

Anti-Inflammatory and Antioxidant Activity of *Acalypha hispida* Leaf and Analysis of its Major Bioactive Polyphenols by HPLC

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Abstract

Purpose: Inflammation and oxidative stress can lead to different chronic diseases including cancer and atherosclerosis. Many medicinal plants have the potential to show as anti-inflammatory activity. Present investigation was performed to investigate anti-inflammatory, antioxidant activity, and quantification of selected bioactive plant polyphenols of the ethanol (EAH) and aqueous (AAH) extracts of *Acalypha hispida* (Euphorbiaceae) leaves.

Methods: Anti-inflammatory activity was evaluated by carrageenan and histamine induced rat paw edema models while antioxidant capacity was evaluated by DPPH free radical scavenging, Fe⁺² chelating ability, reducing power, NO scavenging, total phenolic and total flavonoid content assay. Identification and quantification of bioactive polyphenols was done by HPLC.

Results: At the doses of 200 and 400 mg/kg, both EAH and AAH showed statistically significant inhibition of paw volume in the anti-inflammatory activity test. Both the extracts showed DPPH scavenging (IC₅₀: 14 and 17 µg/ml, respectively), Fe⁺² ion chelating (IC₅₀: 40 and 46 µg/ml, respectively), NO scavenging activity (65.49 and 60.66% inhibition at 100 µg/ml), and concentration dependent reducing power ability. For EAH and AAH, flavonoid content was 126.30 and 149.72 mg QE/g dry extract, while phenolic content was 130.51 and 173.80 mg GAE/g dry extract, respectively. HPLC analysis of EAH and AAH indicated the presence of high content of ellagic acid along with other phenolic constituents.

Conclusion: High content of ellagic acid along with other phenolic constituents might have played an important role in the observed anti-inflammatory and antioxidant activity.

Introduction

Acalypha hispida Burm.f. (Euphorbiaceae) is an erect, sparsely branched shrub, locally known as sibjhal, sibjota or jotamangshi in Bangladesh. It grows in the coastal regions of Bangladesh. The plant is native to New Guinea, the Malay Archipelago and other islands in the East Indies.¹ It is commonly used as an ornamental plant in the garden and house. The leaves are laxative, diuretic and used in the treatment of leprosy and gonorrhoea. Different part of the plant is also used in infectious diarrhoea, pulmonary problems, and as an expectorant in asthma.² The plant contains ellagitannins namely, acallyphidins M₁, M₂, and D₁, anthocyanins namely, cyanidin 3-*O*-(2"-galloyl-β-galactopyranoside), cyanidin 3-*O*-(2"-galloyl-β-galactopyranoside), and cyanidin 3-*O*-β-galactopyranoside.^{3,4} Previous phytochemical screening of *A. hispida* leaves extract indicated the presence of reducing sugars, glycosides, steroids, flavonoids, and saponins.^{3,4,5} The leaves of *A.*

hispida has been reported to possess cytotoxic, antibacterial,⁵ antileprotic,⁶ antimicrobial,⁷ and antifungal⁸ properties, while the flower extract was reported to have DPPH free radical scavenging and cytotoxic activity.⁹ As a part of the continuation of our research on bioactivity screening of Bangladeshi medicinal plants,^{10,11,12} here we report the anti-inflammatory, antioxidant activity and quantification of the major polyphenols of ethanol and water extracts of *A. hispida* leaves.

Materials and Methods

Plant collection and extraction

The leaves of *A. hispida* was collected from Khulna, Bangladesh during July, 2012 and identified by the experts at Bangladesh National Herbarium (voucher specimen no.: DACB 34471). Shade-dried leaves were ground into coarse powder with the help of a grinder.

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The powdered plant material was kept in an airtight container and preserved in a cool, dark and dry place until the extraction commenced. Powdered plant material (150 g) was soaked in 900 ml of ethanol and kept for 72 h with occasional shaking and stirring. The solvent was filtered through a cotton plug. The filtrate was evaporated to dryness using a rotary vacuum evaporator to get the ethanol extract (EAH). The plant material was dried from the residual solvent and macerated with MilliQ water. Upon filtration, the filtrate was freeze dried to get the aqueous extract (AAH).

