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Research Article

Preliminary Pharmacological Screening of *Eulophia herbacea* Lindl. Tubers Extracts for Anti-inflammatory Potential in Experimental Animals

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ABSTRACT

Aim of the current study was to screen various extracts of *Eulophia herbacea* tubers for anti-inflammatory effects on carrageenan-induced paw edema (acute models) and cotton pellet granuloma model (chronic model) in experimental animals. Anti-inflammatory effects were observed by percent reduction in paw edema, assessment of granuloma weight and proinflammatory cytokine level. Diclofenac sodium (10 mg/kg bw) was utilized as the standard drug for comparison. Study observation confirmed that ethanol extract (EEH) and aqueous extracts of *E. herbacea* (AEH) tubers were reduced higher percentage of edema inhibition and comparable to the standard group. The granuloma weight of the control group was increases after the implantation of sterile pellet and was measured on after 8th day. A substantial (p <0.05) reduction in weight of granuloma was witnessed in EEH and AEH groups of animals at dose-dependent manner. The level of proinflammatory markers (TNF-α, IL-6 and PGE2) decreased significantly when compared to granuloma tissue of the control group of animals. In conclusion, ethanol and aqueous extracts of *E. herbacea* tubers may reduce the proliferative phase as proven by a decrease in granuloma weight. Flavonoids present in ethanol extract and polysaccharides present in aqueous extract may be responsible for the anti-inflammatory potential of *E. herbacea* tubers.

Introduction

The body utilizes inflammation as a protective response to eliminate harmful stimuli and kickstart the healing of tissues. However, if left unattended, it could result in the advancement of different conditions like atherosclerosis, vasomotor rhinorrhea and rheumatoid arthritis.[1] The main symptoms of inflammation are redness, swelling, pain, heat, and immobility. Thus, inflammation is thought to serve as the body's defense system against these harmful stimuli.[2,3]

Different medicinal plants contain different amounts of phytochemicals, which are important for the treatment of various illnesses. They have a clear physiological effect on the human body. The biological functions of plant-derived components such as flavonoids, quinine, and terpenoids include enhancing therapeutic actions, like having anti-mutagenic, anti-inflammatory, antioxidant and anti-carcinogenic properties. These ingredients have the power to control inflammation-related illnesses as well as chronic illnesses brought on by oxidation and stress such as cardiovascular diseases, arthritis, and diabetes, because of they possess powerful antioxidant activity.[4]

The *Eulophia* genus covers 200 orchid species, with 26 of these species being indigenous to India. Eulophia is a genus of perennial, terrestrial orchids equipped with fleshy tubers that are found worldwide in warm regions,

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particularly in Asia and Africa. *Eulophia herbacea* Lindl. (Orchidaceae) is generally referred to as Kukad-kand or umarkand. Plant tubers have various uses, such as treating tumors in the neck's scrofulous glands, serving as an aphrodisiac and appetizer and addressing cardiac issues.[5] Amarkand has traditionally been utilized in Ayurveda as a tonic, astringent, diuretic, digestive, expectorant and mild purgative. Additionally, ancient literature has emphasized its effectiveness in treating joint edema, ear discharge, debility, and blood clotting.[6] They are also used for blood disorders, bronchitis, stomatitis, purulent cough, heart issues, dyscrasia, and scrofulous diseases of the neck.

Nutritional components from *E. ochreata*, including carbohydrates (59.31%), crude fibers (22.90%), proteins (5.44%), and lipids (3.25%), have been isolated and their significance demonstrated by a range of activities. Glucomannan is a water-soluble polysaccharide present in *E. herbacea* tubers. Normalizing blood sugar levels and lowering oxidative stress and other abnormalities in the body are the major advantages.[7] *E. herbacea* Lindl. tubers reported the presence of flavonoids, carbohydrates, vitamins, proteins and nutritional elements that make it higher medicinal value for application.[8] Tuber extracts of *E. herbacea* were reported to have a hepatoprotective effect in rats,[9] and ethanol extract of tubers showed potent effect in inflammatory conditions.[10] Accordingly, on the basis of traditional claims and chemical components, the intention of current study was to screen various extracts of *E. herbacea* tubers for therapeutic use as an anti-inflammatory agent.

