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Research Article

A PRELIMINARY STUDY ON THE EFFECTS OF PETROLEUM ETHER & ALCOHOLIC BARK EXTRACT OF *Anthocephalus cadamba* (Roxb.) MIQ. IN ACUTE AND CHRONIC *IN-VIVO* INFLAMMATORY RAT MODELS

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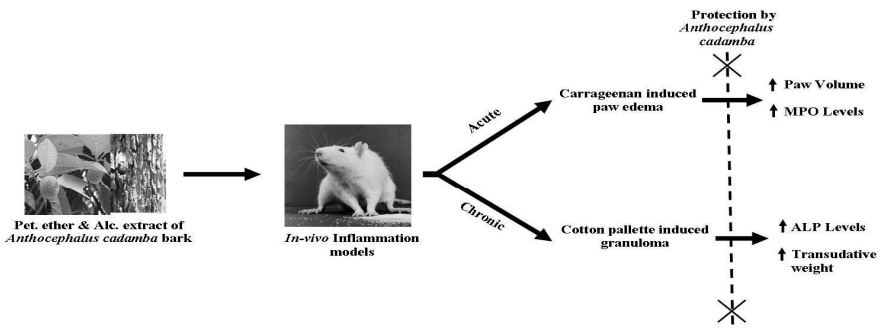
ABSTRACT



In order to appraise the traditional use of *Anthocephalus cadamba*, the petroleum and alcoholic extracts of stem bark of *Anthocephalus cadamba*, were investigated for their acute and chronic anti-inflammatory activity. The anti-inflammatory activity of petroleum ether and alcoholic extract were evaluated using the Carrageenan induced paw oedema (acute inflammation) and Cotton pellet induced granuloma (chronic inflammation) in rats. In the carrageenan induced rat paw edema model, both the extracts were found to exhibit significant reduction in paw size. The groups treated with alcoholic extract at the dose 667 mg/kg showed maximum effect at 4th hour and the group treated with petroleum ether extract at the dose of 286 mg/kg has shown the maximum effect in 4th hour of carrageenan administration. In cotton pellet granuloma the group treated with petroleum ether extract at the dose of 286 mg/kg suppressed the transudative, exudative and proliferative phases of chronic inflammation. The anti-inflammatory effect produced by both petroleum ether and alcoholic extract at the dose of 667 mg/kg, 286 mg/kg, and 200 mg/kg was compared with the reference drug diclofenac sodium. Over the study period there was no mortality or toxic signs & symptoms recorded in the group of mice given 2000 mg/kg p.o of petroleum ether, alcoholic extract. The results demonstrate that though both the extracts are equally effective in acute inflammatory condition but petroleum ether extract of stem bark of *Anthocephalus cadamba* has showed more significant anti-inflammatory activity in chronic inflammatory condition than alcoholic extract.

KEYWORDS: *Anthocephalus cadamba*, Anti-inflammatory, Carrageenan, Cotton pellet granuloma

INTRODUCTION

In modern medicine practice, Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medications due to their efficacy for a wide range of pain and inflammatory conditions.[1] However, there are well known side effects of NSAIDs reported, most commonly gastro-intestinal tract discomfort viz. mucosal damage, dyspepsia, ulceration and upper gastro-intestinal bleeding summarized as "NSAIDs gastropathy". Others includes renal disorders, coronary heart disease, myocardial infraction, cardiovascular death and stroke.[2,3] Due of the deleterious side effects attributed to the prolonged antirational use of NSAID and their limited effectiveness in some exceptional cases like neuropathic pain, the safe control of inflammatory pain is still a major challenge.

Since ancient times, medicinal and aromatic plants have been considered an excellent source of steroids and triterpenes and have been used to treat different types of inflammation and pain[4] Kadamba belonging to family rubiaceae, is one such example of plant containing steroids and triterpenes, widely distributed throughout the greater part of Asia and was used as a folk medicine in the treatment of fever, anaemia, uterine & blood disorders, skin diseases, dysentery and for improvement of semen quality [5]. Several scientific studies had been carried out to reveal its antimalarial[6] and hepatoprotective activities[7]. The major constituents of its bark are steroids viz. sitostenone, 3b-ergost-5-en-ol, g-sitosterol, stigmasterol and 4, 22-cholestadien-3-one, b-sitosterol, triterpenes, tripernoid glycosides, saponins, indole alkaloids cadambine, 3a-dihydrocadambine, cadamine, isocadamine and isodihydrocadambine.[8,9,10] Limited scientific

work has been done on anti-inflammatory activity of the *Anthocephalus cadamba*. Hence, the present work has been undertaken to validate the folklore use of *A. cadamba* bark extracts on experimental acute and chronic inflammation in rats.

