ORIGINAL ARTICLE

Effect of hydroalcoholic extract of aerial parts of *Leucas aspera* (Willd.) Link on inflammatory markers in complete Freund's adjuvant induced arthritic rats

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The plant *Leucas aspera* is claimed to possess anti-inflammatory and anti-rheumatic potential by traditional practitioners. The aim of this study is to validate the traditional claim. The hydroalcoholic extract of aerial parts of *L. aspera* (HAELA) was orally tested at a dose of 100 mg/kg bodyweight for anti-arthritic effect in adjuvant-induced arthritic rats. Group I rats served as vehicle control group [0.2% carboxyl methyl cellulose (CMC) p.o.]. The test groups were injected with 0.1 ml of complete Freund's adjuvant into the subplantar region of right hind paw. Group II animals served as disease control, while the group III and group IV arthritic rats were treated with standard drug diclofenac sodium (0.3 mg/kg) and HAELA (100 mg/kg) for 21 days. Activities of inflammatory markers such as tumour necrosis factor-α, C-reactive protein, interleukin-2, cathepsin D and antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were estimated in plasma/haemolysate and tissue of control and arthritic animals. Histopathological analysis of knee joints was also performed. Statistical analysis of the biochemical parameters was performed by one way analysis of variance (ANOVA) using SPSS software package. Results were expressed as mean±SEM for six rats in each group. $P<0.05$ was considered to be significant. HAELA showed significant anti-inflammatory ($P<0.001$) and antioxidant activity ($P<0.001$) at the specified dose. It did not show mortality up to 2000 mg/kg body weight. Histopathological studies confirmed cartilage regeneration and near normal joint in HAELA treated rats. It can thus be concluded that HAELA possesses significant antioxidant and anti-arthritic potential.

Key words: Anti-inflammatory activity, C-reactive protein, interleukin-2, *Leucas aspera*, rheumatoid arthritis, tumour necrosis factor-α

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by uncontrolled proliferation of synovial tissues. Prevalence is estimated to be 0.8% worldwide with women twice as likely to develop the disease as men. Genetic and environmental factors play a role in pathogenesis.[1]

Experimental and clinical data prove the involvement of free radicals in inflammation accompanying RA.[2] Increased formation of reactive oxygen species (ROS) can be attributed to different pathways. The activated neutrophils in inflamed joints liberate $O_2^{\circ-}$ (superoxide radical), H_2O_2 (hydrogenperoxide), elastase, hypochlorous acid and eicasonoids.[3] ROS is also produced by macrophages, lymphocytes and endothelial cells, which contribute to the destruction of cartilage. Endogenous antioxidant systems fail to keep pace with the increased oxidant generation. Accordingly, reduced or altered concentration of antioxidants has been reported in RA.[4] Therapy necessitates prevention of disease and regeneration of joint destruction. This includes the usage of disease modifying anti-rheumatoid drugs (DMARDS) and nonsteroidal anti-inflammatory drugs (NSAIDS), which are not effective universally and have serious side effects. Because of these limitations, the use of alternative therapies such as the usage of medicinal herbs is on the rise. This has led to evaluate the role of herbs in anti-arthritic therapy in RA. Recently, there have been studies concerning natural products with anti-inflammatory activity like that of *Polygonum tinctorium, Melia azadirachta, Cyperus rotandus, Cudraniatri cuspidata, Curcuma rhizomes,* etc.[5] *Leucas aspera* (thumbai), belonging to the family Labiateae, grows as a herbaceous, branched annual on wastelands. The plant is used as an insecticide and indicated in traditional medicine for painful swellings, snakebites and chronic rheumatism. Leaf extracts of *L. aspera* have shown anti-fungal, analgesic, anti-inflammatory, anti-pyretic and hepatoprotective efficacies. The root part has shown significant antinociceptive, antioxidant

