Research Paper

Evaluation of Antiinflammatory Activity of *Centratherum anthelminticum* (L) Kuntze Seed

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In the present study petroleum ether and alcoholic extracts of *Centratherum anthelminticum* (L) Kuntze seed (100 mg and 200 mg/kg p.o.) were evaluated for antiinflammatory activity in acute and subacute models of inflammation. It was found that both petroleum ether and alcoholic extracts showed significant reduction in paw oedema in carrageenan-induced model. In subchronic inflammatory phase both extracts provoked a significant reduction of transudation phase and too little extent proliferative phase when tested in cotton pellet-induced granuloma model. Both the extracts also reduced alkaline phosphatase activity in serum. The histopathology of granuloma tissue showed significant inhibition of lymphocytes, neutrophils, exudates, necrosis and giant cell when compared with control without ulcerogenic effect. The results suggest that petroleum ether and alcoholic extracts may exert antiinflammatory activity through prostaglandin inhibition, reduced myeloperoxidase and antitransudation.

Key words: Antiinflammatory, alkaline phosphatase, *Centratherum anthelminticum*, myeloperoxidase

Centratherum anthelminticum (L.) Kuntze is used in the indigenous system of medicine for the treatment of various ailments. The seeds are used as an anthelmintic against earthworm and tapeworm infestations; to cure ulcers, *vata* and *kapha*; used in skin disease, leucoderma; also used as emetic, purgative, for asthma, kidney trouble, as a blood purifier, for hiccoughs and in inflammatory swelling, good for sores and itching of the eyes and as depilatory. The seeds are also credited with tonic, stomachic and diuretic properties. In Ceylon, the plant is used for febrile convulsions. In Travancore, the seeds are grounded up to a paste with lime juice and are employed as a means of destroying pediculi, also given in anasarca and abscesses. They are also administered in case of intestinal colic, dysuria and snake bite[1].

The phytochemical constituents in the *C. anthelminticum* (L) Kuntze seeds are flavonoids 7,3',4'-trihydroxydihydroflavone (butin)[2], sterols-4α-methyl vernosterol[3], (24a/S)-stigmasta-5,22-dien-3ß-ol and (24a/S)-stigmasta-7,22-dien-3ß-ol[4], brassicasterol, stigmasterol and alkaloid-vernonine. Constituents such as stearic, palmitic, myristic, oleic, monohydroxy-oleic acids, linoleic, vernolic acid and resins have also been reported[5].

Centratherum (genus) with different species like *Centratherum cinerea*, containing chemical constituent like sterol, flavonoids, resins and alkaloids have been reported to have antipyretic, analgesic and antiinflammatory action[6,7]. These constituents are also present in *Centratherum anthelminticum* (L) Kuntze and may exhibit antiinflammatory activity. Analgesic and antipyretic activities of *Centratherum anthelminticum* have been reported in our earlier study[8]. Hence the present work has been undertaken to substantiate the folklore use of *Centratherum anthelminticum* seeds on experimental inflammation in rats.

MATERIALS AND METHODS

Diclofenac sodium (SGS Pharmaceutical Pvt. Ltd., Ghaziabad, India), carrageenan and o-dianisidine dihydrochloride (Himedia Ltd., Mumbai, India), petroleum ether and ethanol (Nice Chemical Pvt Ltd., Kochi, India), Hexadecyl-trimethylammonium bromide (Acros Organics, Fisher Scientific Mumbai,

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India) were procured from the sources mentioned in parenthesis.

Animals:

Wistar rats (150-200 g) and albino mice (20-30 g) of either sex were procured from K. L. E. S College of Pharmacy, Hubli, India. Animals were kept for one week to acclimatize to laboratory conditions before starting the experiment; they were given free access to water and standard rat feed but 12 h prior to an experiment, the rats were deprived of food but not water. The study was conducted after obtaining clearance from the Institution Animal Ethical Committee (KLESCOPH/IAECClear/2006-2007/05).

