ORIGINAL ARTICLE

Therapeutic Effect of *Aegiceras corniculatum* in Chronic Granulomatous Inflammation and Arthritis

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ABSTRACT

Objective: Chronic inflammation is a result of long lasting of acute inflammation, if not treated or resolved, this condition is a cause of many inflammatory and autoimmune diseases. *Aegiceras corniculatum* a mangrove plant has been using for many years for the treatment of many inflammatory and autoimmune diseases like atherosclerosis, rheumatoid arthritis, asthma and is well known as a folklore medicine constituting various novel chemical constituents.

Methodology: Methanol and ethyl acetate extracts derived from *A. corniculatum* were evaluated against chronic inflammation by using cotton pellet induced granuloma and Adjuvant induced arthritis models in rats. Vascular permeability and leukocyte migration were studied in the presence of extracts.

Results: In cotton pellet granuloma, both the methanol and ethanol extracts were found to highly effective in reducing granulomatous tissue formation in a dose dependent manner. These extracts also suppressed the paw thickness induced by using adjuvant in rat arthritis model, at maximum oral dose of 50 mg/kg methanol extract inhibited swelling in paw by 67%, whereas ethyl acetate extract caused 75% of inhibition in paw swelling at 200 mg/kg. Additionally, ethyl acetate extract has shown inhibition in leukocytes migration (63% at 50 mg/kg) against LTB_4 and carrageenan. However, methanol extract failed to produce inhibitory effect against cell infiltration induced by LTB_4. Interestingly, methanol and ethyl acetate extract has shown remarkable reduction in the acetic acid induced vascular permeability.

Conclusion: *A. corniculatum* extracts has shown remarkable effect as an anti-arthritic and anti-inflammatory agent to combat with chronic inflammatory diseases, this study provides significant justification for its folklore medical use against rheumatism interfering with inflammatory and cellular immune responses.

Key words: Chronic inflammation, *Aegiceras corniculatum*, anti-inflammatory, immuno-modulatory activity.

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INTRODUCTION

Inflammation is a natural defense mechanism of human body against various external stimuli, injuries and infections however it's a protective phenomenon but when inflammatory condition exceeds beyond its usual limit it can cause harm to the body and this term is usually called Chronic Inflammation, which is the result of repeated prolonged infections and autoimmunity. Chronic inflammation is silent and persistent biological condition endorsed other inflammatory diseases like rheumatoid arthritis, atherosclerosis, cardio vascular diseases, diabetes and asthma etc[1-3]. *Aegiceras corniculatum* is a mangrove plant grows in the wetland of tropical and subtropical regions of Indus Delta valley of Pakistan, has a potential effect in many diseases like diabetes, inflammation, rheumatism, cardio vascular diseases etc. *A. corniculatum* has been used as folklore medicine since long time but there is not much scientific evidence available to justify its medicinal use[4]. Various chemical constituents have been isolated from *A. corniculatum* so far, including lignin, tannin, saponins, flavonoids etc. which have been scientifically testified against many inflammatory models recently[8,9]. In our previous findings we have investigated the effect of methanolic extract of *A. corniculatum* in carrageenan-induced peritonitis model and discovered that it act as anti-inflammatory agent *via* inhibiting cyclooxygense pathway which causes suppression of 12-HHT while

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rise in production of 12-lipooxygenase metabolite (12-HETE) which is most probably due to diversion of Arachidonic Acid (AA) metabolism towards LOX pathway, thus increasing 12-HETE levels[5].

We also found that its methanol and hexane extract possesses antioxidant property because it inhibited lipid peroxidase activity, chelate metals ions, have the potential to scavenge free radicals and conceals the respiratory burst which provide the protective effect against oxidative damage, hence proved as the hepatoprotective and anti-inflammatory agent[6]. *A. corniculatum* extract(s) have shown analgesic properties by acting on central nervous system during chemical and thermal pain in rodents[7], its methanol extract have exclusive ability to reduce prostanoids biosynthesis, whereas hexane extract can magnificently reduce the LTB_4 and 12-HETE levels. By the virtue of aforementioned properties, effect of *A. corniculatum* extract(s) on chronic inflammatory model of rats has been evaluated in present study and its protective effect against chronic granulomatous inflammation will be justified.

MATERIALS & METHODS

Animal for Experiment:

Swiss albino mice and rats of both sexes were used in this study taken from International Center for Chemical and Biological Sciences facilitated animal house, University of Karachi, Pakistan. Animals were housed according to the European Community guidelines for the ethical handling and use of laboratory animals.