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Received: 19-01-2010; **Accepted:** 09-08-2010; DOI: 10.4103/0973-8258.74139

and cytotoxic activities. Pharmacognostical evaluation of the plant collected from various regions of northern India has been reported. Alpha-farnesene, alpha-thujene and menthol were isolated from the leaf volatiles, and amyl propionate and isoamyl propionate were isolated from the flower volatiles of *L. aspera*.[6-12] Prostaglandin inhibitory and *in vitro* antioxidant activity guided fractionation of shoots of *L. aspera* yielded eight lignans and four flavonoids.[13] Baicalin and Baicalein isolated from the flowers of *L. aspera* were found to stabilise RBC membrane integrity in hypotonicity-induced haemolysis.[14] The present study was designed to determine the anti-arthritic potential and anti-inflammatory activity of *L. aspera* on inflammatory markers in adjuvant-induced arthritis.

MATERIALS AND METHODS

Plant Material

The aerial parts of the plant *L. aspera* (Family: Labiatae) were collected from Kanchipuram district of Tamil Nadu, India. The plant material was taxonomically identified by the National Institute of Herbal Science, Plant Anatomy Research Centre, Tambaram, Chennai. Voucher specimen (PARC/2007/362) has been deposited in our college herbarium for future reference.

Preparation of Extract

The aerial parts were dried under shade and then powdered. The dried powder (1 kg) was subjected to exhaustive cold maceration in 50% ethanol for 72, 48 and 24 hours, respectively. The solvent was filtered, distilled under vacuum and dried in a vacuum dessicator. The yield of the extract was 9.5% w/w. Hydroalcoholic extract of aerial parts of *L. aspera* (HAELA) was suspended in 0.2% carboxyl methyl cellulose (CMC) and used for the present study.

Phytochemical Screening

The extract was screened for the presence of various constituents using the standard screening tests. The presence of steroids, alkaloids, tannins, flavonoids, glycosides and phenols was determined by employing conventional protocols.[15] Heavy metal content and microbial contamination were also determined.

High Performance Thin Layer Chromatography Fingerprint of Hydroalcoholic Extract of Aerial Parts of *L. Aspera*

High performance thin layer chromatography (HPTLC) fingerprinting was performed on 20×20 cm aluminium sheets precoated with silica gel F_{254} Merck plates of 0.2 mm thickness (Merck Limited, Worli, Mumbai, India). CAMAG HPTLC (Anchrom Enterprises Pvt. Ltd., Mumbai, India) linear thin trough (20×20 cm) was saturated with the developing solvent consisting of a mixture of chloroform:methanol:formic acid:acetic acid (80:10:5:5), for 2 hours. The sample 2.5–20µl was applied in a 5-mm width band through LINOMAT IV in a space of 5 mm. Development was carried out in an ascending mode and the peaks were detected at a scanning wavelength of 295 nm. The R_f values and finger print data were recorded using WIN CATS software.

High Performance Liquid Chromatography Profile of Hydroalcoholic Extract of Aerial Parts of *L. Aspera*

HAELA was subjected to high performance liquid chromatography (HPLC) analysis (Lachrom L-7000 system, Hitachi, Chennai, India) to estimate the amount of total flavonoids. Flavonoids were detected using an analytical column (C-18, 4.6 mm×25 cm), with stationary phase being octadecyl silica gel and mobile phase being 2.5 volumes of glacial acetic acid, 40 volumes of tetrahydrofuran and 60 volumes of water. Standard flavonoid markers were used at a concentration of 1 mg/ml. Injected sample volume was 20 µl. Elution was carried out at a flow rate of 1.5 ml/min. The liquid chromatograph was equipped with a 254-nm UV detector to detect the eluents.

In vivo Studies

Animals

The efficacy study was performed with female Wistar rats (since statistical incidence proves that females are twice as likely to develop RA[1]) weighing 125–150 g, and toxicity study was done with Swiss albino mice of both sexes, weighing 25–30 g. The rats were acclimatised for a week in a light and temperature controlled room with a 12-hour dark-light cycle. They were fed commercial pelleted feed (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. The experimental protocols were approved by the Committee for the purpose of Control and Supervision of experiments on animals (CPCSEA), New Delhi, India (IAEC-XII/SRU/73/2008).

