

Original Research Paper

Anti-Inflammatory, Analgesic and Antioxidant Activities of *Allophylus Cobbe* Leaves

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Abstract: *Allophylus cobbe* L. Raeuschel (Family-Sapindaceae) is a medicinal plant used traditionally for the treatment of various health risks like pain, inflammation, ulcers and wounds in Bangladesh. This study determined the polyphenolic compounds and evaluated the analgesic, anti-inflammatory and antioxidant effects of the ethanol extract of *Allophylus cobbe* leaves. High Performance Liquid Chromatography (HPLC) analysis was used to determine the polyphenolic compounds present in the extract. The analgesic activity was evaluated by hot plate and acetic acid induced writhing in mice at two different doses of 250 and 500 mg kg⁻¹ body weight. The extract was also investigated for the anti-inflammatory effect on rats at above mentioned doses using carrageenan induced rat paw edema method. The antioxidant potential of the extract was determined in terms of radical scavenging ability of the stable 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) free radical. Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins and glycosides in the extract. High Performance Liquid Chromatography (HPLC) analysis also confirmed the presence of polyphenolic compounds such as (+)-catechin hydrate, (-)-epicatechin, caffeic acid, *p*-coumaric acid and quercetin. The extract increased the licking time of hind paw when placed on a hot plate by 35.31% at 250 mg kg⁻¹ dose which is comparable to the increase shown by diclofenac sodium (42.73%) at the 3rd h of study in hot plate test. Moreover, the extract also showed good analgesic effect in acetic acid induced writhing test. The percent inhibition of writhing response by the extract was 85.96 and 78.07% at 250 and 500 mg kg⁻¹ doses respectively while that of the standard drug was 66.67%. Furthermore, the extract also reduced carrageenan induced paw edema formation; the most prominent inhibition was found to be 58.88% (250 mg kg⁻¹) at the 3rd h of study. The DPPH radical scavenging activity of the extract increased markedly with increasing concentrations. At a concentration of 200 µg mL⁻¹, the scavenging activity of the ethanol extract (91.53% inhibition) was comparable to that of the standard ascorbic acid (99.3% inhibition). Our results suggest that *Allophylus cobbe* extract possesses significant antinociceptive, anti-inflammatory and free radical scavenging activities which may justify the folkloric use of the plant in several communities for conditions such as colic, fever, inflammatory rheumatic pains and other oxidative stress associated disorders.

Keywords: Phytochemical Screening, Acetic Acid, Medicinal Plant, Carrageenan, Analgesia, HPLC

Introduction

Inflammation and pain are local responses to living tissue injury and are common manifestations of many diseases affecting millions of people worldwide. Inflammation is a complex phenomenon. Several

components such as edema formation, leukocyte infiltration and granuloma formation are elicited in response to an inflammatory reaction (Beg *et al.*, 2011). In inflammation, several potent chemical mediators such as prostaglandins, leukotrienes, prostacyclins, lymphokines and chemokines like Interferon- α (IFN- α),

γ , Interleukin (IL)-1, IL-8, histamine, 5-Hydroxytryptamine (5-HT) and tissue necrosis factor- α are released in the inflamed tissues (Serhan and Savill, 2005). Many synthetic drugs, mainly Nonsteroidal Anti-Inflammatory Drugs (NSAIDs), steroidal drugs and immunosuppressant drugs are reported to be used for the treatment of inflammatory disorders. However, these drugs are not devoid of adverse effects and they are considered to be highly unsafe for long term treatment (MacLennan *et al.*, 1996; Lanas, 2009). Nevertheless, the existing synthetic therapeutic agents are also expensive and not easily available to the rural people of developing world. On the other hand plant derived preparations are gaining much importance in therapeutical applications because they are cheap, easily available and have little side effects. Thus, the use of traditional medicine and medicinal plants in most developing countries has been widely observed for the treatment of various ailments including prevention and management of pain, inflammation and other oxidative stress associated diseases such as cancer, atherosclerosis, aging and neurodegenerative disorders (Beg *et al.*, 2011; Jain *et al.*, 2014).

