VALIDATION OF THE POTENTIAL OF *EULOPHIA OCHREATA* L. TUBERS FOR ITS ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY

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Summary

The anti-inflammatory activity of *Eulophia ochreata* L. tubers extract was studied in carrageenan induced rat paw model of acute inflammation. Subplantar injection of 0.1 ml of 1 % carrageenan induced a progressive swelling of the rat paw at all the time points, that reached to a maximal volume in control between 1 and 2 h. We could demonstrate in present study that pre treatment of animals with methanolic extract of *E. ochreata* tubers prevented progresson of oedema up to 48 % at dose of 0.5 gm/kg b.w. and 53 % at dose of 1 gm/kg b.w., where as diclofenac showed 61 % inhibition. The tuber extract also showed significant antioxidant activity as indicated by DPPH radical scavenging capacity, antilipid peroxidation activity, reducing power capacity and nitric oxide radical inhibition asssay.

Key words: *Eulophia ochreata*, anti-inflammation, carrageenan, antioxidant activity.

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Introduction

Medicines derived from plant extracts are being increasingly utilized to treat a wide variety of diseases, though relatively little knowledge about their mode of action is available. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional systems of medicine (1). Thus, the present investigation was carried out to evaluate the antioxidant and anti-inflammatory potential of *E. ochreata* in experimental animal models. *E. ochreata*, commonly known as 'Amarkand' or 'Singadya kand', is a ground orchid in the family Orchidaceae. It is a perennial tuberous herb and usually appears in the forest during rainy season in shady rainforests (2). Ehanobotanical survey of the forest areas of Maharashtra revealed that these tubers are used as a specialty food, general tonic and as rejuvenating herb. It has been used by the tribes for properties like astringent, antifatigue, aphrodisiac, anthelminthic, and as a blood purifier. The tubers are also used in cough, cold and heart troubles (3).

Carrageenan-induced inflammation in the rat paw represents a classical model of oedema formation and hyperalgesia. This model has been extensively used in the development of NSAIDs and selective COX-2 inhibitors, as well as for screening herbals having anti-inflammatory properties. Several lines of evidence indicate that the COX-2-mediated increase in prostaglandin (PG) E_2 production in the central nervous system (CNS) contributes to the severity of the inflammatory and pain responses in this model. COX-2 is rapidly induced in the spinal cord and other regions of the CNS following carrageenan injection in the paw (4). The administration of selective COX-2 inhibitors, but not COX-1 inhibitors, reduces the levels of PGE_2 in the cerebrospinal fluid (CSF) and hyperalgesia (5-8).

Reactive oxygen species are produced in the human body as a consequence of metabolic processes. If not inactivated, these free radicals can damage all types of cellular macromolecules including proteins, carbohydrate, lipids and nucleic acids leading to score of degenerative diseases (9). Herbal intervention is well accepted to be the most potent in scavenging free radicals and thus has a prophylactic role in the management of non-communicable diseases. The antioxidant activity of methanolic extract of *E. ochreata* L. tubers assessed by DPPH radical scavenging capacity, reducing power capacity and nitric oxide radical scavenging capacity validated its potential as medicine and as functional food.

In the present work, we have evaluated the anti-inflammatory effect and antioxidant activity of the methanolic extract of *E. ochreata.* There is no such study carried out on this plant based on our literature survey and probably it is for the first time such studies are reported in this plant.

Material and Methods

Collection of plant materials:

Tubers of *E. ochreata* were collected in Satpura hills of Maharashtra. The plant was identified and authenticated by Dr. Sathish Kumar, Tropical Botanical Garden and Research Institute, Kerala, India. Voucher specimen (MPCC 3125, 2007) of this plant material has been retained in the Medicinal Plants Conservation Centre, Pune, Maharashtra, India.

Plant extract:

Tubers of *E. ochreata* were cut into pieces and shed dried. The dried samples were reduced to a fine powder with a mechanical grinder. The powdered plant material (200 g) was extracted in methanol. The extract was concentrated to dryness using a rotary evaporator attached to a vacuum pump and stored at a temperature of -4^0C until use.

Anti-inflammatory study:
Animals

Wistar rats of either sex weighing 200 g, respectively, were purchased from National Toxicological Centre, Pune. They were housed in polypropylene cages at 25$\pm$ 2^{o}C with relative humidity of 45-55 % under 12 h light and 12 h dark cycles and had unlimited access to food and water. Experimental procedures were conducted in accordance with the regulations of Animal Ethical committee. (CPCSEA/ 41/ PCP/2008-09)

Toxicity study

Toxicity testing was carried out according to OECD guidelines. For acute toxicity studies, the mice were divided into four groups each containing 10 mice and they were treated with graded doses. After treatment, the animals were observed for behavior changes and their mortality. From the study it was revealed that the methanolic extract was found to be safe up to 5000 gm/kg orally since there was no mortality. Further work was carried out by selecting doses as 0.5 gm/kg and 1 gm/kg b.w. orally.