Drugs and chemicals

Complete Freund's adjuvant (CFA) was purchased from Sigma-Aldrich Corporation Bangalore, India. Enzyme-linked immunosorbent assay (ELISA) kits for tumour necrosis factor-alpha (TNF-α) were from Pierce Endogen, Rockford, IL 61105, USA; C-reactive protein (CRP) was obtained from Immunology Consultant Laboratory, Newberg, OR, USA; and interleukin-2 (IL-2) was obtained from Bender Med Systems Gmbh, Vienna, Austria, Europe. All other chemicals used were of analytical grade.

Preliminary acute toxicity studies

The acute toxicity studies were carried out as per OECD 423 (Organisation for Economic Cooperation and Development) guidelines. Mice were divided into two groups of three mice each. The control group received 0.2% CMC and the second group 2000 mg/kg of HAELA. Immediately after administration of the dose, the animals were observed continuously for the first 4 hours and next 14 days of drug administration to record mortality.

Dosage studies

Preliminary studies with different dosages (25, 50 and 100 mg) of HAELA were performed on female Wistar rats and the dose that produced significant anti-inflammatory activity was considered for the anti-arthritic study.

Anti-arthritic studies

The animals were divided into four groups each comprising six animals. Rats of the control group (group I) were orally treated with vehicle (0.2% CMC p.o.) and those of the test groups were injected with 0.1 ml of CFA into the subplantar region of right hind paw. Group II animals were treated as disease control, while the group III and group IV arthritic rats were treated with standard drug diclofenac sodium (0.3 mg/kg body weight p.o.) and HAELA (100 mg/kg body weight p.o. as determined by the preliminary dosage studies) from day 15 to day 35 of adjuvant administration. Inflammation was assessed by measuring the paw volume every week using a plethysmograph (Janani Scientific Co. Ltd., Chennai, India).

On the 42nd day, the animals were sacrificed by cervical decapitation and the blood was collected. The liver was immediately dissected out, homogenised in ice-cold Tris HCl buffer (0.01 M, pH 7.4) to give a 10% homogenate. Haemolysate/plasma and liver homogenate were used for carrying out the various biochemical estimations.

Biochemical estimations

Lipid peroxidation was estimated by the method of Ohkawa *et al.*[16] Cathepsin D activity was determined based on the method of Sapolsky *et al*.[17] Superoxide dismutase (SOD) activity was assayed by the method of Marklund and Marklund,[18] glutathione peroxidase (GPx) activity was assayed based on the method of Rotruck *et al*.[19] and catalase (CAT) was assayed by the method of Sinha *et al*.[20] Reduced glutathione (GSH) levels were measured by the method of Moron *et al*.[21] The levels of inflammatory markers TNF-α, CRP and IL-2 in plasma were assayed by ELISA using reagent kits according to the manufacturer's instructions. The protein content of the tissue homogenate was determined by the method of Lowry *et al.*[22]

Histopathological examination

Histopathological studies were done in the knee joints of the animals. The tissues were fixed in formalin, decalcified and embedded in paraffin blocks. Sections prepared with microtome were stained with haematoxylin and eosin and examined under a microscope. Photomicrographs were taken.

Statistical Analysis

The results of the physical parameters such as paw volume were analysed using student's "*t*" test. Statistical analysis of the biochemical parameters was performed by one way analysis of variance (ANOVA) using SPSS software package. Results were expressed as mean±SEM for six rats in each group. $P<0.05$ was considered to be significant.

RESULTS

Preliminary Screening

The preliminary phytochemical screening tests confirmed the presence of flavonoids, phenols, glycosides, saponins, steroids, alkaloids and tannins. The concentrations of heavy metals such as mercury, lead, cadmium and arsenic were below the WHO/FDA permissible limits.[23] The presence of pesticide residues and pyrethroids was not detected in the plant samples. The tests for *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Pseudomonas aeruginosa* and Enterobacteria showed their absence.

