) as well as sub-acute (cotton pellet induced granuloma model) inflammatory experimental models. The anti-inflammatory activity of HECO is due to its constituents, flavonoids and triterpenoids. They decrease the chemical mediators of inflammation. Further studies can give more strong correlation and association of HECO with anti-inflammation and can be utilize in Health care.

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