MATERIALS AND METHODS

Plant material

The stem bark of plant *Anthocephalus cadamba*, were collected from Siwan, Bihar, India and was botanically identified by Dr. Jawahar, Senior taxonomist, herbarium division, FRLHT, Bangalore. A voucher specimen has been deposited at the Herbarium, FRLHT, Bangalore for the same.

Chemicals

Alcohol, Petroleum ether, Anesthetic ether (Karnataka Fine Chemicals, Bangalore, India), Tween 80 (Rolex chemical, Atla's trademark, Mumbai, India), Carrageenan (Hi-media, Mumbai, India) and Diclofenac (Voveran, Novartis, Thanem, India) were used in this study and was used as standard drug.

Preparation of the petroleum ether (PEAC) and alcoholic (AEAC) extracts of *Anthocephalus cadamba* stem bark

The shed dried crude drug was subjected to pulverization and was passed through sieve no. 40. Then the powder was packed into a soxhlet apparatus and exhaustively extracted with petroleum ether (60-80ºC) for 18 hours. The same marc was successively extracted with alcohol for 18 hours. The solvent was removed under reduced pressure on a rotary evaporator at 40-45^{0}C. The obtained crude extract was stored in airtight containers for further experimental use. The percentage yield for PEAC and AEAC were found to be 1.67 % and 7.85 % respectively. The extracts thus obtained were subjected to phytochemical analysis, toxicity studies and anti-inflammatory activity.

Phytochemical screening of the extracts

The extracts of *Anthocephalus cadamba* were screened for the presence of various constituents employing standard screening test[9,11]. Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc., was used.

Experimental Animals

Wistar albino rats (150-200 g) and mice (20-30 g) of either sex were procured from ABMRCP animal house, India. Animals were kept for one week to acclimatize the laboratory conditions before starting the experiment. They were given free access to water and standard rat feed but were deprived of food 12 h prior to experiments. The study was conducted after obtaining clearance from Institutional Animal Ethical Committee (997/C/06/CPCSEA). Animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA under standard husbandry conditions: Room temperature 26$\pm$2^{0}C, Relative humidity 45-55%, 12 hours light and dark cycle).

Acute toxicity studies

The acute oral toxicity study were carried out as per the standard guidelines set by Organization's 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. The mice were divided into six groups containing six animals in each group. PEAC and AEAC were suspended in vehicle. The PEAC and AEAC were administered orally as a single dose at different concentrations (500, 750, 1000, 1250, 1500 and 2000 mg kg^{-1} b.w.). The mortality, morbidity, food and water intake, toxic symptoms and the general behavior of mice were observed continuously during the study period of 72 hrs. The number of mortality rate was expressed as a percentile and the LD_{50} was determined by earlier standard protocols[12]. The maximum non lethal dose was found to be 2000 mg/kg body weight; hence 1/10th, 1/7th & 1/3rd doses were taken to evaluate the effective dose for both the extracts of *Anthocephalus cadamba* to evaluate acute and chronic anti-inflammatory activity.

Anti-Inflammatory Activity

Carrageenan induced rat hind paw oedema model (Acute inflammation)

Rats were divided into eight groups (n=6). Before any treatment, size of the right paw of each animal was determined thrice using a vernier caliper. The mean of these determinations constituted the Vo, basal paw size. Group 1 served as control and received 0.5 ml of vehicle (DMSO and distill water in the ratio of 1:1 and two drops of Tween 80 per ml) orally. Group 2 received the standard drug, diclofenac (100 mg/kg) orally. Group 3, 4 and 5 received the alcoholic extract (suspended in vehicle), as pretreatment, 30 minutes before sub-plantar injection of 0.1 ml carrageenan solution (0.1%), administered orally at 667, 286 and 200 mg/kg respectively. Similarly, group 6, 7 & 8 received petroleum ether extract, suspended in vehicle, was administered orally at 667, 286, and 200 mg/kg respectively 30 minutes before the carrageenan injection. After 30 minutes of drug treatment, paw edema was induced by sub-plantar injection of 0.1 ml carrageenan solution (0.1%) into the plantar surface of the right hind paw of each rat. After carrageenan administration, the paw size was again measured at 0, 1, 2, 3, and 4 hour[13]. The paw size value, which was measured at different time intervals after treatment represent (V_t) for each treated rat. The mean paw size value (V_t) of treated group were compared against control subsequently to determine percentage protection. Percentage inhibition (*I*%) of inflammation in each treated group was determined using the following formula:

$$I\,\% = \frac{(Vt - Vo)\ \text{control} - (Vt - Vo)\ \text{treated}}{(Vt - Vo)\ \text{control}} \times 100$$