Plant extraction:

The seeds of *C. anthelminticum* (L.) Kuntze were collected from Hubli, Karnataka, and were authenticated at Department of Botany, S. K. Arts and H. S. Kothambri Science Institute, Hubli. Coarse powder of desired particle size was subjected to successive extraction in a Soxhlet apparatus using petroleum ether (60-80°) and alcohol. Appearance of colorless solvent in the siphon tube was taken as the end point of extraction. The extracts were concentrated to ¾ of its original volume by distillation. The concentrated extracts were taken in a china dish and evaporated on a thermostat controlled water bath till it forms a thick paste.

Acute toxicity study:

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD), revised draft guidelines 423, revised by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Acute toxicity studies were performed on albino mice of either sex. Mice were fasted over night prior to subjecting them to the up and down procedure. In addition, source of animal, sex, age, bodyweight, route of administration, solvent, presence and absence of any immediate reactions were also recorded for further references. The maximum non lethal dose was found to be 2000 mg/kg body weight; hence $1/10^{th}$ dose was taken as effective dose for both petroleum ether and alcohol extracts of *Centratherum anthelminticum* (L) Kuntze seed to evaluate antiinflammatory activity.

Carrageenan-induced paw oedema:

Wistar rats were divided into six groups, of six rats each. Oedema was induced by injecting 0.1 ml of 1% carrageenan suspension into the subplantar region of the right hind paw of the rats[9]. Control group rats received saline solution (0.9% w/v, NaCl) 2 ml/kg and reference group of rats received 40 mg/kg sodium diclofenac, orally. The test groups of rats were treated orally with 100 and 200 mg/kg of the petroleum ether and alcohol extracts 60 min before carrageenan injection. The paw volume was measured plethysmometrically before administering carrageenan (V_0) and 0.5, 1, 2, 3, 4, 5, 6 and 24 h after (V_t)[10]. Inflammation was calculated as the increase in volume (ml) of the paw after treatment subtracted of the basal volume. Results were expressed as percentage of inhibition of oedema, calculated according to the formula[11], percent inhibition = $[(V_t–V_0)_{control} – (V_t–V_0)_{treated}]/(V_t–V_0)_{control}\times100$.

Subplantar tissue obtained from animals per group, killed 3 h after injection of carrageenan or saline[12]. Myeloperoxide (MPO) was extracted from homogenized tissues by suspending the material in 0.5% hexadecyl-trimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. The specimens were freeze-thawed 3 times. Suspensions were then centrifuged at 3000 rpm for 10 min at room temperature, and the supernatant fraction was used for MPO activity determination. A 100 µl aliquot of supernatant was mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide[12,13]. Incubation was performed at room temperature for 10 min, and then change in the absorbance at 460 nm over 1 min was measured with a spectrophotometer. Units of MPO were calculated considering that 1U MPO= 1 µmol H_2O_2 gives split, and 1 µmol gives a change in absorbance of 1.13×10^{-2} nm/min. MPO activity was expressed as unit of MPO per milligram of protein.

Cotton pellet method:

Wistar rats were divided into six groups of six rats each. Adsorbent cotton wool was cut into pieces weighing 20±1 mg and made into pellets. The pellets were then sterilized in a hot air oven at 120^0 for 2 h[14,15]. The abdomen was shaved cleanly, swabbed with 70% ethanol and two sterilized cotton pellets were implanted subcutaneously, one on each side of the abdomen of the animal under light ether

anaesthesia. Test drugs were administered once daily throughout the experimental period of 7 days. On the 8th day after implantation, rats were anaesthetized with pentobarbital sodium. The pellets were dissected and dried at 60^0 for 18 h, weighed after cooling. The mean weight of the cotton pellets of the control group as well as of the test groups was calculated. The transudative weight, granuloma formation and percent granuloma inhibition of the test compound were calculated.

Biochemical analysis

Alkaline phosphatase (ALP)[16] was estimated using ALP kit in Auto-analyzer. Blood was collected by retro-orbital, centrifuged and 1000 µl of supernatant serum was collected. The serum ALP reagent is added and estimated in an auto-analyzer. After collectiing blood, the abdomen of the animals from the cotton pellet-induced granuloma formation was opened. The stomachs were removed and opened along the greater curvature, rinsed in normal saline and pinned out on wax plate and ulcer index was measured[17].