Test animals

For the *in vivo* anti-inflammatory activity study, male rats of Wister strain weighing 179-205 g were used. The animals were housed at the Pharmacology Research Laboratory of Bangladesh Council of Scientific and Industrial Research (BCSIR), Chittagong under standard laboratory conditions maintained at $25\pm 1^\circ\text{C}$ and fed with rodent food and water *ad libitum*. Protocols approved by BCSIR Ethics Committee on research in animals were followed during all experimental procedures that involve animals.

Chemicals and drug

Indomethacin, carrageenan, histamine phosphate, (+)-catechin hydrate, gallic acid, vanillic acid, caffeic acid, *p*-coumaric acid, (-)-epicatechin, rutin hydrate, quercetin, ellagic acid, ascorbic acid, DPPH, and Folin-Ciocalteu's reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tween 80, chloroform (analytical grade), ethanol (analytical grade), acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (HPLC grade), trichloroacetic acid, phosphate buffer (pH 6.6), potassium ferricyanide, ferric chloride, sodium phosphate, EDTA, ammonium molybdate, and sodium carbonate were purchased from Merck (Darmstadt, Germany).

Tests for anti-inflammatory activity

Carrageenan-induced rat paw edema test

The rats were divided into control, positive control and test groups, consisting of five animals in each. Control and positive control groups were orally administered with 1% Tween-80 in normal saline (10 ml/kg), and indomethacin (10 mg/kg), respectively. Test groups received EAH and AAH orally at the doses of 200 and 400 mg/kg, respectively. Acute inflammation was induced in all the rats by injecting 0.1 ml of carrageenan (1% w/v in 1% Tween-80 in normal saline) in the right hind paw of the rats, 1 h post exposure of the treatments. The paw volume was determined with a micrometer screw gauge at 1, 2, 3, 4 and 5 h after carrageenan administration.¹³

Histamine-induced rat paw edema test

The rats were divided into groups, and treated in the same fashion as that of carrageenan-induced edema test. Acute inflammation was initiated in all test animals by

injecting 0.1 ml of histamine (1% w/v in 1% Tween-80 in normal saline) in the right hind paw of the rats. The paw volume was measured with a micrometer screw gauge at 1, 2, 3, 4 and 5 h after histamine administration.¹⁴

Tests for antioxidant activity

DPPH free radical scavenging assay

Aliquots (1 ml) of EAH, AAH and ascorbic acid at different concentrations were added to 3 ml of a 0.004% w/v DPPH solution. The mixture was allowed to stand in the dark at 25°C for 30 min and the absorbance of the mixture was recorded against blank at 517 nm using a double beam UV/Visible spectrophotometer (Analykjena, Model 205, Jena, Germany). The IC_{50} was determined from the absorbance versus concentration plot.¹⁵

Fe^{2+} ion chelating assay

Aliquots of (5 ml) different concentrations (5-100 $\mu\text{g/ml}$) of EAH, AAH and standard (EDTA) were taken and 0.1 ml solution of 2 mM ferrous chloride was added to it, followed by the addition of 0.2 ml of 5 mM ferrozine. After an interval of 10 min to complete the reaction, the absorbance of the solution was measured at 562 nm.¹⁶

Reducing power assay

Different concentrations of EAH, AAH and standards (ascorbic acid and BHA) (5-100 $\mu\text{g/ml}$) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1 %). The mixture was incubated at 50°C for 20 min. A 10 % solution of trichloroacetic acid (2.5 ml) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl_3 (0.5 ml, 0.1 %) and the absorbance of the mixture was measured at 700 nm.¹⁷

Nitric oxide scavenging assay

Sodium nitroprusside in phosphate buffer was mixed with different concentrations of EAH and AAH (5-100 $\mu\text{g/ml}$), and incubated at 25°C for 30 min. The incubated solution (1.5 ml) was diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the resulting reaction mixture was measured at 546 nm.¹⁸