Animals were sacrificed 3h after carrageenan injection and sub-plantar tissue were obtained.[14] Tissue was homogenized and Myeloperoxide (MPO) was extracted by suspending the homogenate in 0.5% hexadecyl-trimethylammonium bromide in 50 mM potassium phosphate buffer (pH 6.0). Suspensions were centrifuged (3000 rpm for 10 min), and supernatant fraction was further used for determination of MPO activity. After mixing 100 µl aliquot of supernatant fraction with 2.9 ml of 50 mM phosphate buffer (pH 6.0), containing o-dianisidine dihydrochloride (0.167 mg/ml) and hydrogen peroxide (0.0005%)[15], incubation was done for 10 min at normal room temperature. A Hitachi (u 2900) spectrophotometer was used to measure the change in the absorbance at 460 nm over 1 min. MPO activity was expressed as unit of MPO/milligram of protein; 1U MPO=1 µmol H_2O_2 gives split, and 1 µmol gives alteration in absorbance of 1.13×10^{-2}nm/min.

Cotton pellet-induced granuloma (Chronic inflammation)

The cotton pellets-induced granuloma in rat was studied according to pervious method.[16,17] The animals were divided into eight groups (n= 6). The rats were anaesthetized and autoclaved (sterile) cotton pellets; weighing 10±1 mg were

implanted sub-cutanueously into both sides of the groin region of each rat. Group 1 served as control and received 0.5 ml of vehicle (DMSO and distill water in the ratio of 1:1 and two drops of Tween 80 per ml) orally. Group 2 received the standard drug, diclofenac (100 mg/kg) orally. Group 3, 4 and 5 received the alcoholic extract, suspended in vehicle, administered orally at 667, 286, and 200 mg/kg respectively. Group 6, 7 and 8 received the petroleum ether extract, suspended in vehicle, was administered orally at 667, 286 and 200mg/kg respectively, daily for seven consecutive days. On 8th day the animals were anaesthetized and the cotton pellets along with granuloma tissue were removed carefully and made free from extraneous tissues. The wet pellets were weighed and were dried in an oven at 60ºc for 24 h to get constant weight. Increment in the dry weight of the pellets was taken as a measure of granuloma formation.[18] The antiproliferative effect of PEAC and AEAC were compared with control.

Biochemical Analysis

Blood was collected through retro-orbital route, blood allowed to clot properly at room temperature for approximately 30-60 min then it was centrifuged (Remi-Microcentrifuge CM-12 plus) at 3000 rpm for 15 min to separate serum from blood. Alkaline phosphatase (ALP) was estimated using erbachem ALP kit.[19] The serum ALP reagent was added to supernatant serum and ALP was estimated using a semi auto analyzer (RA 50 BAYER).

Statistical Analysis

Analysis of each data set was performed by student-t test, and one-way analysis of variance (ANOVA). Statistically significant effects were further evaluated with Dunnett's multiple comparison tests. Differences were considered significant at $P < 0.05$. Results are expressed as means ± SEM.

RESULTS AND DISCUSSION

Preliminary phytochemical screening of *Anthocephalus cadamba* extract revealed the presence of steroids, tannins, alkaloids, saponins and absence of carbohydrate, proteins, inulin, flavonoids and amino acids in both PEAC and AEAC extracts. The percentage yield for PEAC was found to be 1.67 % (brown color), where as for AEAC was found to be 7.85 % (dark brown-black color).

In the acute toxicity assay no mortality were observed at the tested dosage levels. Even with 2000 mg/kg dose, neither deaths nor stereotypical symptoms associated with toxicity occurred during 72 hrs of observation period. Hence three doses, 200, 286 & 667 mg/kg were selected in the present study for both the extracts.

Edema formation due to carrageenan in the rat paw is the biphasic event. In early phase (1-2 hrs) of the carrageenan model, mediated by histamine, serotonin and increased synthesis of prostaglandins in the oedematous tissue while later phase (3-5 hrs) was sustained by prostaglandin release, oxygen derived free radicals, production of inducible cyclooxygenase, protease, lysosome and mediated by bradykinins, leukotrienes, polymorph nuclear cells and prostaglandins produced by tissues macrophages[20].