Rat Paw Oedema Model

The animals were maintained under standard environmental conditions and had free access to standard diet and water. Anti-inflammatory activity was measured using carrageenan induced rat paw oedema model (10,11). Groups of 6 rats were given a dose of the extract (plant extracts were dissolved in sterile distilled water and administered orally at different dose levels). After 1h, 0.1 ml, 1% carrageenan suspension in 0.9% NaCl solution was injected into the sub-plantar tissue of the hind paw. The linear paw circumference was measured at time interval (minutes) of 0, 30, 60, 120, 180, 240, 360. Anti-inflammatory activity was measured as the percentage reduction in oedema level. Dichlofenac was used as a standard at 10 mg/kg , (13) as shown in Table 1.

Statistical analysis
All data were expressed as mean ± SD and one-way ANOVA was applied to determine the significance of the difference between the control groups and rat treated with the test compounds. GRAPHPAD (Instat USA Ver. 3) Statistical software was used for analysis of data obtained.

Antioxidant Study:
Free Radical Scavenging Activity by DPPH Method

The free radical scavenging capacity of the extracts was determined using DPPH. A methanol DPPH solution (0.33%) was mixed with serial dilutions (10µg to 50 µg) of *E. orchreata* tuber extracts and after 10 min; the absorbance was read at 517 nm. Vitamin C was used as a standard. The inhibition curve was plotted and IC50 values obtained (14).

Nitric Oxide Radical Inhibition Assay Activity

Nitric oxide radical inhibition was estimated by the use of Griess reagent. The reaction mix containing sod nitroprusside, phosphate buffer saline and different concentrations (10µg to 50 µg) of *E. orchreata* tuber extract was incubated at 30°C for 10 min. After incubation, 0.2 mL of Griess reagent B was added and incubated at 30°C for 20 min. Absorbance was measured at 542 nm against blank (15).

Reducing Power Assay

The reducing capability was measured by the reduction of Ferric to ferrous in the presence of different concentrations of methanolic extract at 700nm as per the standard method (16).

Anti lipid peroxidation activity

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using colon tissue homogenate as lipid media. Lipid peroxidation was induced by ferric chloride. Malondialdehyde (MDA), produced by the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) to yield a pink red complex measured at 532 nm using UV-visible spectrophotometer. Percentage inhibition of lipid peroxidation by different concentrations (100-500µg/ml) of *E. ochreata* methanolic extract was calculated. The colon 10% (w/v) homogenate was prepared using a tissue homogenizer under ice-cold conditions. The mixtures containing 0.5 ml of homogenate, 1 ml of 0.15 M KCl, and 0.5 ml of different concentrations (100-500µg/ml) of *E. ocherata* extract were prepared. Along with this, ascorbic acid was taken in different vials as standard. Lipid peroxidation was initiated by adding 100 µl of 1mM ferric chloride. The reaction mixtures were incubated for 30 min at 37˚C. After incubation, the reaction was stopped by adding 2 ml of ice–cold 0.25 N HCl containing 15% trichloroacetic acid (TCA) and 0.38% thiobarbituric acid (TBA) and 0.2 ml of 0.05 % butylated hydroxyl toluene (BHT). These reaction mixtures were heated for 60 min at

80˚C, cooled and centrifuged at 5000 g (≈6900 rpm) for 15 min. The absorbance of the supernatant was measured at 532 nm against a blank, which contained all reagents except colon homogenate and *E. ochreata* extract. Identical experiments were performed to determine the normal (without drug and $FeCl_3$) and induced (without drug) lipid peroxidation level in the tissue. The percentage of anti-lipid peroxidation effect (%ALP) was calculated by the following formula (17).

$$\% \text{ ALP} = [A_{FeCl3} - A_{test.} / A_{FeCl3} - A_{Normal}] \times 100$$

Where, A_{FeCl3}: Absorbance of $FeCl_3$, $A_{Normalt:}$ Absobance of control reaction,
$A_{test.}$: Absorbance of test reaction.