Total phenolic content assay

Aliquots (0.5 ml) of the extracts (1 mg/ml) and various concentrations of gallic acid solutions (500-15.62 mg/L) were mixed with 5 ml of Folin-Ciocalteu's reagent (1:10 v/v in distilled water) and 4 ml of sodium carbonate. The mixture was then vortexed for 15 sec and kept for 30 min at 40°C for the reaction to complete and the absorbance was measured at 765 nm. Total phenol content of the extracts were determined from the standard curve and

expressed as mg of gallic acid equivalent (GAE) per gram extract.¹⁹

Total flavonoid content assay

The extracts (5 ml, 1 mg/ml) were mixed with 2.5 ml of aluminium chloride reagent (133 mg of aluminium chloride and 400 mg of sodium acetate in 100 ml of de-ionised water) and kept for 30 min at room temperature. The absorbance of the reaction mixture was measured at 430 nm. Total flavonoids content was determined from the standard curve and expressed as mg of quercetin equivalent (QE) per gram extract.²⁰

Quantification of polyphenolic compounds by HPLC

HPLC chromatographic analysis was done using a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) system (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary rapid separation pump (LPG-3400RS), rapid separation diode array detector (DAD-3000RS) and ultimate 3000RS auto sampler (WPS-3000). Separation was accomplished on Acclaim[®] C18 (4.6 x 250 mm; 5 μ m) column (Dionex, USA), controlled at a constant temperature of 30°C. The separation was carried out using a gradient elution programme (0-10 min, 5%A/95%B; 10-20 min, 10%A/80%B/10%C; 20-30 min, 20%A/60%B/20%C; and 30 min, 100%A, where A is acetonitrile, B is acetic acid solution in water at pH 3.0, and C is methanol). The flow rate was maintained at 1 ml/min, and the injection volume was 20 μ l. For the detection, UV detector was set to 280 nm and held for 18 min, changed to 320 nm and

held for 6 min, finally to 380 nm and continued for the rest of the analysis while the diode array detector was set at an acquisition range of 200-700 nm. The standard solution was made by diluting the standard stock solutions in methanol to give a concentration of 20 μ g/ml for each polyphenols, except for caffeic acid (8 μ g/ml) and quercetin (6 μ g/ml). Solution of the test extracts were prepared in ethanol having a concentration of 5 mg/ml. Spiking the sample solution with phenolic standards was done for further confirmation of individual polyphenols.²¹

Statistical analysis

Data were presented as mean \pm standard deviation (SD). One-way ANOVA followed by Dunnett's test was performed and the results were considered statistically significant when $p < 0.05$.

Results

Anti-inflammatory activity test

Carrageenan-induced paw edema

At the doses of 200 and 400 mg/kg, both EAH and AAH significantly decreased the volume of carrageenan-induced rat paw edema. At the above dose levels, the maximum decrease in paw volume was 26 and 36%, respectively for EAH, in contrast to 29 and 40%, respectively for AAH as compared to control. Indomethacin showed statistically significant anti-inflammatory activity with the highest inhibition (64%) of paw edema at 5 h after carrageenan injection (Table 1).

Table 1. Effect of ethanol and aqueous extracts of *A. hispida* leaves on carrageenan-induced rat paw edema volume.

Treatment	Dose (mg/kg)	Right hind paw volume (% Inhibition)				
		1 h	2 h	3 h	4 h	5 h
Vehicle	10	0.95 \pm 0.15	1.11 \pm 0.21	1.28 \pm 0.19	1.45 \pm 0.27	1.51 \pm 0.24
Indomethacin	10	0.51 \pm 0.12 (46)*	0.53 \pm 0.18 (52)*	0.55 \pm 0.25 (57)*	0.59 \pm 0.16 (59)**	0.60 \pm 0.17 (60)**
Ethanol extract	200	0.82 \pm 0.17 (14)*	0.95 \pm 0.19 (14)*	1.04 \pm 0.21 (19)*	1.13 \pm 0.10 (22)*	1.12 \pm 0.15 (26)**
	400	0.78 \pm 0.17 (18)*	0.85 \pm 0.19 (23)*	0.91 \pm 0.13 (29)*	0.97 \pm 0.20 (33)*	0.96 \pm 0.14 (36)**
Aqueous extract	200	0.81 \pm 0.18 (15)	0.92 \pm 0.16 (17)*	1.01 \pm 0.19 (21)	1.07 \pm 0.21 (26)*	1.08 \pm 0.14 (29)**
	400	0.73 \pm 0.19 (23)	0.77 \pm 0.16 (31)*	0.84 \pm 0.15 (34)*	0.92 \pm 0.17 (37)*	0.90 \pm 0.13 (40)**

Value presented as the mean \pm SE ($n=5$); * $p < 0.05$, ** $p < 0.01$ versus control (Dunnett's test).