Plant material:

This plant grows in the coastal area of Indus delta, Gharo district Thatta, Sindh, Pakistan and collected with the co-operation of International Union of Conservation of Nature from the lowest tide which was identified by the taxonomist from the Department of botany, University of Karachi. Voucher specimen represent 68219 label mentioned.

Preparation of extracts from *A. corniculatum*:

The whole plant was kept at room temperature (25-30 °C) for three days process of air drying. Leaves (8.2 kg) were separated from plant and soaked in 10 L hexane for a week. After a week the extract was filtered and evaporated with vacuum, a gummy hexane extract (8.0 g) was obtained. The material left after hexane extract was soaked in 10 L ethyl acetate for another week and processed same as above procedure to obtain ethyl acetate extract (9.5 g). The residues were that soaked in butanol and after getting its extract finally the material left were soaked in the 10 L methanol and methanol extract (7.0 g) collected with the aforementioned procedure. This extraction procedure is brief but clearly indicates that from non-polar to highly polar extracts sequentially obtained.

Acute toxicity testing:

Acute toxicity testing was performed on albino mice of both sexes, 20-25g of weight (n=10 mice), treated with different extracts of *A. corniculatum* including methanol extract (1-500 mg/kg), acetyl acetate (100-1000 mg/kg) and hexane extracts (1-1000 mg/kg) intraperitonially. Changes in behavior, gross effect and mortality were observed for 15 days[10,11]. According to the results of these extract toxicity, doses of test was selected.

Cotton pellet granuloma test:

Albino rats were used in the study of chronic inflammation, divided into 11 groups (n=10) to study effect of methanol extract (10, 25, 50 and 100 mg/kg) and acetyl acetate extract (10, 25, 50, 100 and 200 mg/kg) derived from *A. corniculatum*. Control groups were given 10 % DMSO, while Dexamethasone (5 mg/kg) was used as a standard drug. Rat's fur was shaved and methanol, acetyl acetate extract, control and standard drug were orally administered. After thirty minutes autoclaved cotton pellet of weighted 30mg were implanted subcutaneously under sterile conditions. The administration of extracts, standard drug and control were continued till 1 week, on eighth day animals were sacrificed with the high dose of thiopental sodium and pellet surrounded by granuloma were taken out and weight than dried in oven at 60 °C overnight. After that again weight of the cotton was calculated and mean weight of granuloma was determined[12].

Leukocyte migration test:

Albino mice weighing 20-25g were pretreated intraperitoneally with phenidone, hexane and ethyl acetate extract as well as methanol extract derived from *A. corniculatum* at the concentration of 100mg/kg. After 1 hour of respective treatment mice received injection of 0.2 ml of 1.25µg LTB4 /ml (250ng /mouse) in normal saline into the peritoneal cavity and in other group rats were slightly anaesthetized by using diethyl ether and injected 300µl of 1% carrageenan (suspension in saline) into the peritoneal cavity[15]. After 2 hours of LTB4 injection into the peritoneum, the mice were sacrificed by excessive inhalation of diethyl ether and peritoneal cavity was injected with 5.0 ml of phosphate buffered saline without Ca^{2+} and Mg^{2+}. Peritoneal fluid (5 ml) was withdrawn and centrifuge at 5000rpm for 15 min. Supernatant was discarded and cell pellet was washed twice with PBS. Total infiltrated leukocytes

count and differential count was determined using a hemocytometer by light microscope and differential count determined by microscopic counting of giemsa stained slides under an oil immersion[16].

Vascular permeability test:

Extract of methanol (25-50 mg/kg) and acetyl acetate (50-200 mg/kg) were orally administered in albino mice of either sex, in control mice were receive dexamethasone 50 mg/kg. One hour later of the dose, Evans's blue dye (0.25 % in saline) purchased from Sigma Aldrich will be intravenously injected via tail vein. After thirty minutes, rats were injected acetic acid 0.6% v/v intraperitonially and rested for another 30 minutes. Treated animals were sacrificed and peritoneal cavity were washed with normal saline and collected in heparinized tube than centrifuged. The solution was proceeded for the spectrometer analysis at the wavelength of 610 nm[14].

Freund's Adjuvant-induced arthritis:

Effect of *A. corniculatum* extracts of chronic arthritis will be determined by using Adjuvant induced arthritis model described by Babu *et al*[13]. Freund's adjuvant (0.1 ml), which contains 10 mg of Mycobacterium tuberculosis (heat killed) suspended in paraffin oil purchased from Sigma Aldrich (USA) is injected right hind paw. The methanol and ethanol extracts of *A. corniculatum* were orally administered daily for the next 14 days after adjuvant injection. Volume of paw was determined before and after the treatment with extract by using water displacement method (plethysmometer).