Results and Discussion

Monocytes/ macrophages, polymorphonuclear leucocytes (PMNs) and endothelial cells are mainly involved in inflammatory response. Activation of these cells triggers production of proinflammatory cytokines, ROS and other mediators of inflammation (18). Initiation and perpetuation of inflammatory cascade by ROS causes subsequent tissue damage through activation of nuclear factor kappa B (NF-kB) which is a ubiquitous transcription factor involved in regulation of several genes in immune and inflammatory response(19). Oxidants are capable of activating NF-kB and a group of structurally diverse anti-oxidants is capable of inhibiting NF-kB activation (20). Human body has anti-oxidant defense system, which involve enzymes such as superoxide dismutases, catalases and glutathione peroxidases. However, this anti-oxidant defense system is not sufficient and leads to increased free radical level and oxidative stress ultimately resulting in severe inflammation including cell death (21).

Not surprisingly, ROS has been implicated in the initiation and perpetuation of inflammatory disorders. It is therefore, well accepted that ROS play an important role in pathogenesis of Inflammatory responses. Concomitant to the increased ROS levels, antioxidant defense is found to be altered in inflammatory disorders. For example enzyme activities of Catalase, Superoxide dismutase and Glutathione are low. Increased ROS and lowered antioxidant defense leads to unrestricted oxidative stress which appears to be the major factor in pathogenesis of Inflammation (22). The excess ROS results in damage to cellular mechanism, ultimately disruption of cellular integrity and triggering inflammation mediated by NFkB. Plant products that have strong anti oxidant activity are, therefore, considered to have potential beneficial role in Inflammatory treatments.

Based on the properties of *E. ochreata*, it is clear that it can potentially cure local inflammation, can modulate antioxidant defense system and can be a logical choice in reliving symptoms in inflammatory disorders.

Rat Paw Oedema Model:

It is now well established that in Carrageenan-induced rat paw oedema model, Cox-2 mediated increase in prostaglandin production in the central nervous system contributes to the severity of inflammation and pain responses. Cox-2 is rapidly induced in the spinal cord and other regions of central nervous system following carrageenan injection in paw. Selective Cox-2 inhibitors that do not inhibit Cox-1 reduce levels of prostaglandin E2 in CSF. Any plant extract that shows effective reduction of oedema in this model has to be effectively and selectively inhibiting Cox-2 or PGE 2 and should prove to be effective against EP 3 receptor. The result of anti inflammatory activity of the, methanolic extract of *E. ochreata* is shown in Table 1. The extracts were tested at two different dose levels. The results showed that the methanolic extract with a dose of 0.5 gm/kg b.w showed 48 % of inhibition on carrageenan induced rat paw oedema after 6 hours, whereas reference drug dichlofenac showed 61 % inhibition. Methanolic extract with a dose 1 gm/kg b.w produced 53 % of inhibition. The development of odema in the paw of the rat after the injection of carrageenan is due to mediators of inflammation such as histamine, serotonin and prostaglandin like substances (23). Anti-inflammatory activity of methanolic extract (0.5 gm/kg b.wt) of *E. ochreata* may thus be due to inhibition of these mediators. The present result indicates the efficacy of methanolic extract (0.5 gm/kg b.wt) of *E. ochreata* as an efficient therapeutic agent in acute anti-inflammatory conditions. When the statistical analysis was carried out, it was observed that *E. ochreata* showed anti-inflammatory activity when compared with the standard. From Table 1. it is clearly seen that the anti-inflammatory activity of *E. ocheata* extract is very close to that of control drug used in the experiment.

Table 1. Percent reduction in paw volume as a marker of anti inflammatory activity.

Time (min.)	**Diclofenac cm**	**Eulophia ochreata cm 1 gm/kg**	**Negative cm**	**Eulophia ochreta cm 0.5 gm/kg**
0	3.39±0.01	3.72±0.01	2.37±0.01	3.21±0.03
30	3.34±0.02	3.72±0.01	2.55±0.03	3.17±0.01
60	3.27±0.01	3.69±0.02	2.79±0.01	3.09±0.015
120	3.19±0.15	3.57±0.01	2.93±0.01	2.91±0.01
180	3.17±0.02	3.48±0.03	3.16±0.02	2.74±0.01
240	3.09±0.01	3.34±0.01	3.52±0.01	2.68±0.01
360	2.89±0.01	3.11±0.02	3.66±0.01	2.59±0.01
% Inhibition	61%	53%**	----------	48%**

P<0.001 **
(n=6)

Antioxidant Study:

This study was designed to determine the antioxidant and free radical scavenging properties of methanolic extract of tubers of *E. orchreata* in various *in vitro* systems. The tubers showed significant result of DPPH radical scavenging capacity, Lipid Peroxidation, reducing power capacity and nitric oxide radical (table 2).