It is known that the third phase of the edema-induced by carrageenan, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction [21]. In carrageenan-induced paw oedema activity, the paw volumes and percentage of inhibition of control, standard and test compounds are shown in Fig. 1.

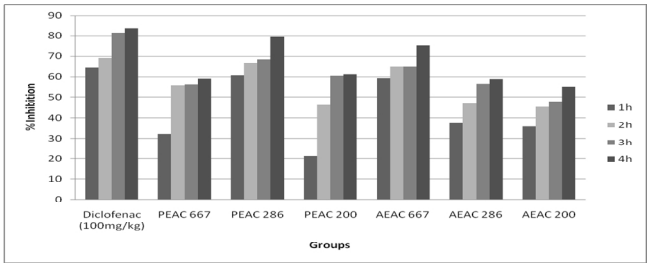


Fig 1: Effect of stem bark extract of Anthocephalus cadamba on carrageenan induced paw edema in rats

The total investigational extracts were compared with diclofenac (100 mg/kg) as a standard for anti-inflammatory activity. Diclofenac showed maximum inhibition (83.67%) of inflammation at 4th h when compared to control.

Petroleum ether and alcohol extracts of *Anthocephalus cadamba* bark (667 mg/kg) showed significant inhibition of inflammation with 59.18% (4th h) and 74.97% (4th h), respectively. 286 mg/kg petroleum ether and alcohol extracts showed 79.63% (4th h) and 58.97% (4th h) respectively, whereas 200 mg/kg petroleum ether and alcohol extracts showed 61.10% (4th h) and 54.89% (4th h) inhibition of oedema, when compared with control.

Both the extracts PEAC and AEAC have significant effect on both the phases but with different doses. The suppression of the 1st phase may be due to inhibition of release of early mediators such as histamine, serotonin and inhibition of phospholipase A_2 (PLA2) activity or cyclooxygenase pathway and the suppression of 2nd phase was observed with all the doses of PEAC and AEAC very significantly. Maximum suppression of 2nd phase was observed with the dose of PEAC 286 mg/kg after 4th h of carrageenan induced edema, probably by blocking the release of vasoactive substance (histamine, serotonin and kinins). Both the extract showed significant protection against oedema.

Neuropeptides such as SP and CGRP are released from peripheral terminals of primary afferent sensory nerves and contribute significantly to the inflammatory response of a variety of diseases including rheumatoid arthritis. These neuropeptides have been shown to be capable of producing vasodilation, increasing vascular permeability, attracting and activating phagocytic white blood cells, releasing cytokines, lysosomal enzymes and prostaglandins from these cells, increasing the expression of adhesion molecules as well as causing the activation of synoviocytes.

Polymorphonuclear cell (PMNs) accumulation in subplantar areas was evaluated by estimation of MPO activity in control, standard and treated groups. The results are shown in Table 1.

Table 1: Effect of *Anthocephalus cadamba* bark extract on Alkaline Phosphatase and Myeloperoxidase in cotton pellet

Group	Control	Diclofenac (100 mg/kg)	PAEC (mg/kg b.w.) 667	286	200	AEAC (mg/kg b.w.) 667	286	200
ALP (IU/L)	659.8±38.29	225.7±17.51***	398.3±6.0***	317.8±9.22***	508.5±15.13***	458.3±10.5***	470.2±14.05***	541.7±18.63***
ALP % Inhibition	--	65.80	39.63	51.83	22.93	30.54	28.73	17.90
MPO (U/mg)	1382±37.11	454.2±17.37***	656.2±17.54***	568.3±14***	746.3±15.84***	683.3±14.87***	695±19.32***	900.7±28.78***
MPO % Inhibition	--	67.13	52.52	58.88	46.00	50.56	49.71	34.83

ALP: Alkaline phosphatase; MPO: Myeloperoxidase; PAEC: Petroleum ether extract of Anthocephalus cadamba; AEAC: Alcoholic extract of Anthocephalus cadamba. Values expressed as mean+SEM, n=6 animals in each group. The results were analyzed using One way ANOVA by Dunnett's multiple comparision tests. ***p<0.001 was used to indicate statistical signicance when compare to control

In the present analysis, diclofenac showed 67.13% inhibition of MPO which was highly significant (P<0.001) when compared with control. Both extracts (petroleum ether and alcoholic) at the dose of 667 mg/kg showed 52.52 and 50.56% inhibition in MPO, while petroleum ether extract 286 & 200 mg/kg and alcohol extract 286 & 200 mg/kg showed 58.88 & 46.00% and 49.71 & 34.88% when compared with control. The maximum significance was observed in the petroleum ether extract 286 mg/kg and alcohol extract 667 mg/kg.