Materials And Methods

Acquisition and Verification of Botanical Specimens

E. herbacea Lindl, (Orchidaceae) tubers were gathered from the subtropical hilly region of Nandurbar (Maharashtra) in the month of October. They were identified and authenticated, Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur (M.P.) India. For removal of dirt and soil, the *E. herbacea* tubers were thoroughly cleaned with water. In order to facilitate further extraction, the plant components were dried in the shade and stored in closed tight-pack containers.

Phytochemical Screening of Different Extracts

The dried tubers of *E. herbacea* were size reduced with mortar and pestle made into a coarse powder and then used for extraction using a soxhlet apparatus with the help of various organic solvents. The extraction was done with various solvents with sequential arrangement of polarity, i.e., petroleum ether, chloroform, ethyl acetate and ethyl alcohol using a soxhlet apparatus. Aqueous extract was obtained by the maceration process. These extracts were concentrated at lower pressure using a vacuum rotary evaporator after being filtered at room temperature using a Buckner funnel and Whatman filter paper. Using several chemical tests according to the described procedure, all extracts were qualitatively tested for a variety of chemical constituents, including carbohydrates, lipids, tannins, flavonoids, glycosides, alkaloids, saponins, proteins, and amino acids.[11]

Acute Toxicity Study

Different extracts of *E. herbacea* tubers were subjected to acute oral toxicity trials in rats as per regulations of OECD (No. 423). The Wistar rats (150–200 g) of both sexes were chosen and oral administration of different extracts with dose of 500, 700, 1000, 1500, 2000, 2500 and 3000 mg/kg wt. All animals were observed during the first 48 hours and up to the next 14 days for abnormal behavior and any mortality. All experimental protocol was followed as per the Institutional Animal Ethics Committee (Reg. No. 1693/PO/Re/S/13/CPCSEA).

Animals

Healthy albino Wistar rats between 150 to 200 g were acquired from College of Veterinary Science & Animal Husbandry, Mhow (M. P.) and were kept within a typical environment 22 ± 1°C temperature and 55 ± 1% RH, with dark/light cycle (12h). Rats were granted unlimited access to water *ad libitum* and rat food (Ashirwad brand, Chandigarh, India). All these investigates were conduct after clearance from Institution Animal Ethical Committee. Following the ethical committee's approval, the protocol was implemented in accordance with ethical principles (Reg. No. 1693/PO/Re/S/13/CPCSEA).

Anti-inflammatory Activity

Carrageenan-induced paw edema model

Quantification of anti-inflammatory activity was performed by acute model, i.e., Carrageenan-induced paw edema model previously reported by Sasikala *et al.* (2011)[12] with slight modification. Albino Wistar rats were distributed in 12 groups 6 animals in each group. Different doses of extracts were administered 30 minutes later of inflammation. Left hind paws of all animals were injected 0.05 mL carrageenan (1% w/v) subcutaneously, to cause acute inflammation on the plantar side. The paw that has been treated is leveled with ink up to a level of lateral malleolus and submerged in mercury up to the mark is reached. To facilitate comparison, the right paw was designated as the reference non-inflamed paw. Animals were divided in following groups:

Group I: Vehicle control received normal saline solution (10 mL/kg, p.o.); Petroleum ether extract of *E. herbacea* tubers at dose of 200 and 300 mg/ kg, p.o. were given to groups II and III, correspondingly; Groups IV and V treated with chloroform extract of *E. herbacea* tubers at dose of 200 and 300 mg/kg, p.o., correspondingly; Groups VI and VII administered with ethyl acetate

extract of *E. herbacea* tubers at 200 and 300 mg/kg, p.o., correspondingly; Groups VIII and IX were given ethanol extract of *E. herbacea* tubers at dose of 200 and 300 mg/kg, p.o., correspondingly; Groups X and XI treated with aqueous extract of *E. herbacea* tubers at dose of 200 and 300 mg/kg, p.o., respectively; Group XII is the standard group and given Diclofenac sodium (10 mg/kg), p.o. Percent inhibition was calculated as per following formula:

$$\%\ Inhibition: \frac{Paw\ Volume\ (Control) - Paw\ Volume\ (treated\ group)}{Paw\ Volume\ (Control)} \times 100$$