High Performance Thin Layer Chromatography Fingerprint of Hydroalcoholic Extract of Aerial Parts of *L. aspera*

In this study, the HPTLC fingerprinting of alcoholic extract revealed 11 spots with the following R_f values: 0.07, 0.12, 0.3, 0.38, 0.44, 0.52, 0.55, 0.63, 0.66, 0.78 and 0.85 at a concentration of 0.4 mg/20 μl as shown in Figure 1.

High Performance Liquid Chromatography Profile of Hydroalcoholic Extract of Aerial Parts of *L. aspera*

HPLC with UV detection was employed for the identification and quantification of flavonoids present in the hydroalcoholic extract. Total flavonoid content of HAELA was 5.24 mg%, with quercetin (1.21 mg), apigenin (0.56 mg), kaempferol (1.31 mg) and luteolin (2.04 mg) being the most abundant flavonoids as shown in Figure 2. Appreciable amounts of polyphenols (10.98 mg%) have also been indicated in the extract.

Effect on Paw Volume

HAELA, when given at a dose of 100 mg/kg body weight to arthritic rats, significantly reduced the paw swelling ($P<0.001$) as shown in Figure 3.

Lower doses of HAELA (25 and 50 mg/kg) did not reduce paw swelling (data not shown).

Effect on Inflammatory Markers

In the present study, the levels of inflammatory markers, CRP, TNF-α, IL-2, were significantly raised in plasma of disease control rats, whereas the standard drug treated and HAELA treated rats showed marked decrease in the levels of CRP ($P<0.001$ for HAELA treated and $P<0.05$ for diclofenac treated), TNF-α ($P<0.001$) and IL-2 ($P<0.001$)as shown in Table 1.