The antioxidant activity of methanolic extract increased in a dose dependent manner. About 10, 20, 30, 40 and 50 µg of methanol extract of *E. orchreata* L. showed 12.70, 23.83, 55.76, 62.53, 67.58 % inhibition respectively in DPPH free radical scavenging assay. The effect of methanolic extract on reducing power increases in a dose dependent manner. In nitric oxide scavenging assay, extract exhibited maximum of 89.86% inhibition at the concentration of 50 ug/ml. The IC50 value is 9.48 ug/ml, which indicate good antioxidant activity.

Lipid peroxidation in body involves series of free radical mediated chain reaction processes, which is associated with several biological damages. Lipid Peroxidation mainly affects biological membranes of especially intestine, liver, brain, spinal cord, containing highly oxidizable PUFA (polyunsaturated fatty acids). Inhibitory effects of ascorbic acid, *E. ochreata* extract, on TBARS formed in rat colon induced by $FeCl_3$ *in-vitro* were studied. The concentration range for both ascorbic acid and *E. ochreata* extract was taken in between 100 to 500 µg/ ml. *E. ochreata* extract showed increase in activity with increase in concentration. It was lowest (11.40 %) at 100 µg/ ml which significantly increases to 66.12 % at 500 µg/ml. In case of ascorbic acid, the lipid proxidation inhibition activity was far higher than *E. ochreata* extract at low concentration Ascorbic acid showed 83.58 % activity at 100 µg/ ml. Ascorbic acid showed 93.71 % inhibition of lipid peroxidation at 500 µg/ ml (Table 2). The data obtained in the assay was statistically analyzed by Unpaired T-test**.** Here P value is <0.0001 and is considered exteremly significant. Table 2. clearly shows that percentage inhibition of *E. ochreata* extract as judged by DPPH assay, NO scavenging assay and Reducing power assay is very similar to that of control i.e. Ascorbic acid and Curcumin. Surprisingly the antilipid peroxidation potential of *E. ochreata* extract is excellent as compared to standard and is at least 2-5 time more than that of Ascorbic acid.

Since oxygen radicals have high reactivity and short lifetime, it is difficult to assess the involvement and extent of tissue damage induced by oxygen radicals. Therefore, measuring the effect of radical reaction with biological substances like lipid peroxides was assessed. The degree of inflammation, tissue injury and lipid peroxidation caused was substantially reduced with the help of *E. ochreata*.

Table 2. Anti oxidant activity of methanolic extract of tubers of *E. ochreata*.

Conc. (μM/ml)	**DPPH scavenging assay (% Inhibition)**		**Anti lipid peroxidation**		**NO scavenging assay (% Inhibition)**		**Reducing power assay**	
	Ascorbic acid	***E. ochreata***	**Ascorbic acid**	***E. ochreata***	**Curcumin**	***E. ochreata***	**Ascorbic acid**	***E. ochreata***
10	17.07±0.35	12.70±0.86	83.58±0.0043	11.40±0.0037	78.74±2.43	74.69±2.2	0.6811±0.15	0.6526±0.25
20	26.60±1.14	23.83±0.57	86.14±0.0041	18.97±0.0037	87.74±2.10	76.46±1.01	0.8219±0.09	0.8127±1.74
30	57.68±1.72	55.76±0.43	89.17±0.0056	36.08±0.0052	88.47±0.45	80.68±0.55	0.9915±0.25	0.9813±1.65
40	69.03±1.46	62.53±2.16	91.85±0.0059	58.09±0.0067	89.22±1.12	81.44±1.83	1.1311±1.51	1.1021±1.77
50	71.45±0.93	67.58±0.87	93.71±0.0043	66.12±0.0037	89.86±1.74	82.44±2.09	1.501±0.13	1.4015±0.58
IC50 (μM/ml)	26.00	26.90	16.82	41.57	6.35	6.69	11.35	15.89

Conclusion

The results obtained in the present study indicate that the methanolic extract of *E. orchreata* tubers is a potential source of natural antioxidant and could therefore be exploited for its neutraceutical and medicinal properties. Further investigation on the isolation and identification of antioxidant component(s) in the plant may lead to identification of chemical entities with potential clinical use.

The results of the antiinflammatory study support the traditional use of *E. ochreata* in inflammation. Tubers of the *E. ochreata* possess significant anti-inflammatory activity. This may be due to active secondary metabolite present in the tubers. It is essential to carry out further studies to establish its therapeutic value as well as mechanism of action.

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