Allophylus cobbe L. (Family-Sapindaceae) is a small shrub that grows wild in the hilly regions of Bangladesh. Different parts of this plant are used in traditional medicine in Bangladesh, India, Brazil and Nigeria. It showed antifeedant activity and is used as an anti-diarrheal agent (Jayasinghe *et al.*, 2003). Leaves are used to induce lactation in breast feeding mothers. Leaves and barks are used as a remedy for elephantiasis; decoctions are given in colic and as a drink to cure fever in children (Mandade and Sreenivas, 2012). Hot infusion of the root barks is also used for rheumatic pains. Recent scientific investigations suggest that *Allophylus cobbe* possesses strong antimicrobial (Islam *et al.*, 2012), anti-ulcer (Dharmani *et al.*, 2005) and anti-osteoporosis activities (Kumar *et al.*, 2010). Previous phytochemical studies of the plant have reported the presence of several flavonoids such as quercetin, pinitol, luteolin 7-O- β -D-glucopyranoside, rutin and apigenin-4-O- β -D-glucoside (Kumar *et al.*, 2010). Flavonoids exhibit potent antioxidant and anti-inflammatory activities. The Chakma tribe of Bangladesh has been using this plant for centuries for the treatment of inflammatory disorders. However, no extensive report is found on the pharmacological activities of the leaves of this plant. The current study was thus designed to evaluate the anti-inflammatory, analgesic and free radical scavenging activities of the ethanolic leaf extract of *A. cobbe* plant.

Materials and Methods

Plant Material and Extraction Process

Whole plant *Allophylus cobbe* was collected from Kamalgong, Moulavibazar, Bangladesh in April, 2012 and identified by Md. Abdur Rahim, National Herbarium, Mirpur, Dhaka, Bangladesh. Accession number DACB-37950 is retained there for further

references and the specimen has been preserved in the Phytochemistry and Pharmacology Laboratory, North-South University Bangladesh. The dried powder of leaves (200 g) was extracted with 95% ethanol at room temperature ($25 \pm 2^\circ\text{C}$). The extract was concentrated by evaporation under reduced pressure at 40°C using rotary evaporator (Bibby RE-200, Sterilin Ltd, UK) to have gummy concentrate of dark orange color (8.51 g).

Phytochemical Analysis

The freshly prepared crude extract was qualitatively tested for the identification of chemical constituents, such as, alkaloids, flavonoids, steroids, glycosides, saponins, terpenoids, gums and tannins. The tests were carried out by the method described previously and 10% (w/v) solution of the extract was taken in each test unless otherwise mentioned in individual test (Talukder *et al.*, 2012).

Assay for Total Phenolic and Flavonoid Content

The concentration of total phenol in extract was measured by a UV spectrophotometer based on a colorimetric oxidation/reduction reaction using Folin-Ciocalteu reagent (Majhenič *et al.*, 2007). Gallic acid was used to make the calibration curve and the result was expressed as Gallic Acid Equivalents (GAE) in milligrams per gram extract. Total flavonoid content of the extract was determined by aluminium chloride colorimetric method (Chang *et al.*, 2002). Quercetin was used as standard and the result was expressed as Quercetin Equivalents (QE) in milligrams per gram extract.

High Performance Liquid Chromatography (HPLC) System

The phenolic composition of the ethanol extract of *Allophylus cobbe* was determined by HPLC, as described previously with some modifications (Uddin *et al.*, 2014). The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B) and methanol (solvent C). The system was run with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30 min, 100%A. There was a 5 min post run at initial conditions for equilibration of the column. The flow rate was kept constant throughout the analysis at 1 mL/min and the injection volume was 20 μL . For UV detection, the wavelength program was optimized to monitor phenolic compounds at their respective maximum absorbance wavelengths as follows: λ 280 nm held for 18.0 min, changed to λ 320 nm and held for 6 min and finally changed to λ 380 nm and held for the rest of the analysis and the DAD was set at an acquisition range from 200 to 700 nm. The detection and quantification of GA, CH, VA, CA and EC was done at 280 nm, of PCA, RH and EA at 320 nm and of MC, QU and KF at 380 nm, respectively.