Cotton pellet granuloma model

Inflammation was induced by cotton pellet granuloma model as outlined by Afsar *et al*. (2013)[13] with minor adjustments. Pellets were formed from sterilized adsorbent cotton wool, which was divided into pieces measuring 10 ± 1 mg. After clean shaving then swabbing their abdomens with 70% ethanol, rats had two sterile cotton pellets subcutaneously injected into their groin area. Following this, the groups received daily treatments of 200 and 300 mg of various extracts for seven days in consecutive days. Saline and diclofenac sodium (10 mg/kg) were given to animals in control as well as reference groups, respectively. Animals were given anesthesia on 8th day of treatment. Subsequently, pellets encircled by granuloma tissue were meticulously removed and utilized for additional research. One part of granuloma tissue was used for the estimation of proinflammatory markers.

Measurement of granuloma weight

After the animals were put under anesthesia on 8th day of implantation, these cotton pellets were surgically taken out from superfluous tissue; then their weight was recorded. The wet pellets were then dried in an oven at 60˚C for a whole day. To determine the mean weight of dry cotton, pellets were weighed once more after drying. The %inhibition of granuloma tissue formation in experimental animals was calculated with subtraction of granuloma weight of test group from the control group. The difference was divided by the granuloma weight of control group as formula is given below:

Percent Inhibition (%) =

$$\frac{\text{Granuloma W of Control} - \text{Granuloma weight of Test}}{\text{Granuloma weight of Control}} \text{ x } 100$$

Assessment of proinflammatory markers

Small proteins such as cytokines are proinflammatory indicators, that are essential for cell signaling, especially in the immune system, by regulating inflammation, cell differentiation, and cell proliferation. Proinflammatory markers such as TNF-α, IL-6 and PGE2 play important role in regulating inflammation and immune responses. Numerous diseases, such as autoimmune disorders, inflammatory ailments, and cancer, can be attributed to the pathophysiology of dysregulation of cytokine production or signaling pathways.[14]

After 8th day of implantation, granuloma tissue was collected from anesthetized animals of different groups for the cytokines assay. The levels of various inflammatory indications like TNF-α, IL-6 and PGE2 were estimated by ELISA Kit in serum samples as pictogram per milliliter (pg/mL) utilizing ELISA Reader (Lisa Plus, Germany). Assays were conducted following guidelines provided by the manufacturer.

Statistical Analysis

The one-way ANOVA test and Dunnet's t-test were used to statistically assess the data before comparing each group's results to the control. The findings were presented as Mean ± S.D., with statistical significance denoted by $p < 0.05$.

RESULTS

Phytochemical Studies

Phytochemical analysis of different extracts *E. herbacea* tubers was qualitatively characterized by using different chemical tests and observations were recorded in Table 1. Preliminary screening of different extracts for confirmation of different chemical constituents has exposed the occurrence of steroids, terpenoids in petroleum ether extract, terpenoids in chloroform, flavonoids, glycosides in ethyl acetate and ethanol extract, carbohydrates, proteins, tannins and saponins were found in aqueous extract.

Acute Toxicity

Oral administration of *E. herbacea* tubers extract up to 1000 mg/kg was not able to produce any abnormality in the animal behavior or any toxic effects during the first 48 hours and even up to the next 14 days. At the suggested doses, every extract was confirmed to be safe, and no mortality was noted.

Anti-inflammatory Effects by Carrageenan-induced Paw Edema Method

Carrageenan was used to induce acute inflammation in experimental rats via administration into the hind paw. The effect was produced a progressive edema reaching a maximum at 4 hours in all administered rats. Different extracts of *E. herbacea* tubers indicated as PEEH, CEH, EAEH, EEH and AEH were given in two doses 200 and 300 mg/kg bw, p.o. Observation of the anti-inflammatory effect indicates that the effect of both extract EEH and AEH was in dose-dependent manner. From 1 to 4 hours, there was a substantial ($p < 0.05$) percent inhibition of paw edema detected in comparison to a control group. The highest percentage of paw edema inhibition was noted as 51.80 and 56.34% by EEH at both doses of 200, 300 mg/kg p.o., respectively. AEH was also displayed substantial percent inhibition of edema at both doses (50.46 and 53.56%, respectively). The maximum percent inhibition of paw edema by the diclofenac sodium group showed 56.96%