The histological changes in the granuloma tissue were studied[18]. Under light ether anesthesia the hairs in the axilla and groin were clipped out and a subcutaneous dead space wound were inflicted in the same region by making a pouch through a small nick in the skin. Granuloma formation was induced by implanting grass pith. Test drugs were administered once daily throughout the experimental period of 7 days. On 8th day after implantation, rats were anaesthetized with pentobarbital sodium. The grass pith was dissected and the granuloma tissues were fixed in 10% formalin solution.

Statistical analysis

The results were expressed as the Mean±SEM. Results obtained from the present study were analyzed using One way ANOVA followed by Dunnett's multiple comparison tests. Data was computed for statistical analysis by using Graph pad Prism Software. Differences between the data were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Following the up and down method it was found that both petroleum ether and alcohol extracts of *Centratherum anthelminticum* do not produce any toxic effect at a maximum dose of 2000 mg/kg. Hence two doses, 100 and 200 mg/kg were selected in the present study for both the extracts.

In carrageenan-induced paw oedema activity, the paw volumes and percentage of inhibition of the control, standard and test compounds are shown in Table 1. The entire test compounds were compared with diclofenac as a standard at a dose of 40 mg/kg for antiinflammatory activity. Presently diclofenac showed 50.77% inhibition of inflammation at 3rd h when compared to control.

Petroleum ether and alcohol extracts of *Centratherum anthelminticum* seeds (200 mg/kg) showed significant inhibition of inflammation with 46.15% and 41.54%, respectively. Whereas, 100 mg/kg petroleum ether and alcohol extracts showed 36.92% and 33.85% inhibition of oedema, respectively at 3rd h when compared with control. The results of test compounds were found to be statistically significant at value $P<0.05$.

MPO activity, a marker of polymorphonuclear cell (PMNs) accumulation in subplantar areas, was determined in diclofenac, extracts and control treated groups. The results are shown in Table 2. In the

TABLE 1: EFFECT OF *CENTRATHERUM ANTHELMINTICUM* SEED EXTRACTS IN CARRAGEENIN-INDUCED PAW OEDEMA IN RATS

Treatment	Dose (mg/kg)	30 min (%)	1 h (%)	2 h (%)	3 h (%)	24 h (%)
Control	--	0.43±0.02	0.48±0.02	0.50±0.02	0.65±0.02	0.32±0.02
Diclofenac	40	0.36±0.03* (16.28)	0.39±0.02*(18.75)	0.34±0.02* (32)	0.32±0.02* (50.77)	0.1817±0.01* (43.75)
Pet ether extract	100	0.39±0.02 (9.30)	0.45±0.01 (6.25)	0.43±0.01* (14)	0.41±0.01* (36.92)	0.28±0.01* (12.5)
	200	0.37±0.02 (13.95)	0.41±0.02* (14.58)	0.38±0.02* (24)	0.35±0.02* (46.15)	0.22±0.01* (31.25)
Alcohol extract	100	038±0.01 (11.63)	0.47±0.2 (2.10)	0.44±0.02 (12)	0.43±0.01* (33.85)	0.30±0.01 (6)
	200	0.38±0.02 (11.63)	0.43±0.02 (10.42)	0.40±0.01* (20)	0.38±0.01* (41.54)	0.25±0.01* (21.88)

Values expressed as mean±SEM, n=6 animals in each group. The results were analyzed using One way ANOVA followed by Dunnett's multiple comparison tests. $P<0.05$ was used to indicate statistical significance when compared to control.

current investigation diclofenac showed 53.44% inhibition of MPO when compared with control. Both the extracts at the dose of 200 mg/kg showed 41.23 and 29.98% inhibition in MPO, while petroleum ether extract 100 mg/kg and alcohol extract 100 mg/kg showed 23.28% and 18.27% when compared with control. The petroleum ether extract 200 mg/kg and alcohol extract 200 mg/kg were found to be more significant.