Statistical analysis:

The data was analyzed by using one way ANOVA and compared Dunnet's t-test and presented as mean ± S.E. Simple linear regression was used to evaluate IC50, ED50 and LD50 were performed by using least significance difference (LSD). Data analysis was performed using SPSS version 11.5 software and $p< 0.05$ value was accepted as significant.

RESULTS

Effect of *A. corniculatum* extracts against cotton pellet- induced granuloma in rats:

The methanol extract of *A. corniculatum* have shown dose dependent decrease in granuloma formation, at doses 10, 25, 50 and 100 mg/kg it inhibited granuloma formation by 45, 58,68 and 70 %. While at the dose of 50, 100 and 200 mg/kg ethyl acetate has inhibited 54, 67 and 73 % granuloma formation. Dexamethasone used as Standard drug has inhibited the granuloma *via* 67% as shown in Table-1.

Table 1: Effect of A. corniculatum extracts in cotton pallet granuloma rat model[a]

Groups	Dose (mg/kg body weight)	Wet weight of granuloma (mg)	Dry weight of granuloma (mg)
Control		275.00±13.0	150.00±10.0
Dexamethasone	5	97.00±8.0	50±8.8*** (67%)
Methanol extract	10	200.00±15 (28%)	83.00±5.0* (45%)
	25	185.00±14 (33%)	64.50±6.5** (58%)
	50	120.00±12 (57%)	49.50±4.0*** (68%)
	100	150.00±9.0 (46%)	45.3±7.0*** (70 %)
Ethyl acetate extract	10	300.00±17 (-9%)	110±11.0 (27%)
	25	207.00±19 (25%)	105±8.9 (93%)
	50	160.00±13 (42%)	70.0±30** (54%)
	100	150.00±10 (46%)	50.0±6.0*** (67%)
	200	100±11 (64%)	41.5±4.4*** (73%)

Cotton pellet implanted 30.0 mg = 2.5
a. Each value is a the mean ± S.E.M. ten rats.
The values given in parenthesis represent percent inhibition of wet and dry weight of granuloma *p<0.05, **p<0.01. ***p<0.005 compared to control group.

Non-toxic effect of *A. corniculatum* extract:

The extracts of *A. corniculatum* have shown remarkable non-toxic effects *via* oral and peritoneal doses. Its highly non polar methanol extract was found to be non-toxic above the dose of 200 mg/kg, whereas ethyl acetate was found to be non-toxic up to 1g/kg in oral administration but cause mortality with LD50 value 825 mg/kg. Hexane extract did not cause any mortality in mice up to dose of 500 mg/kg.

Effect of *A. corniculatum* extracts against arthritis induced by Adjuvant in rats:

Dose dependent reduction in rat's foot thickness has been observed in the presence of both methanol and ethanol extracts of *A. corniculatum* (Table-2). Swelling in foot was controlled with these extracts as compared to the control levels. At the lower dose of 10 mg/kg methanol extract has shown 26% of inhibition in the paw swelling whereas, ethyl acetate extract has exhibited 24% of inhibition at similar dose. However, methanol extract at the doses of 25 and 50 mg/kg has inhibited the swelling by 57% and 67% while ethyl acetate shown the highest effect of 75% at the dose of 200 mg/kg.

Table 2: Effect of *A. corniculatum* extracts on adjuvant-induced arthritis in rats

Groups	Dose (mg/kg body weight, oral)	Paw vol in ml		Inhibition %
		Before treatment (Adjuant induced arthritis)	After treatment (Adjuant induced arthritis)	
Control		0.76±1.50	3.9 ± 1.21	
Methanolextract	10	0.59±0.07	2.9 ±1.50	26
Ethyl acelate extract	25	0.89±1.20	1.7 ± 0.77**	57
	50	0.55±0.06	1.3 ± 1.73**	67
	10	0.55±1.7	3.0 ± 2.0	24
	25	0.89±0.08	2.5 ± 1.2	36
	50	0.76±0.89	2.0 ± 0.9*	49
	100	0.66±0.05	1.5 ± 0.55**	62
	200	0.56±0.09	0.99 ± 0.07***	75

Paw volume of rats before induction of arthritis is 0.86±1.6 (n=3-10).
[a]Each value is the mean ± S.E.M. of 8-10 rats.
*p<0.05, **p<0.005, compared to control group; Student's t-test.