Table 1: Observations of percent inhibition by different extract of *E. herbacea* tubers in carrageenan induced paw edema model

Animal groups	*Alteration in paw size (mm) (% inhibition) at different time*			
	1 hour	*2 hours*	*3 hours*	*4 hours*
Control (Normal Saline) (10 mL/Kg)	*1.29 ± 0.24*	*2.11 ± 0.08*	*2.98 ± 0.07*	*3.23 ± 0.09*
PEEH (200 mg/kg)	1.14 ± 0.10 (11.62%)	1.83 ± 0.05 (13.27%)	2.40 ± 0.10 (19.46%)	2.36 ± 0.10 (26.93%)
PEEH (300 mg/kg)	1.09 ± 0.09 (15.50%)	1.75 ± 0.04 (17.06%)	2.28 ± 0.08 (23.48%)	2.27 ± 0.09 (29.72%)
CEH (200 mg/kg)	1.19 ± 0.12 (7.75%)	1.87 ± 0.12 (11.37)	2.43 ± 0.16 (18.45%	2.45 ± 0.12 (24.14%)
CEH (300 mg/kg)	1.15 ± 0.13 (10.85%)	1.78 ± 0.15 (15.63%)	2.32 ± 0.18 (22.14%)	2.24 ± 0.12 (30.65%)
EAEH (200 mg/kg)	1.12 ± 0.15 (13.17%)	1.69 ± 0.17 (19.05%)	2.16 ± 0.14 (27.51)	2.22 ± 0.14 (31.26)
EAEH (300 mg/kg)	1.09 ± 0.06 (15.50%)	1.62 ± 0.15 (23.22%)	2.08 ± 0.15 (30.20%)	2.05 ± 0.17 (36.53%)
EEH (200 mg/kg)	1.05 ± 0.05 (18.60%)	1.55 ± 0.13* (26.54%)	1.63 ± 0.12* (45.30%)	1.56 ± 0.15* (51.80%)
EEH (300 mg/kg)	0.98 ± 0.03* (24.05%)	1.42 ± 0.11* (32.70%)	1.54 ± 0.10* (49.32%)	1.41 ± 0.12* (56.34%)
AEH (200 mg/kg)	1.01 ± 0.07* (21.70%)	1.45 ± 0.15* (31.27%)	1.59 ± 0.14* (46.64)	1.60 ± 0.17* (50.46%)
AEH (300 mg/kg)	0.99 ± 0.09* (23.25%)	1.38 ± 0.16* (34.59%)	1.48 ± 0.13* (50.33%)	1.50 ± 0.16* (53.56%)
Standard (Diclofenac sodium, 10 mg/kg)	0.95 ± 0.02* (26.35%)	1.45 ± 0.03* (31.27%)	1.49 ± 0.07* (50.00%)	1.49 ± 0.06* (53.86%)

Abbreviations: PEEH: indicate to petroleum ether extract of *E. herbacea*; CEH: stand for chloroform Extract of *E. herbacea*; EAEH: ethyl acetate extract of *E. herbacea*; EEH: ethanol extract of *E. herbacea*; AEH: aqueous extract of *E. herbacea*; each value denoted as mean ± SD. for N=6. *p <0.05, values considered as significant when compared with control group.

at 4 hours after its administration (Table 1). Other extracts were not shown a significant reduction in paw edema.