The cotton pellet granuloma method has been widely employed to access the transudative, exudative and proliferative components of subacute inflammation. The results are shown in Table 3. As shown in Table 3, all the extracts and standard drug (diclofenac 40 mg/kg) used in study elicited significant inhibitory activity on the wet weight granuloma. Presently diclofenac showed significant 57.52% inhibition of transudative weight when compared with control.

Both the extracts at the dose of 200 mg/kg showed significant 51.89% and 48.11% inhibition in wet weight granuloma. Whereas, compounds petroleum ether extract 100 mg/kg and alcohol extract 100 mg/kg showed 42.62% and 36.13% inhibition when compared with control (159.84±2.89). These results suggest that the test extracts exhibit antitransudative effect.

TABLE 2: EFFECT OF *CENTRATHERUM ANTHELMINTICUM* SEED EXTRACTS ON MYELOPEROXIDASE

Treatment	Dosage (mg/kg)	MPO (U/mg)	Percentage of inhibition (%)
Control	--	0.09583±0.007	---
Diclofenac	40	0.04467±0.003*	53.44
Pet ether extract	100	0.0735±0.003*	23.28
	200	0.05633±0.003*	41.23
Alcoholic extract	100	0.07833±0.002*	18.27
	200	0.06717±0.003*	29.98

Values expressed as mean±SEM, n=6 animals in each group. The results were analyzed using One way ANOVA followed by Dunnett's multiple comparison tests. *P<0.05 was used to indicate statistical significance when compared to control.

The granuloma weight of standard (diclofenac) and various extract after drying were calculated. The percentage inhibition of granuloma weight or antiproliferative effect of diclofenac, petroleum ether extract 200 mg/kg, alcohol extract 200 mg/kg, petroleum ether extract 100 mg/kg and alcohol extract 100 mg/kg were found to be 24.30, 22.49, 15.05, 11.39 and 8.28%, respectively, when compared with control. Hence, the petroleum ether extract 200 mg/kg as been found to be more significant compared to alcohol extract 200 mg/kg. These extracts were found to be less significant as compared to diclofenac.

Oral administration of petroleum ether and alcohol extracts in two doses of 100 mg/kg and 200 mg/kg for 7 days did not induce gastric lesion in rats. Whereas, diclofenac produced significant gastriomucosal lesions. Cotton pellet implantation caused an increase in serum alkaline phosphatase. As shown in the Table 4, the petroleum ether and alcoholic extracts as well as diclofenac reduced increased serum alkaline phosphatase when compared to control and were found to be statistically significant at value P<0.05.

In the present investigation diclofenac showed significant 66.23% inhibition of alkaline phosphatase when compared with control. Both petroleum ether and alcoholic extracts at the dose of 200 mg/kg showed significant 60.79% and 44.30% inhibition in ALP, while both the extracts at the dose of 100 mg/kg showed 19.26% and 14.51% when compared with control. The petroleum ether and alcoholic extracts at 200 mg/kg were found to be more significant.

Histopathological studies reveal that the tissue of control group shows fibrocollogenous tissue with few proliferating blood vessels and dense infiltration by acute inflammatory comprising of neutrophils and lymphocytes (chronic inflammatory cells).

TABLE 3: EFFECT OF CENTRATHERUM ANTHELMINTICUM SEED EXTRACTS IN COTTON PELLET METHOD

Treatment	Dosage mg/kg	Wet weight (mg)	Dry weight (mg)	Percentage of inhibition	Transudative weight (mg)	Percentage of inhibition
Control	--	217.5±8.81	57.85±1.67	--	159.7±7.64	--
Diclofenac	40	115±1.85*	43.79±1.95*	24.30	67.84±2.89*	57.52
Pet ether extract	100	142.9±1.67*	51.26± 0.64*	11.39	91.64±1.25*	42.62
	200	121.7±1.04*	44.89±1.07*	22.49	76.83±1.51*	51.89
Alcoholic extract	100	153.8±1.79*	53.06±1.77	8.28	102±1.65*	36.13
	200	130.8±1.84*	49.14±1.57*	15.05	81.7±0.98*	48.11

Values expressed as mean±SEM, n=6 animals in each group. The results were analyzed using One way ANOVA followed by Dunnett's multiple comparison tests. *P<0.05 was used to indicate statistical significance when compared to control.