Vascular permeability test:

Methanol extract of *A. corniculatum* has shown effect dose dependently on vascular permeability caused by acetic acid. At the dose of 25 and 50 mg/kg, the percent inhibitions obtained were 33% and 62 %. On the other hand ethyl acetate extract has also shown the similar results by inhibiting the vascular permeability in dose dependent manner at low dose of 50 mg/kg the inhibition in vascular permeability was 24%, while 66% inhibition was calculated at the highest dose of 200 mg/kg.

Table 3: Effect of extract on acetic acid vascular permeability

Groups	Dose (mg/kg)	Absorbance (610mm)	Inhibition %
Control		0.65±0.33	
Methanolextract	25	0.44±0.55	33
	50	0.25±0.23**	62
Ethyl acelate extract	50	0.50±0.11	24
	100	0.35±0.44*	47
	200	0.21±0.11***	66

*P< 0.05, **p<0.01, ***p<0.005 compared with Dexamethasone as a control. Percent inhibition was calculated with respect to the control

Leukocyte migration test

Cellular infiltration (>90% neutrophils) in response to 250 ng LTB4 was 1.97×105±0.28 neutrophils ml^{-1}. Analysis of cell accumulation in the acute phase of this inflammatory reaction demonstrated that methanol, ethyl acetate and hexane extract from *A .corniculatum* along with phenidone have been inactive at 100mg/kg in inhibiting neutrophils recruitment. However hexane extract was the only extract that found to exhibit significant decrease in the number of infiltrating cell (0.89×105±0.05 neutrophils ml^{-1}) by 55 ± 2.7% at 100mg/kg. While in carrageenan model different doses of ethyl acetate has reduced leukocyte migration in dose dependent manner. Ethyl acetate at dose 10-50 mg/kg diminished leukocyte count by 22.0-63.5% with ED50 of 38.6 ± 3.3 mg/kg. Methanol extract has also shown dose dependent (10-100 mg/kg) inhibitory effect upon leukocyte recruitment with the value of 57 ± 5.9% at the highest dose of 100 mg/kg and the ED50 value appears as 72 ± 6.0 mg/kg.

Table 4: Effect of Carrageenan induced peritonitis in mice

Treatment	Dose (mg/kg)	Neutrophils ×06ml^{-1}	Percent inhibition	ED50 (mg/kg)
Phenidone	100	3.0 ±0.14***	60.0 ±1.9	75.5 ±2.1
Ethyl acetate	10	5.8 ±0.58*	22.0 ±7.5	38.6 ±3.3
	25	4.6 ±0.40***	38.0 ±5.4	
	50	2.7 ±0.49***	63.5 ±4.8	
Methanol	10	7.0 ±0.17	5.4 ±2.3	72.2 ±6.0
	25	4.8 ±0.30***	35.2 ±3.5	
	50	3.4 ±0.23***	54.0 ±3.2	
	100	3.2 ±0.46***	57.0 ±5.9	

ED_{50}= the median effective dose that prevent the neutrophils influx into the localized inflammatory site obtained from regression lines showing high coefficient of determination (r^2=0.80).
Value are mean infiltrated neutrophils x10^6 ml^{-1}±SEM of control animals (n=18) ***p<0.005 compared to control group.

DISCUSSION

The current investigation demonstrate that methanol and ethyl acetate extracts derived from *Aegiceras corniculatum* significantly suppress chronic phase of inflammation interfering with the infiltration of mononuclear cells, proliferation of fibroblast, collagen fibers and formation of connective tissues which form granuloma. Additionally, traditional use of this extract against rheumatism was validated in adjuvant induced arthritis rat model.

The cotton pellet granuloma formation in rats is one of the most commonly employed method in animal research to screen for chronic anti-inflammatory activity of the drugs and novel natural product[12]. It has been used to evaluate the transudative, exudative and proliferative component of chronic inflammation because the dried weight of the cotton pallet correlate well with the amount of granulomatous tissue[17], and the moist weight of the cotton pallet correlate well with the amount of transudate. Granuloma also comprises of accumulation of modified macrophages and lymphocytes[18]. Methanol extract of *A. corniculatum* significantly (45%-70%) and concentration dependently (10-100 mg/kg; orally) reduced the dry weight of cotton pellet implanted in rats representing its significant response against granulomatous tissue formation. Moreover, at 50 mg/kg, it exhibited 57% inhibition in wet weight of cotton pellet showing its inhibitory effect

against cellular immune responses as well as exudates formation at the site of inflammation *via* reducing vascular permeability. Likewise, ethyl acetate extract of *A. corniculatum* consisting of chemical constituents including pentacyclic triterpenes (having oleanane skeleton), flavonioids such as quercetin, kaempferol and isorhamnetin significantly inhibit both wet and dry weight of cotton pellet in this rat granuloma model.