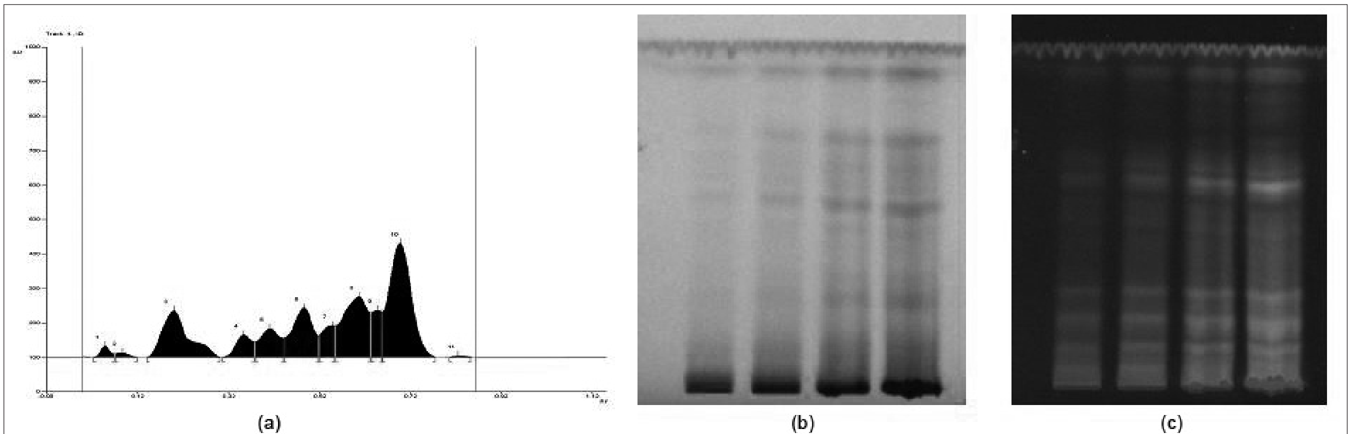
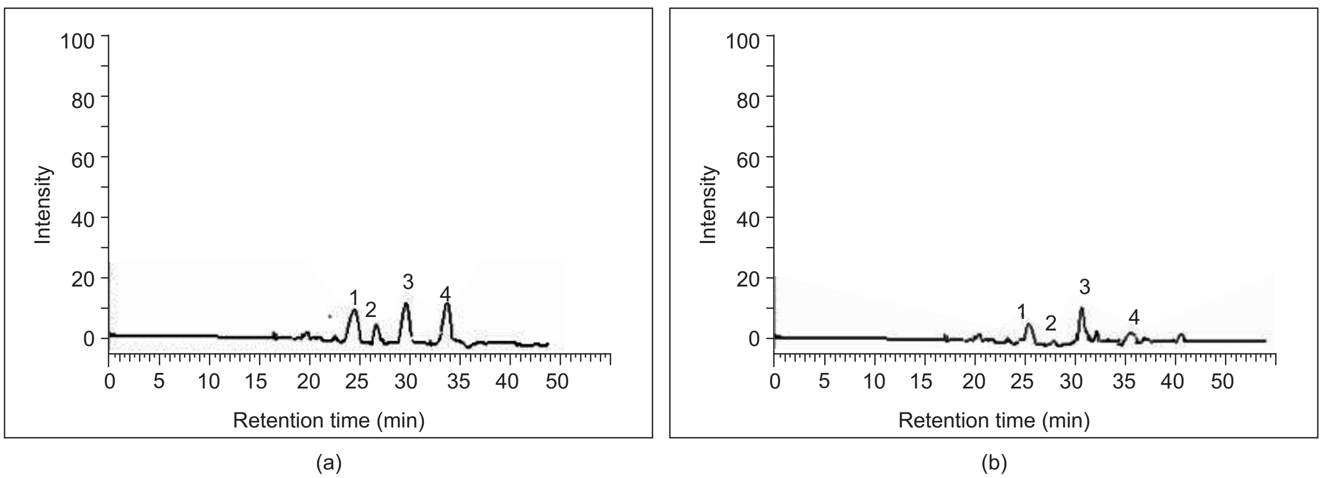


Figure 1: (a) HPTLC fingerprint of HAELA showing 11 different peaks of phytoconstituents when applied at a concentration of 0.4 mg/20 µl, (b) HPTLC chromatogram of HAELA at 254 nm, (c) HPTLC chromatogram of HAELA at 366 nm



(a)

	NAME	R.T	AREA	AREA %
1	QUERCETIN	25.76	167.8	3.230
2	APIGENIN	29.8	1144.5	21.52
3	KAEMPFEROL	30.9	2067.8	38.560
4	LUTEOLIN	34.8	1986.7	36.780
			5366.8	100.000

(b)

	NAME	R.T	AREA	AREA %
1	QUERCETIN	25.80	89.6	4.990
2	APIGENIN	29.6	1567.7	87.4
3	KAEMPFEROL	30.67	78.7	3.660
4	LUTEOLIN	34.6	56.5	3.850
			1792.5	100.000

Figure 2: HPLC-UV chromatogram of HAELA (a) HPLC-UV graph of the standard flavonoids (b) HPLC-UV graph of the flavonoids in hydroalcoholic extract of *L. aspera*

Cathepsin D, an important protease involved in cartilage degradation, showed a marked increase in activity in RA and decreased activity in both standard drug treated and HAELA treated rats ($P<0.001$) as shown in Table 2.

Effect on Antioxidant Status

Elevated lipid peroxidation and decreased antioxidant status were observed in arthritic rats. Malondialdehyde (MDA) levels of plasma and tissue homogenate were increased in group II rats while it was brought down significantly ($P<0.001$) in group III and IV rats. Similarly, administration of HAELA increased the enzymatic activities of SOD ($P<0.001$), GPx ($P<0.001$) and CAT ($P<0.001$) significantly in haemolysate and tissue besides GSH levels, and the increase is comparable to that of the standard drug treated group, suggesting the *in vivo* antioxidant potential of *L. aspera* as shown in Table 3.