Macrophage, giant cells, necrosis and exudates were also noted. Where as, the tissue from the standard diclofenac (40 mg/kg) groups showed many proliferating blood vessel along with minimal number of acute inflammatory cells and lymphocytes as shown in fig. 1.

The tissue from petroleum ether and alcohol extracts treated groups showed few proliferating blood vessel along with minimal number of acute and chronic inflammatory cells, necrosis, giant cell and exudates. The vasculature changes were less compared to the control and the microscopical changes seen were similar to diclofenac treated group. Alcohol extract 100 mg/kg treated group did not show much of the characteristics explained above showing weak antiinflammatory activity. The histopathological feature obtained with petroleum ether extract 200 mg/kg and alcohol extract 200 mg/kg showed more significant antiinflammatory activity when compared with control.

Antiinflammatory effect of *Centratherum anthelminticum* (L) Kuntze seeds extracts was investigated in present study. The carrageenin test was selected because of its sensitivity in detecting orally active antiinflammatory agent particularly in acute phase of inflammation[19]. The intraplantar injection of carrageenin in rats leads to paw oedema and is believed to be biphasic. Its first phase (0-2 h after injection of carrageenin) results from the concomitant release of mediators: Histamine, serotonin and kinins on the vascular permeability. The second phase is correlated with the elevated production of prostaglandins, oxygen- derived free radials and production of inducible cyclooxygenase[20]. Oral administration of the petroleum ether extract and alcohol extract of *Centratherum anthelminticum* seeds suppressed the oedematous response after 2 h and this effect continued up to 5 h. The observed effect was similar to that of diclofenac. Based on the results obtained, it is likely that the mechanisms of action of the seed extracts are similar to that of Non-steroidal antiinflammatory drugs, namely inhibition of prostaglandins biosynthesis.

TABLE 4: EFFECT OF *CENTRATHERUM ANTHELMINTICUM* SEED EXTRACTS ON ALKALINE PHOSPHATASE IN COTTON PELLET METHOD

Treatment	Dosage mg/kg	ALP (IU/L)	% Inhibition of ALP
Control	--	379±27.43	--
Diclofenac	40	128±4.44*	66.23
Pet ether extract	100	306±10.44*	19.26
	200	148.6±4.70*	60.79
Alcoholic extract	100	324.2±3.93*	14.51
	200	211.1±5.95*	44.30

Values expressed as mean±SEM, n=6 animals in each group. The results were analyzed using One way ANOVA followed by Dunnett's multiple comparison tests. *P<0.05 was used to indicate statistical significance when compared to control.