Anti-inflammatory Effects by Cotton Pellet-induced Granuloma Method

Effect of different extracts of *E. herbacea* tubers, i.e., PEEH, CEH, EAEH, EEH and AEH on granuloma formation were observed at two doses 200 and 300 mg/kg p.o. A substantial (p <0.05) percent reduction of the granuloma weight was observed by the EEH and AEH groups at both doses. EEH was showed as 43.11 and 48.1%, for 200 and 300 mg/kg doses, correspondingly (Table 2). Maximum percent reduction in granuloma weight by standard group diclofenac sodium was 46.26% which was also similar to the AEH group. EEH and AEH groups reduced granuloma weight significantly when compared to a control group of animals. A significant reduction in granuloma weight by

Table 2: Effect of different extract of *E. herbacea* tubers on granuloma weight in cotton pellet induced granuloma model

Animal groups	*Granuloma weight (mg)*	*Percent inhibition*
Control (Normal Saline; 10 mL/Kg)	72.51 ± 3.42	-
PEEH (200 mg/kg)	70.21 ± 3.20	3.17
PEEH (300 mg/kg)	68.53 ± 2.85	5.48
CEH (200 mg/kg)	71.32 ± 3.14	1.64
CEH (300 mg/kg)	70.62 ± 3.08	2.60
EAEH (200 mg/kg)	63.55 ± 2.75	12.35
EAEH (300 mg/kg)	60.42 ± 2.64	12.09
EEH (200 mg/kg)	41.25 ± 1.75*	43.11*
EEH (300 mg/kg)	37.62 ± 1.34*	48.11*
AEH (200 mg/kg)	43.57 ± 2.67*	39.91*
AEH (300 mg/kg)	39.14 ± 2.75*	46.02*
Diclofenac sodium (10 mg/kg)	38.96 ± 1.29*	46.26*

Abbreviations: PEEH: Petroleum ether extract of *E. herbacea*; CEH: Chloroform Extract of *E. herbacea*; EAEH: ethyl acetate extract of *E. herbacea*; EEH: ethanol extract of *E. herbacea*; AEH: aqueous extract of *E. herbacea*; Value denoted as mean ± SD. for N=6. * p <0.05, values considered as significant in comparison to the control group.

EEH was shown in dose-dependent manner. Other extracts of *E. herbacea* tubers PEEH, CEH and EAEH were not shown a significant reduction in granuloma weight at both doses. The anti-inflammatory impact of different extracts was also confirmed by marked expression of proinflammatory markers, i.e., TNF-α, IL-6 and PGE2 in granuloma tissue. Inhibitory effect of different extracts on level of proinflammatory markers (TNF-α, IL-6 and PGE2) were observed at two doses 200 and 300 mg/kg p.o. A significant reduction of TNF-α (from 41.52 ± 2.08 to 21.37 ± 1.34), IL-6 (32.46 ± 1.97 to 17.23 ± 1.62) and PGE2 (55.27 ± 2.68 to 32.69 ± 1.75) were observed by EEH at 300 mg/kg (Table 3). Results were confirmed that only ethanolic and aqueous extracts of *E. herbacea* tubers showed a significant reduction in cytokines and prostaglandins at dose-dependent manner. Other extracts PEEH, CEH and EAEH were not shown substantial (p <0.05) decrease in level of proinflammatory markers of granuloma tissue in comparison with a control group. Ethanolic and aqueous extracts were discovered to be most efficient at a dosage of 300 mg/kg bw.

Discussion

According to numerous reports on *E. herbacea* Lindl., it is an abundant source of proanthocyanidin, phenolic acid, carbohydrates, and flavonoids, which may be the main cause of the plant's notable biological activity.[9] Additionally, some historical literature has emphasized the benefits of using this species to cure blood coagulation, joint edema, debility, and ear discharge.[15]

Table 3: Table 3: Observation of proinflammatory markers level after treatment with different extract of *E. herbacea* tubers in cotton pellet induced granuloma model in rats