Histamine-induced paw edema

Rats pre-treated with EAH at 200 and 400 mg/kg, showed decrease in the histamine-induced paw edema volume with the maximum inhibition of 27 and 39%, respectively, while that of AAH was 31 and 45% inhibition, respectively as compared to control. Indomethacin showed a maximum inhibition of 64% at 5 h after histamine administration, and the results were statistically significant (Table 2).

Antioxidant activity test

DPPH free radical scavenging assay

In the quantitative DPPH free radical scavenging assay, EAH and AAH showed strong antioxidant activity with

IC₅₀ values of 14 and 17 μ g/ml, respectively, while that of ascorbic acid was 10 μ g/ml.

Fe²⁺ ion chelating ability

Both EAH and AAH showed Fe²⁺ ion chelating ability with IC₅₀ values of 40 and 46 μ g/ml, respectively. The IC₅₀ value of EDTA, used as the standard in this assay showed an IC₅₀ of 17 μ g/ml.

Reducing power assay

Concentration dependent reducing power was observed for both EAH and AAH with the maximum absorbance of 1.13 and 0.88, respectively at the highest concentration tested (100 μ g/ml) (Figure 1).

Nitric oxide scavenging assay

Both the extracts EAH and AAH significantly scavenged NO radical. The IC₅₀ value for EAH and AAH were found to be 43 and 50 µg/ml, respectively and were comparable to that of ascorbic acid (17 µg/ml) (Figure 2).

Total phenolic content assay

The total phenolic content of EAH and AAH were 130.5 and 173.8 mg GAE/g of dry extract, respectively (Figure 3).

Table 2. Effect of ethanol and aqueous extracts of *A. hispida* leaves on histamine-induced rat paw edema volume.

Treatment	Dose (mg/kg)	Right hind paw volume (% Inhibition)				
		1 h	2 h	3 h	4 h	5 h
Vehicle	10	1.10 ± 0.13	1.27 ± 0.11	1.36 ± 0.17	1.41 ± 0.22	1.48 ± 0.27
Indomethacin	10	0.54 ± 0.17 (51)*	0.58 ± 0.13 (54)*	0.59 ± 0.18 (57)*	0.56 ± 0.15 (60)**	0.53 ± 0.14 (64)**
Ethanol extract	200	0.92 ± 0.15 (16)*	1.01 ± 0.13 (20)*	1.04 ± 0.21 (24)	1.07 ± 0.12 (24)*	1.08 ± 0.16 (27)**
	400	0.82 ± 0.18 (26)	0.91 ± 0.15 (28)*	0.92 ± 0.19 (32)	0.89 ± 0.17 (37)*	0.90 ± 0.13 (39)**
Aqueous extract	200	0.87 ± 0.21 (21)	0.96 ± 0.13 (24)*	0.99 ± 0.20 (27)	1.01 ± 0.15 (28)*	1.02 ± 0.11 (31)**
	400	0.81 ± 0.19 (26)	0.88 ± 0.13 (31)*	0.89 ± 0.17 (35)*	0.84 ± 0.18 (40)*	0.82 ± 0.17 (45)**

Value presented as the mean ± SE (n=5); *p<0.05, **p<0.01 versus control (Dunnett's test).