Neutrophils accumulation is a key aspect of a number of inflammatory disorders. The intensity of inflammation can be evaluated by measuring the extent of neutrophils in inflamed tissue.[22] MPO enzymes enclosed in azurophilic granules of PMNs, plays important role in the generation of reactive oxygen species.[23] Proinflammatory cytokines[24] and free radicals are released from PMNs which further leads to increase in plasma exudation.

In present study, the elevated MPO activity in experimental inflamed paws was significantly reduced by standard and treated groups, suggesting neutrophils infiltration inhibition might be another mechanism by which experimental test compound attain its antiinflammatory effect.

Chronic inflammation is the reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and infiltration of neutrophils with exudation of fluid. It occurs by means of development of proliferative cells which can either spread or form granuloma.

Efficacy of anti-inflammatory agents in chronic inflammatory states is indicated by their ability to inhibit the increase in the number of fibroblasts during granular tissue formation.[25]

The cotton pellet granuloma method has been widely employed to access different components of subacute inflammation (transudative, exudative and proliferative). The results are shown in Fig. 2.

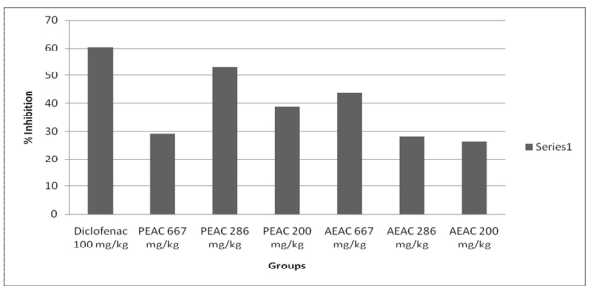


Fig. 2. Effect of stem bark extract of *Anthocephalus cadamba* on cotton pellet method (transudative weight)

As shown in the Fig.2, all the extracts and standard drug (diclofenac 100 mg/kg) used in study elicited significant inhibitory activity on granuloma formation. Presently diclofenac showed significant 60.42% inhibition of transudative weight when compared with control.

Both extracts at dose of 667 mg/kg showed significant 29.22% and 43.80% inhibition in wet weight granuloma. Whereas, both extract at dose 286 mg/kg and 200 mg/kg showed 53.17 & 28.18% and 38.75 & 26.16 % inhibition when compared with control. The present results suggeted antitransudative effects of the extract.

Inflammatory granuloma is a distinctive trait of subacute inflammatory reaction. NSAIDs like diclofenac possess less efficacy in inhibition of granuloma formation. On the other hand, Steroidal drugs, exhibits profound diminution of the inflammatory 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. The deranged ALP concentration was normalized by diclofenac and both the extracts (Table 1).

The mechanisms underlying the anti-inflammatory effect of tannins include the scavenging of radicals (antioxidant effect)[26] and inhibition of inflammatory mediators, such as TNF. It should be pointed out that plant species rich in phenolic compounds, like tannins and flavonoids, are currently being investigated regarding their anti-inflammatory activity[27]. In the recent report, polyphenols possess anti-inflammatory and analgesic effects[28]. The suppression of inducible cyclooxygenase-2 enzymes has been shown for polyphenols.[29]

The present study clarifies that both the petroleum ether and alcoholic extracts of stem bark of *Anthocephalus cadamba,* possess saponins, steroids, alkaloids and tannins and possessed an anti-inflammatory property in both acute and chronic phases of inflammation.

The anti-inflammatory effect of it could be due to either inhibition of phospholipase A_2 (PLA_2) activity or cyclooxygenase pathway and by blocking the release of vasoactive substances such as histamine, serotonin and kinins. Even the anti-inflammatory effect of extracts might be due to inhibition of release of TNF-α and/or NO in turn inhibition PGE_2 production. The other possible mechanisms of anti-inflammatory effect of extracts might be inhibition of free radicals formation and also by reducing the levels of proinflammatory enzymes like myeloperoxidase and adenosine-deaminase activities hence preventing the activation of neutrophils and macrophages thus inhibiting the

release of proinflammatory mediators and their consequences, hence exhibited the anti-inflammatory effect.

Further investigations are needed to know the active principles from the stem of *Anthocephalus cadamba* that possess anti-inflammatory effects.

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