Histopathological Analysis

Group I rats showed normal cartilage and a normal synovium [Figure 4a]. The group II arthritic rats showed thinning of cartilage plates, bone erosion, irregular bone remodelling and alterations in bone structure of knee joints [Figure 4b]. The diclofenac treated rats showed a reduction in inflammation but the cartilage showed degenerative changes and irregular bone remodelling [Figure 4c]. HAELA treated rats showed regeneration of cartilage and new bone formation with mild changes in the synovium, which proves the anti-arthritic potential of HAELA [Figure 4d].

DISCUSSION

The present study demonstrates that the HAELA attenuated adjuvant induced arthritis and facilitated recovery as measured by the decrease in paw volume, markers of inflammation and improved antioxidant status.

Adjuvant induced arthritis is an immune response to an antigen present on the capsule of *Mycobacterium tuberculosis*. Following the induction of adjuvant arthritis with CFA, rats

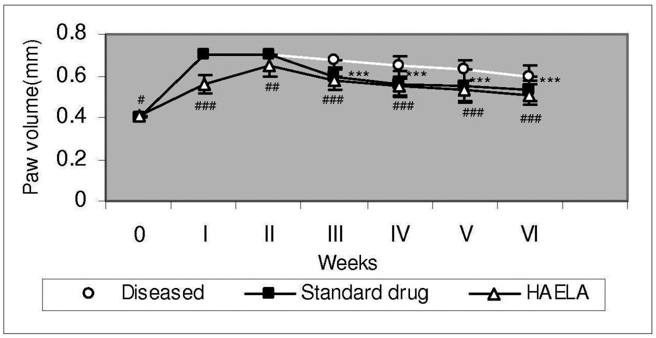


Figure 3: Effect of HAELA on paw volume of arthritic rats. Each value represents mean±SD, *n*=6. Effect of diclofenac (0.3 mg/kg, p.o.) and HAELA (100 mg/kg, p.o.) on paw volume in adjuvant arthritic rats. **P*<0.05; ***P*<0.01; ****P*<0.001 as compared between arthritic and diclofenac; #*P*<0.05; ##*P*<0.01; ###*P*<0.001 as compared between arthritic and HAELA

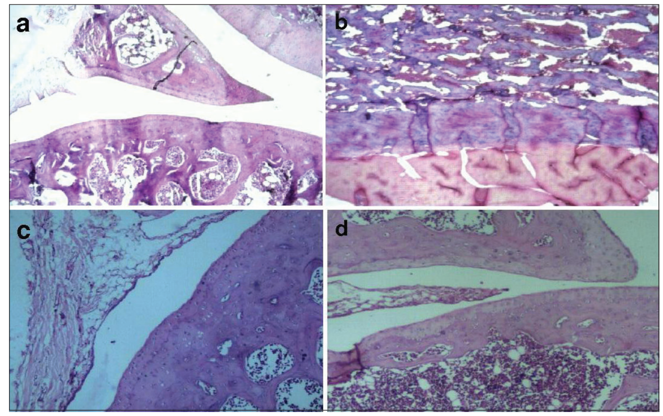


Figure 4: Histopathological changes in knee joints of experimental animals. Method of staining: stained with haematoxylin and eosin. Magnification: ×20. (a) normal cartilage, bone and synovium (b) thinning of cartilage plate, bone erosion and irregular bone remodelling seen in arthritic rats (c) reduction of synovial inflammation but degenerative changes seen in cartilage in the standard drug treated group (d) new bone formation with mild changes in synovium in HAELA treated rats

Table 1: Effect of HAELA on pro-inflammatory cytokines in adjuvant induced arthritic rats