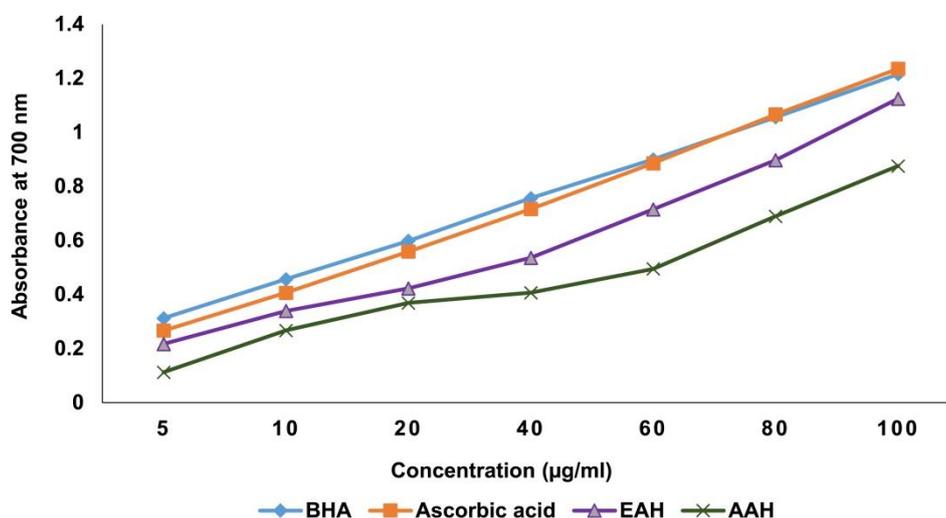


Figure 1. Reducing power of ethanol and aqueous extracts of *A. hispida* leaves.

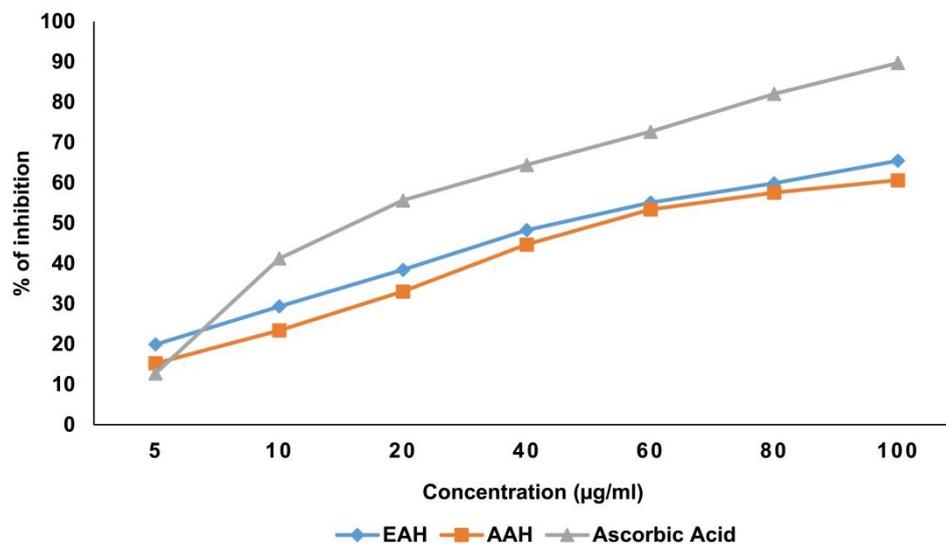


Figure 2. Nitric oxide (NO) scavenging activity of ethanol and aqueous extracts of *A. hispida* leaves.

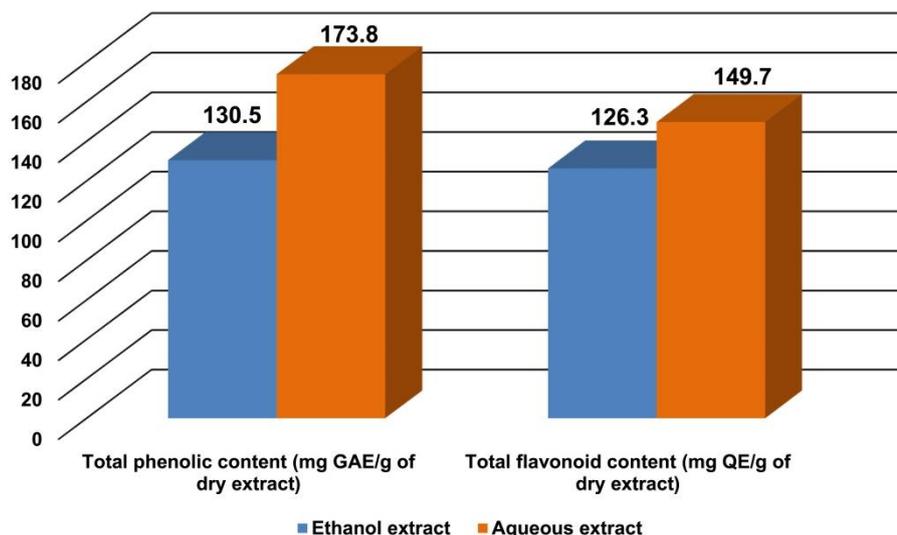


Figure 3. Total phenolic and flavonoid content of ethanol and aqueous extracts of *A. hispida* leaves.