Experimental Animals

Swiss-Albino mice aged about 4-5 weeks with average weight of 25-35 g and Long Evans rats of either sex having average weight of 100-130 g were used for the experiments and maintained in the animal house of the Department of Pharmaceutical Sciences, North South University Bangladesh for acclimation. These animals were originally obtained from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). They were housed in standard cages under standard environmental conditions of room temperature at $24\pm 1^\circ\text{C}$ and 55-65% relative humidity with 12 h dark light cycle and provided with standard food for rodents and water *ad libitum*. All experiments involving animals were conducted according to the UK Home Office regulations (UK Animals Scientific Procedures Act 1986) and the 'Principles of Laboratory Animal Care' (National Institutes of Health publication no. 86-23, revised 1985).

Analgesic Activity Studies

Hot Plate Test

The hot plate test method was employed to assess the analgesic activity in accordance with the method described previously with minor modification (Williamson *et al.*, 1996). The experimental animals were divided into control, positive control and test groups with six mice in each group. The animals of test groups received test samples at the doses of 250 and 500 mg kg^{-1} body weight, positive control group was administered diclofenac sodium at the dose of 10 mg kg^{-1} body weight and vehicle control group was treated with 1% Tween 80 solution in distilled water at the dose of 10 mL kg^{-1} body weight orally. In this test, the animals were positioned on Eddy's hot plate kept at a temperature of $55\pm 0.5^\circ\text{C}$. The test samples and the standard drug were administered 30 min before the beginning of the experiment. Reaction time was recorded when animals licked their fore or hind paws, or jumped at 0, 60, 120, 180 and 240 min. A cut-off period of 20 sec was observed to avoid paw damage. The antinociceptive response latency was recorded from the time between placement and licking of fore or hind paws or jumping movements of the animals. Percent Analgesic Score (PAS) was calculated as:

$$\text{PAS} = (T_a - T_b) / T_a \times 100$$

Where:

T_b = Reaction time (sec) before drug administration;

T_a = Reaction time (sec) after drug administration.

Acetic Acid Induced Writhing Test

The methods of (Williamson *et al.*, 1996; Amabeoku and Kabatende, 2012; Koster *et al.*, 1959) were used for the assessment of the antinociceptive

activity of *Allophylus cobbe* extract. Different groups containing six mice each, received normal saline solution (10 mL kg^{-1}) (i.e., control), diclofenac sodium (10 mg kg^{-1}), or plant extract (250 and 500 mg kg^{-1}) orally. Thirty minutes later, 0.6% acetic acid (10 mL kg^{-1}) solution was injected intraperitoneally to all animals in the different groups. The number of writhes (abdominal constrictions) occurring between 5 to 15 min after acetic acid injection was counted. The number of writhes in each treated group was compared to that of a control group while diclofenac sodium served as a reference standard (positive control).

The percentage inhibition of writhing was calculated using the following formula:

$$\text{Percent Inhibition} = (1 - W_t/W_c) \times 100$$

where, W_c and W_t represent the average number of writhing produced by the control and the test group, respectively.

Evaluation of Anti-Inflammatory Effect by Carrageenan Induced Paw Edema Method

The anti-inflammatory activity of the ethanol extract of *Allophylus cobbe* was investigated on carrageenan induced inflammation in rat paw following an established method (Winter *et al.*, 1962; Williamson *et al.*, 1996). Rats were randomly divided into four groups, each consisting of five animals, of which group I was kept as control that received only distilled water. Group II received standard, diclofenac sodium (10 mg kg^{-1}) as the reference drug for comparison while Group III and Group IV were given the test extract at a dose of 250 and 500 mg kg^{-1} body weight respectively. Half an hour after the oral administration of the test materials, 1% carrageenan was injected to the right hind paw of each animal (Winter *et al.*, 1962). Paw edema volume was measured at 0, 60, 120 and 180 min using a Plethysmometer (Model 7141, UGO Basile, Italy) after administration of carrageenan. The left hind paw served as a reference non-inflamed paw for comparison. The average percent increase in paw volume with time was calculated and compared against the control group. Percent inhibition was calculated using the formula:

$$\text{Percent Inhibition} = (1 - V_t/V_c) \times 100$$

where, V_c and V_t represent the average paw volume of control and treated animal respectively.