Group and dose	CRP (ng/ml plasma)	TNF-α (pg/ml plasma)	IL-2 (pg/ml plasma)
Vehicle control	356.28±16.55***	0.476±0.03***	0.362±0.004***
Arthritic	482.93±9.7	4.566±0.08	2.596±0.05
Arthritic+diclofenac (0.3 mg/kg p.o.)	421.43±8.26*	0.403±0.03***	0.623±0.01***
Arthritic+HAELA (100 mg/kg p.o.)	254.35±9.52***	2.324±0.13***	0.856±0.09***

Each value represents mean±SEM; *n*=6; **P*<0.05; ****P*<0.001; as compared to disease control

Table 2: Effect of HAELA on lipid peroxidation and cathepsin D activity in adjuvant-induced arthritic rats

Group and dose	Lipid peroxidation		Cathepsin D	
	Plasma (nM/dl)	Tissue (nM/mg)	Plasma (μM/hour/ml)	Tissue (μM/hour/mg)
Vehicle control	0.23±0.04***	0.033±0.01*	1.1±0.61***	17.46±0.83***
Arthritic	0.35±0.07	0.04±0.01	2.18±0.44	23.89±1.87
Arthritic+diclofenac (0.3 mg/kg p.o.)	0.227±0.06***	0.028±0.01**	1.02±0.05***	15.29±3.14***
Arthritic+HAELA (100 mg/kg p.o.)	0.223±0.01***	0.020±0.001***	1.2±0.01***	18.32±0.28***

Each value represents mean±SEM; *n*=6; **P*<0.05; ***P*<0.01; ****P*<0.001 as compared to disease control

Table 3: Effect of HAELA on antioxidant status in adjuvant-induced arthritic rats

Group and dose	SOD		GPx		CAT		GSH	
	Haemolysate (Units/ml)	Tissue (Units/mg)	Haemolysate (nM/mg/ml)	Tissue (nM/mg/mg)	Haemolysate (mM/min/ml)	Tissue (mM/min/mg)	Haemolysate (μg/dl)	Tissue (μg/mg)
Vehicle control	3.00±0.60	0.03±0.02*	11.40±1.02	0.15±0.04***	1.047±0.71	0.136±0.08***	10.24±0.61***	1.45±0.03***
Arthritic	2.44±1.83	0.02±0.08	2.54±0.29	0.04±10.01	0.722±0.36	0.026±0.02	5.67±0.2	0.28±0.02
Arthritic+diclofenac (0.3 mg/kg p.o.)	17.67±6.60***	0.052±0.03	3.72±0.29*	0.126±0.02***	1.373±3.77*	0.032±0.03*	9.07±0.54***	0.89±0.02***
Arthritic+HAELA (100 mg/kg p.o.)	27.35±1.75***	0.043±0.002***	4.42±0.32***	0.047±0.002^NS	3.31±0.36*	0.25±0.03***	13.64±1.06***	2.54 ±0.06***

Each value represents mean±SEM; *n*=6; **P*<0.05; ***P*<0.01; ****P*<0.001 as compared to disease control develop arthritis and other systemic features of inflammation such as body weight loss and increase in paw volumes.[24] Paw swelling is a major factor in evaluating the degree of inflammation and also the therapeutic efficacy of the administered drugs. The initial phase of the inflammatory response (first 2 weeks after adjuvant induction) showed a steady increase in paw swelling [Figure 2] in all the induced rats. This early inflammatory response was mitigated in the treated groups, namely, group III (NSAID diclofenac) and Group IV (HAELA) rats. The anti-inflammatory effect of diclofenac is mediated chiefly through its inhibition of cyclooxygenase (COX) and prostaglandin (PG) production,[25] while that of HAELA might be due to the significant reduction of total leukocyte migration as well as the migration of lymphocytes and monocytes from blood into the synovial cavity as these inflammatory cells are considered to be the major contributors to the inflammatory response.[26]

The damaging effect of oxygen free radicals and the accompanying lipid peroxidation *in vivo* plays a very important role in mediating pathological processes.[27] Lipid peroxidation, the oxidative breakdown of polyunsaturated fatty acids, produces free radicals which bring about severe cellular damage. Malondialdehyde (MDA), a major end product of reaction is an index of lipid peroxidation and is estimated as thiobarbituric acid reactive substances TBARS.[28] In our study, there was an increase in the level of TBARS in arthritic joints; HAELA and diclofenac significantly brought down its level both in tissue and plasma (P<0.001).