Total flavonoid content assay

The total flavonoid content for EAH and AAH were 126.3 and 149.7 mg QE/g of dry extract, respectively (Figure 3).

Quantification of polyphenolic compounds by HPLC

The HPLC analysis indicated that both EAH and AAH are rich in ellagic acid content (119.40 and 540.90

mg/100 g dry extract). Gallic acid and quercetin were detected in both extracts but concentration was in lower amount (31.60 and 5.80 mg of gallic acid/100 g EAH extract, 0.50 and 0.60 mg of quercetin/100 g AAH extract, respectively). *p*-Coumaric acid and rutin were detected only in AAH (0.90 and 14.30 mg/100 g AAH, respectively) (Figure 4, Figure 5 and Figure 6).

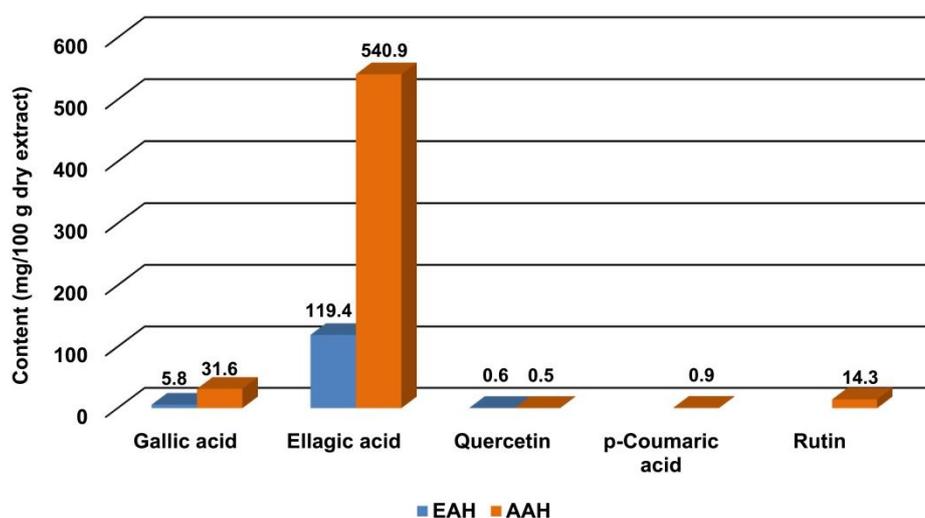


Figure 4. Polyphenolic contents of ethanol and aqueous extracts of *A. hispida* leaves.

Discussion

The inflammatory reaction induced by carrageenan in rats is recognised by a biphasic response. The key feature of marked edema formation is mediated by histamine, serotonin and bradykinins in the first phase, while the second phase is associated with the release of prostaglandins and nitric oxide, which is generated by cyclooxygenase-2 (COX-2), and inducible isoform of nitric oxide synthase (iNOS).^{22,23} Histamine is an important mediator of inflammation, and act as a potent vasodilator, and vascular permeability

facilitator.²⁴ Administration of histamine in test animals results in an increase in the vascular penetrability of the host capillary venules at the site of injection. Agents that oppose the activity of histamine receptors reduce the swelling triggered by inflammation. In the present investigation, both the ethanol and aqueous extracts of *A. hispida* leaves significantly reduced the edema formation in carrageenan, as well as in histamine induced rat paw edema. Thus the anti-inflammatory activity of the extracts might involve antihistaminic activity. In

addition, inhibition of other inflammatory pathway cannot be ruled out, since the extracts also inhibited

carrageenan induced inflammatory response, which involves several inflammatory pathways.