Animal Groups	*TNF-α (pg/mL)*	*IL-6 (pg/mL)*	*PGE2 (pg/mL)*
Control (Normal Saline) (10 mL/Kg)	41.52 ± 2.08	32.46 ± 1.97	55.27 ± 2.68
PEEH (200 mg/kg)	40.63 ± 1.56	31.27 ± 1.24	52.67 ± 2.05
PEEH (300 mg/kg)	40.69 ± 1.75	30.58 ± 1.45	51.38 ± 2.17
CEH (200 mg/kg)	38.49 ± 1.62	31.62 ± 1.69	50.75 ± 2.64
CEH (300 mg/kg)	37.22 ± 1.58	30.27 ± 1.88	48.99 ± 2.34
EAEH (200 mg/kg)	35.41 ± 1.67	28.67 ± 1.62	45.01 ± 2.18
EAEH (300 mg/kg)	34.69 ± 1.96	28.63 ± 1.20	44.08 ± 2.07
EEH (200 mg/kg)	25.46 ± 1.08*	23.34 ± 1.68*	38.20 ± 1.99*
EEH (300 mg/kg)	21.37 ± 1.34*	17.23 ± 1.62*	32.69 ± 1.75*
AEH (200 mg/kg)	29.88 ± 1.75	24.55 ± 1.42	36.16 ± 2.61
AEH (300 mg/kg)	23.28 ± 1.96	20.45 ± 1.62	33.31 ± 2.07
Standard (Diclofenac sodium, 10 mg/kg)	22.61 ± 1.25*	16.22 ± 1.16*	33.62 ± 1.85*

Abbreviations: PEEH: Petroleum ether extract of *E. herbacea*; CEH: Chloroform Extract of *E. herbacea*; EAEH: ethyl acetate extract of *E. herbacea*; EEH: ethanol extract of *E. herbacea*; AEH: aqueous extract of *E. herbacea*; Value denoted as mean ± SD. for N=6. * p <0.05, values in comparison to the control group and considered as significant.

The rat paw edema model caused by carrageenan is a model of acute inflammation that can be affected by the anti-inflammatory properties of experimental medications. Exposure of carrageenan to the human body has a number of symptoms that are closely related to acute inflammation, in addition to higher vascular permeability, release of plasma content and rapid edema.[16] It can release various inflammatory mediators (histamine, bradykinin, prostaglandins, leukotrienes, TNF- α, etc.) during inflammation reactions.

Outcomes of the current study discovered that two to three hours after administration of injection of carrageenan, paw thickness significantly decreased in response to the ethanol and aqueous tuber extracts of *E. herbacea*, which demonstrated extremely consistent anti-inflammatory activity. In this paradigm, the mechanism of inflammation may be separated into two phases. The initial stage is associated with the synthesis of mediators such as kinins, serotonin, and histamine. Conversely, the second stage is linked to mediators such as prostaglandins being released.[17-19] Carrageenan-induced paw edema is linked to proinflammatory and inflammatory mediators like TNF-α, prostaglandins, histamine, and leukotrienes being modulated.[20] Ethanol and aqueous extracts of *E. herbacea* tubers was demonstrated to be beneficial in both stages of inflammation in the present study.

Rats exhibit at least three distinct phases in their reaction to a subcutaneously implanted cotton pellet: transudative, exudative, and proliferative phases. A common method for evaluating the proliferative and transudative aspects of chronic inflammation is the cotton pellet-induced granuloma.[21] Additionally, the material that has been implanted triggers an inflammatory reaction in the host and controls releasing of inflammatory mediators, which ultimately results in granular development and growth of tissue. Transude material and the amount of granulomatous tissues is correlated with the weight of wet cotton pellets and dry pellets, respectively.[22] Anti-inflammatory medications may be able to lower transudative weight by preventing the blood vessels surrounding the cotton pellet implantation from responding to permeability. They may also successfully prevent the formation of granulomas, most likely by interfering with the proliferative elements of inflammatory processes. Ethanol and aqueous extracts of *E. herbacea* tubers may reduce the proliferative phase which was proved by the reduction of granuloma weight.

Conclusion

Results of the present study confirmed that *E. herbacea* tubers extracts has been observed to have anti-inflammatory potential through significant %inhibition of paw edema in a dose-dependent manner of ethanol extracts. It was able to inhibit the weight of granuloma tissue in a higher dose of ethanol extract as well as very close to the inhibitory effect of diclofenac sodium. These observations confirmed that ethanol extract of *E. herbacea* tubers showed an anti-inflammatory effect in both stages of inflammation (2–3 hours) and may reduce the release of prostaglandins, leukotrienes, histamine, and TNF- α. In addition, cycloxygenase inhibition, which prevents prostaglandin synthesis, maybe a root cause of the possible inhibitory effect of *E. herbacea* tubers extracts in carrageenan-induced inflammation, it might be concluded.

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