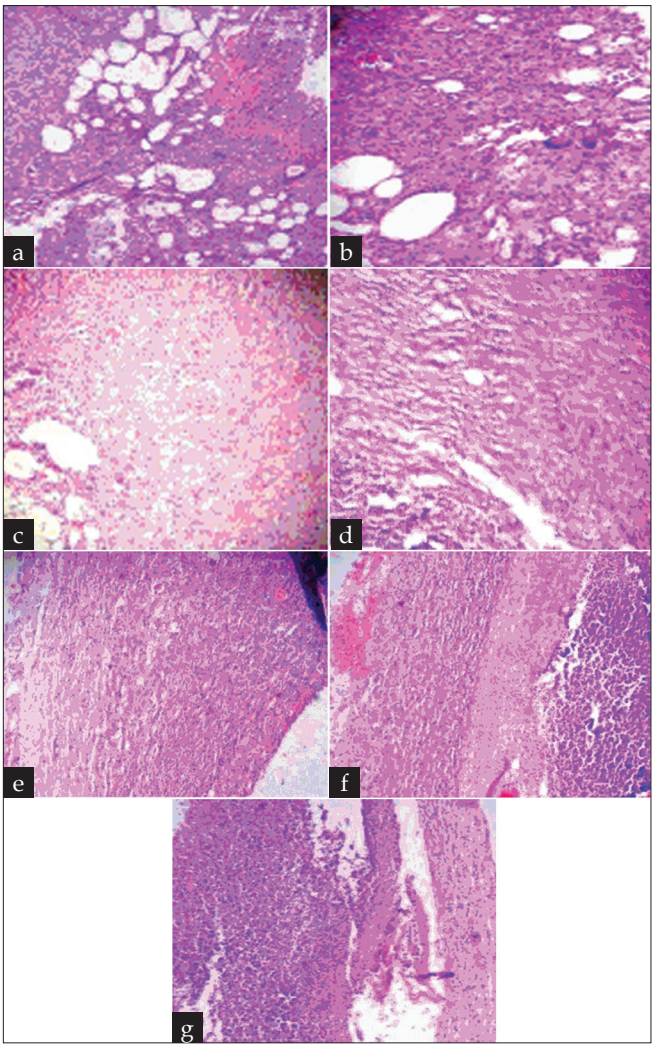

Fig.1: Histological sections of granuloma tissue
(a and b). control group; (c) standard group; (d) petroleum ether extract 100 mg/kg group; (e) petroleum ether extract 200 mg/kg group; (f) alcohol extract 100 mg/kg group; (g) alcohol extract 200 mg/kg group. (a) Histological section of granuloma tissue in control group showing severe acute inflammation with necrosis, exudates and fats. (b) Histological section of granuloma tissue in control group showing acute inflammation with giant cells and lymphocyte. (c) Histological section of granuloma tissue in standard group showing mild chronic inflammation with granulation tissue and blood vessels. (d) Histological section of granuloma tissue in petroleum ether extract 100 mg/kg group showing mild-moderate chronic inflammation with exudates and necrosis. (e) histological section of granuloma tissue in petroleum ether extract 200 mg/kg group showing mild chronic inflammation with granulation and blood vessels. (f) Histological section of granuloma tissue in alcohol extract 100 mg/kg group showing moderate to severe acute inflammation with exudates, necrosis (g) Histological section of granuloma tissue in alcohol extract 200 mg/kg group moderate chronic inflammation with exudates.

Accumulation of neutrophils is a prominent feature of a number of inflammatory diseases. Indeed, an ability to estimate the quantity of neutrophils in inflamed tissue might prove useful to judge the intensity of inflammation[21]. MPO enzymes, contained in azurophilic granules of PMNs, are involved in the formation of reactive oxygen species[22]. The PMNs release oxygen–derived free radicals and pro–inflammatory cytokines[23]. These factors from PMNs also cause the increase of plasma exudation[24].

In the present study, diclofenac and both petroleum ether and alcohol extracts significantly decreased the elevated MPO activity in inflamed paws, suggesting that inhibition of neutrophils infiltration may be another mechanism by which test compound achieve its antiinflammatory effect.

The inflammatory granuloma is a typical feature of subacute inflammatory reaction[25].The cotton pellet granuloma method has been widely used to assess the transudative, exudative and a proliferative phase of subacute inflammation. The fluid adsorbed by the pellet greatly influences the wet weight of the granuloma, whereas the dry weight correlate well with the amount of granulomatous tissue formed. Most of the NSAIDs like diclofenac possess only slight inhibition on the granuloma formation. The steroidal drug on the contrary, exhibits profound reduction of the granuloma. As both test extract with different dose used in this study elicited significant inhibitory activity on the wet weight of granuloma. This suggests an inhibitory effect of the compounds on vascular permeability. When assessment was made on the dry weight of granuloma, which projects the effect of test substances on the proliferative phase of inflammation, it was found that only petroleum ether extract 200 mg/kg appeared to be effective in inhibition of granuloma formation. The raise in ALP was normalized by diclofenac and both extracts. Histopathological report reveals that extracts inhibited migration of leukocytes and other inflammatory cells like neutrophils, lymphocytes, giant cells, necrosis and exudates at site of inflammation. Thus the present study concludes that the petroleum ether and alcoholic extracts have anti-inflammatory activity in acute and subacute phase of inflammation in laboratory animals at the doses of 100 mg/kg and 200 mg/kg without ulcerogenic effect. Further investigation are needed to know the protective effect of *Centratherum anthelminticum* (L) Kuntze seeds using different animal models and different extracts of the seeds.

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