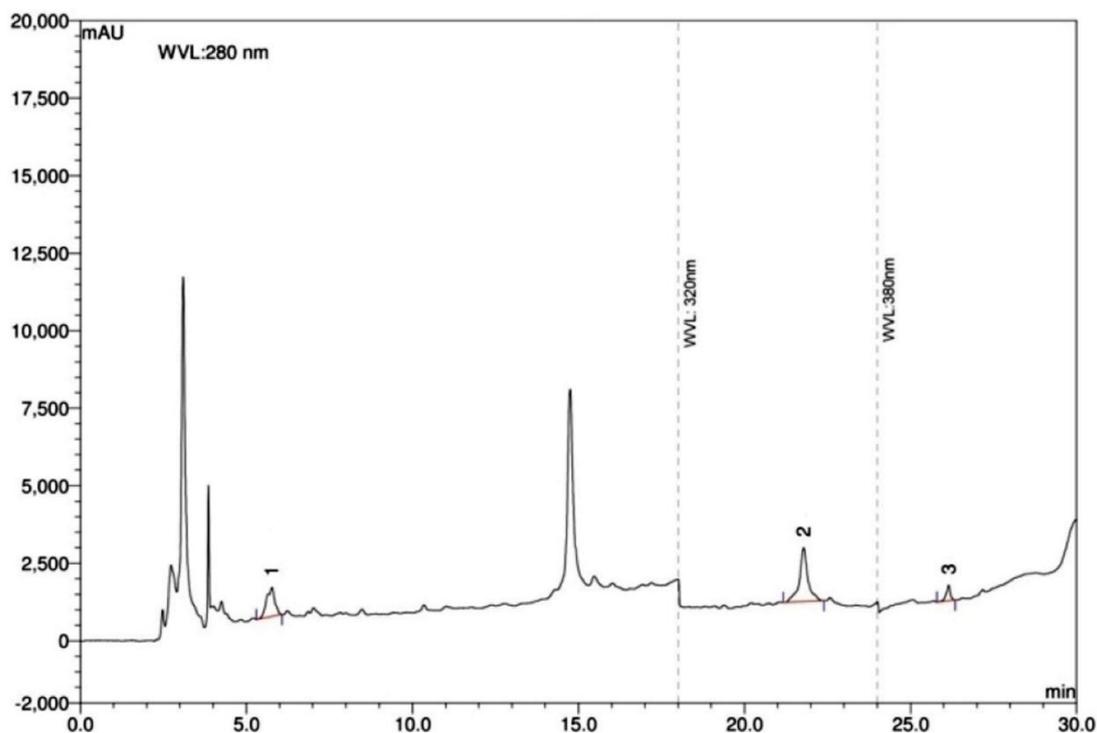


Figure 5. HPLC chromatogram of ethanol extract of *A. hispida* leaves. Peaks 1: gallic acid, 2: ellagic acid, 3: quercetin.