Freund's adjuvant induced-arthritis is a model used for exploring the effect of new molecules against chronic inflammation and rheumatism[19]. Freund's adjuvant is consisting of an antibody stimulator such as tubercle bacilli and an emulsifying agent such as lanolin solubilized in mineral oil. The Complete Freund's Adjuvant (CFA)-induced arthritic share many features of human rheumatoid arthritis, it can be induced in rat hind paw by the injection of various bacterial cell walls or their components; however, the exact immunogen remains unknown. We investigate the paw volume of rats before treatment and after treatment by inducing the arthritis in the absence and presence of both methanol and ethyl acetate extracts of *Aegiceras corniculatum* exhibited significant anti-arthritic effect in a dose dependent manner and compared with standard drug indomethacin (0.5 mg/kg, oral) that reduced arthritic phenomenon by 50%. Methanol extract was found to be highly effective (67%) against arthritis at 50 mg/kg, this inhibitory action with related to reduction in paw edema by extract is attributed to inhibition of pro-inflammatory prostaglandin synthesis. In our previous study methanol emerged as an impressive anti-inflammatory agent as it was ~7x better than non-selective cyclooxygenase inhibitor aspirin. It is also known that prostaglandinE2-induced paw edema enhances the expression of cyclooxygenases with a concomitant rise in PGE2 levels[20] inhibitory effect of methanol extract in PGE2-induced paw edema reveals its anti-inflammatory effect *via* cyclooxygenase pathway. The anti-inflammatory property of methanol extract might be related to lignans and triterpenoidal saponins reported earlier to be present in it[21,22].

The arthritic disease progression was correlated with the fragility of the lysosomal membranes and the subsequent discharge of the lysosomal hydrolases[23,24]. In our previous study, ethyl acetate extract have shown to reduce vascular permeability induced by hyaluronidase enzyme and hence it may act by modulating lysosomal membrane thereby preventing the release of hydrolases. Furthermore, in arthritic condition macrophages in the synovium secrete IL-1 and TNF-á that stimulate the synthesis and release of collagenase and PGE2 which degrade collagen and thereby leading to the destruction of cartilage in the joints. The ethyl acetate extracts reduced the levels of these pro-inflammatory cytokines (data not shown) and its anti-arthritic activity may also be attributed to its antagonistic action against aforementioned cytokines and thereby decreasing the inflammatory process like lysosomal membrane instability in arthritic condition.

The inflammatory response is a physiological characteristic of vascularized tissues[25]. Increased vascular permeability seen in the inflammatory reaction leads to exudation of fluid rich in plasma protein including immunoglobulins (antibodies), coagulation factors and cells[26] into the injured tissues (with subsequent edema at the site). Exudation, which is a consequence of increased vascular permeability, is considered a major feature of acute inflammation[27]. Oral administration of the extract methanol and ethyl acetate derived from *Aegiceras corniculatum* evoked a significant ($P<0.05$) dose-related inhibition of vascular permeability. Increased vascular permeability occurs as a result of contraction and separation of endothelial cells at their boundaries to expose the basement membrane, which is freely permeable to plasma proteins and fluid[28]. Histamine and other mediators of inflammation increase vascular permeability at various times after injury.

Carrageenan-induced inflammation is useful in detecting orally active anti-inflammatory agents. Edema formation due to carrageenan in the rat paw is a biphasic event[29,30]. The initial phase is attributed to the release of histamine and serotonin. The edema produced at the peak (3 h) is thought to be due to the release of kinin-like substances, especially bradykinin. The second phase of edema is due to the release of prostaglandins, protease, and lysosome[30,31]. The second phase is sensitive to most clinically effective anti-inflammatory drugs[29,30,10]. Leukotriene B4 (LTB4) induced peritonitis is the most potent chemoattractant involved in cell in filtration, activation of leukocytes and promotion of neutrophils adhesion to endothelial cells. In the present investigation methanol and ethyl acetate derived from *Aegiceras corniculatum* showed reduction in neutrophils influx *in vivo* study, because it interfere with various level of cells infiltration including (LTB4) receptor antagonism present on leukocytes and suppressing the expression of specific inflammatory adhesion molecule integrins (CD11b/CD 18) on leukocytes and intracellular adhesion molecule-1 (ICAM-1) and E–selectin on vascular endothelial cells.

It is concluded that methanol and ethyl acetate extract significantly inhibit chronic inflammation interfering with exudative and cellular phase of inflammation. Inhibitory effect of these extracts will be validated against rheumatism in adjuvant-induced arthritis and can be incorporated in drug discovery program as an anti-arthritic and anti-inflammtory agent.

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