Antioxidant Activity

The antioxidant activity of the extract was measured in terms of hydrogen donating or radical scavenging ability of the stable 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) free radical (Braca *et al.*, 2001). Briefly, 0.1 mL of plant extract at various concentrations was added to 3

mL of a 0.002% methanolic solution of DPPH. The reaction mixtures were incubated for 30 min at room temperature and the absorbance at 517 nm was measured against a blank. A low absorbance of the reaction mixture indicated a high free radical scavenging activity. Ascorbic acid was used as standard in the experiment. The radical scavenging activity was calculated using the following formula:

$$\text{Percentage of inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \right] \times 100$$

Statistical Analysis

The data are expressed as the mean \pm SEM analyzed by one-way Analysis of Variance (ANOVA) and Dunnett's *t*-test was used as the test of significance. *P* value < 0.05 was considered as the minimum level of significance. All statistical tests were carried out using SPSS statistical software.

Results

Phytochemical Analysis

Preliminary phytochemical screening of the ethanol extract of *Allophylus cobbe* revealed the presence of various bioactive components of which flavonoids, alkaloids, terpenoids, tannins, gums and carbohydrates were the most prominent. Total phenolic content present in the extract was 55 mg GAE/g extract while total flavonoid present was 30 mg QEE/g extract.

HPLC-DAD Analysis of Phenolic Compounds Present in *Allophylus Cobbe*

The chromatographic separation of polyphenols in ethanol extract of *Allophylus cobbe* is shown in Fig. 1. The experimental results indicated that the extract contains (+)-catechin hydrate, (-)-epicatechin, caffeic acid, *p*-coumaric acid and quercetin (Table 1). However, (+)-catechin hydrate and (-)-epicatechin seems to be plenty in the extracts.

Analgesic Activity

Hot Plate Test

The ethanolic extract of the plant significantly increased the reaction time of heat sensation in mice at the doses of 250 and 500 mg kg⁻¹ (Table 2). In the 3rd h

of study, the extract increased the reaction time of heat sensation to 35.31 and 20.44% at the doses of 250 and 500 mg kg⁻¹ BW respectively while that of the standard drug was 42.73% and the results were found to be highly statistically significant (*p* < 0.001). However, the extract exhibited no dose dependent increase in latency time when compared with the control.

Acetic Acid-Induced Writhing Test

Inhibition of licking response in mice due to the administration of the test drugs during acetic acid-induced writhing test is shown in Fig. 2. The oral administration of both doses of extract significantly (*p* < 0.001) attenuated the acetic acid-induced abdominal writhes in mice. The percent inhibition of writhing response by the extract was 85.96 and 78.07% at 250 and 500 mg kg⁻¹ doses respectively while the standard diclofenac sodium (10 mg kg⁻¹) showed 66.67% inhibition in comparison with the control.

Anti-Inflammatory Activity

Results from carrageenan induced paw edema test, showed that ethanolic extract of *A. cobbe* leaves possess significant anti-inflammatory activity (Fig. 3). The extract was administered orally at the doses of 250 and 500 mg kg⁻¹ body weight of the animals. The most prominent inhibition of 58.88% at 250 mg kg⁻¹ was observed at the 3rd h of study, while the standard diclofenac sodium (10 mg kg⁻¹) showed 41.62% inhibition in comparison with the control. The result was found to be highly statistically significant at the 3rd h after administration of the sample drugs (*p* < 0.001).