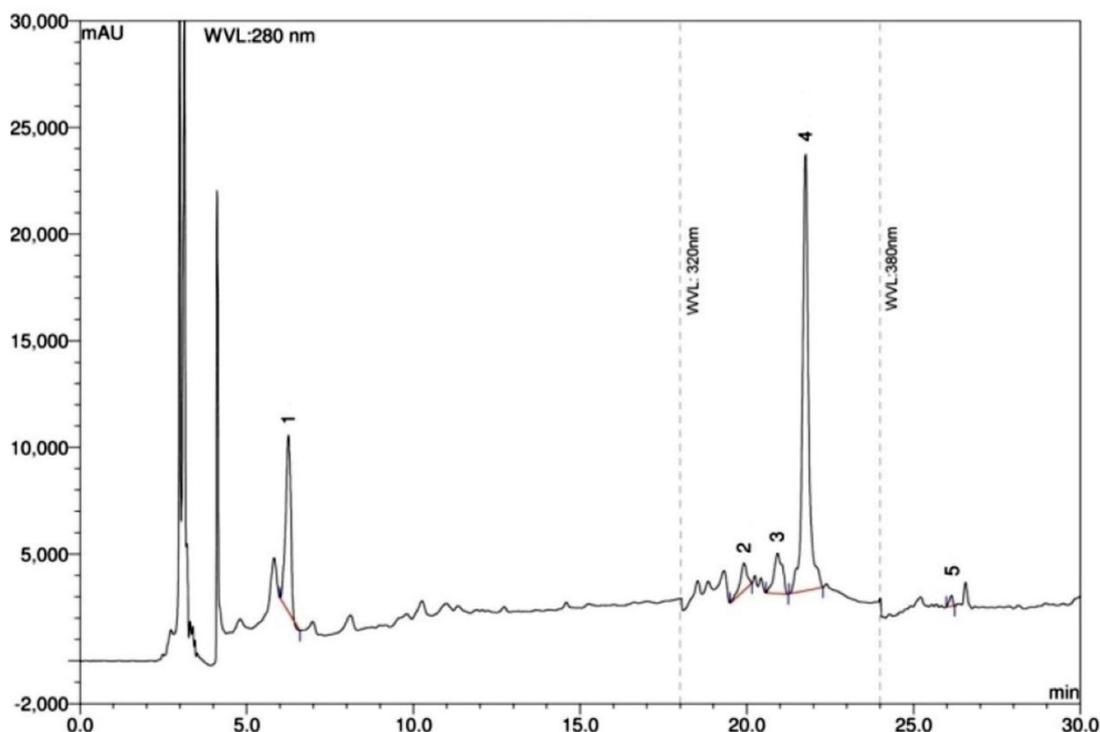


Figure 6. HPLC chromatogram of aqueous extract of *A. hispida* leaves. Peaks 1: gallic acid, 2: *p*-coumaric acid, 3: rutin, 4: ellagic acid, 5: quercetin.

Plant derived compounds with antioxidant activity often show anti-inflammatory activity.²⁵ The ability of antioxidants in protecting the cells from oxidative stress

can contribute towards the anti-inflammatory activity.²⁶ Commonly occurring plant phenolics and flavonoids are often associated with antioxidant activity.²⁷ Thus a

number of assays were performed to investigate the antioxidant capacity of *A. hispida* leaves. In the DPPH radical scavenging assay, *A. hispida* leaves extracts showed a concentration dependent free radical scavenging activity. Saponins, flavonoids and tannins, which are also present in this plant, are known to show DPPH radical scavenging activity by donating proton to neutralise the free radicals.^{28,29} Agents that form σ bond with a metal are active as secondary antioxidants, because they decrease the redox potential and steadying the oxidised form of the metal ion.³⁰ Results demonstrated that *A. hispida* leaf extracts have the capacity to reduce ferric ion, a further proof to the antioxidant potential of the extracts. Nitric oxide (NO) works as a neural modulator, which is involved in neurotransmitter release, neuronal excitability and other intellectual activities including learning and memory.³¹ Though it has a role in physiologic processes, it also participates in pathogenic pathways underlying a large group of disorders including inflammation, inflammatory bowel disorder, primary headaches and stroke.³² *A. hispida* leaf extracts showed NO scavenging activity suggesting the ability to ameliorate inflammatory response associated with NO.

With a view to justify the correlation between pharmacological activity and phenolic constituents of *A. hispida* leaf extracts, HPLC analysis was conducted to identify some of the biologically important antioxidant principles from plants. The HPLC analysis showed a high content of ellagic acid in both extracts. Previous studies revealed that ellagic acid possesses strong antioxidant and anti-inflammatory activities.^{33,34} The anti-inflammatory activity of ellagic acid can be attributed due to its ability to inhibit one or more inflammatory pathways including inhibition of COX-2 and NO synthase expression.³⁵ Presence of phenolic hydroxyl group in the structure of ellagic acid enables the donation of proton to neutralise free radicals.³⁶ Trace amount of gallic acid, rutin and quercetin were also present in the leaf extracts. In addition to their antioxidant property, aforementioned compounds are also reported to have anti-inflammatory activity.³⁷⁻³⁹

The anti-inflammatory activity observed for the aqueous extract was higher than that of the ethanol extract. In the HPLC analysis, ellagic acid content, along with other phenolics were found higher for aqueous extract than the ethanol extract and it is also reflected by the total phenol and total flavonoid content assay. Therefore, stronger anti-inflammatory activity of the aqueous extract may be due its higher phenol and flavonoid content.

Conclusion

The ethanol and aqueous extracts of *A. hispida* leaf exhibited significant anti-inflammatory activity through the reduction of carrageenan and histamine induced edema formation in test rats. The extracts also showed potent antioxidant activity in a number of methods. The observed anti-inflammatory activity may be due to the presence of high concentration of ellagic acid in the plant

extracts. In addition, other phenolic constituents present in the plant might have assisted towards the anti-inflammatory activity.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors declare no conflict of interests.

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