Oxygen-derived free radicals like superoxide ($O_2^{\circ-}$) hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) formed in all aerobic cells have deleterious effect in inflammatory conditions like arthritis. Polymorphonuclear leucocytes (PMNL) produces these reactive free radicals in excessive amounts. Since the synovial fluid has lesser activities of SOD and CAT, the oxygen-derived species are not scavenged and react with the joint components causing significant damage.[29] The scavenging antioxidant enzymes present in the circulatory system are utilised at higher levels, thereby showing a reduced activity in arthritic rats. The H_2O_2 formed by the action of SOD is decomposed by CAT or by GPx which uses GSH as a substrate. These enzymic antioxidants and the nonenzymic antioxidant GSH are also reduced in arthritic rats. HAELA played a significant role in maintaining the oxidative homeostasis as is manifested by the decrease in MDA and increase in GSH, along with increased activity of SOD, GPX and CAT, indicating its promising role as an antioxidant. This is in harmony with earlier studies done on other plant extracts.[24,30]

CRP is a prototypic marker of inflammation. It is a prognostic indicator of disease progression and is considered to be a real time measure of disease activity in RA.[31] Synovitis causes the release of proinflammatory cytokines such as TNF-α and IL-1 β from monocytes and macrophages. These cause the release of IL-6 which stimulates the liver to secrete CRP.[32] HAELA greatly suppressed the inflammatory process by reducing the production of CRP and other inflammatory cytokines in arthritic rats, indicating the anti-inflammatory role of *L. aspera*.

Cathepsin D, a protease actively involved in cartilage destruction,[33] showed a marked increase in activity in RA and its decreased activity in both standard drug treated and HAELA treated rats indicates the curative potential of HAELA on cartilage and bone damage.

The antioxidant and anti-inflammatory potential can be attributed to the presence of flavonoids, phenols and tannins in HAELA, as has been proved in the preliminary phytochemical analysis. HPTLC is an invaluable quality assessment tool for the evaluation of botanical materials, thus serving as a reference standard for quality control of this extract. It allows the analysis of a large number of compounds both efficiently and cost effectively.[34] In our study, HPTLC fingerprint revealed the presence of 11 different phytocompounds.

HPLC studies have indicated the presence of luteolin in significant quantities, and kaempferol, quercetin and apigenin in considerable amounts in HAELA. These are potent antioxidants. Earlier studies on the methanolic extract of *L. aspera* have indicated the presence of macelignan, nectandrin B, machilin C, chrysoerol, acacetin and apigenin, along with various phytoconstituents.[13]

Histopathological analysis of arthritic knee joints in general indicates knee effusions and synovial thickening which may lead to atrophy and progressive loss of cartilage and ligament weakening. In this study, erosions which represent the destruction of bones were seen in arthritic rats and reduction of inflammation and inflammatory cells is seen in both standard drug treated and HAELA treated rats. However, the persistence of mild changes in the synovial region of HAELA rats suggests that the treatment period (21 days) can be extended further for complete regeneration.

In conclusion, the results of the present study indicate the anti-arthritic potential of HAELA against joint damage induced by CFA. Thus, on the basis of the phytochemical, physical, biochemical and histopathological studies, it can be concluded that the HAELA possesses significant anti-arthritic activity. These results seem to support the traditional use of the plant in chronic rheumatism.

ACKNOWLEDGEMENT

The authors are grateful to Dr. N. Jayaraman for authenticity of the plant and also wish to thank the Department of Science and Technology for financial grant and A-Z Laboratories, Chennai for technical assistance in performing HPLC.

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Source of Support: Funded by the Department of Science and Technology, **Conflict of Interest:** None declared.