Antioxidant Activity

The antioxidant activity of the extracts was determined using DPPH method because it is one of the most effective methods for evaluating radical-scavengers. The DPPH radical contains an odd electron which is responsible for the absorbance at 517 nm and also for a visible deep purple color. Figure 4 shows the DPPH radical scavenging activity of the extract and ascorbic acid which was used as standard in this study. At a concentration of 200 $\mu\text{g mL}^{-1}$, the scavenging activity of the ethanol extract (91.53% inhibition) was comparable to that of the standard ascorbic acid (99.3% inhibition).

Table 1. Contents of polyphenolic compounds in the ethanol extract of *Allophylus cobbe*

Polyphenolic compound	Ethanolic extract of <i>Allophylus cobbe</i>	
	Content (mg/100 g of dry extract)	% RSD
CH	175.47	2.01
CA	4.61	0.32
EC	67.74	1.68
PCA	4.01	0.63
QU	4.35	0.71

CH, (+)-catechin hydrate; CA, caffeic acid; EC, (-)-epicatechin; PCA, *p*-coumaric acid; QU, quercetin

Table 2. Effect of the ethanol extract of *Allophylus cobbe* on latency to hot plate test

Group	Reaction time at different time intervals (in sec)				
	0 min	60 min	120 min	180 min	240 min
Control	10.70±0.85	8.00±0.81	6.58±0.64	5.52±0.55	5.00±0.44
Standard	9.14±0.52	12.60±0.94 (27.46)	14.16±1.08*** (35.45)	15.96±0.68*** (42.73)	12.48±0.69 (26.76)
<i>Allophylus cobbe</i> 250 mg kg ⁻¹	9.38±1.04	12.36±2.14 (24.11)	13.66±1.14** (31.33)	14.50±1.39*** (35.31)	12.30±0.81** (23.74)
<i>Allophylus cobbe</i> 500 mg kg ⁻¹	10.20±0.87	11.82±0.65 (13.71)	12.26±1.03*** (16.80)	12.82±1.39*** (20.44)	11.78±0.68 (13.41)

Data are represented as the mean ± SEM, (n = 5); Values in parentheses indicate percent increase in reaction time; *p<0.05, **p<0.01, ***p<0.001 were considered statistically significant as compared to control

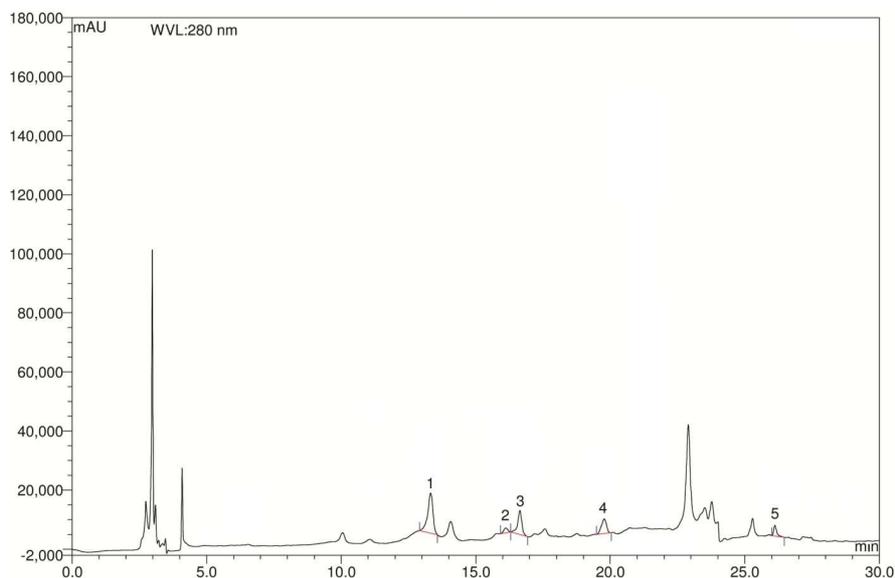


Fig. 1. HPLC chromatogram of ethanol extract of *Allophylus cobbe* L. Peaks: 1, (+)-catechin; 2, caffeic acid; 3, (-)-epicatechin; 4, *p*-coumaric acid; 5, quercetin

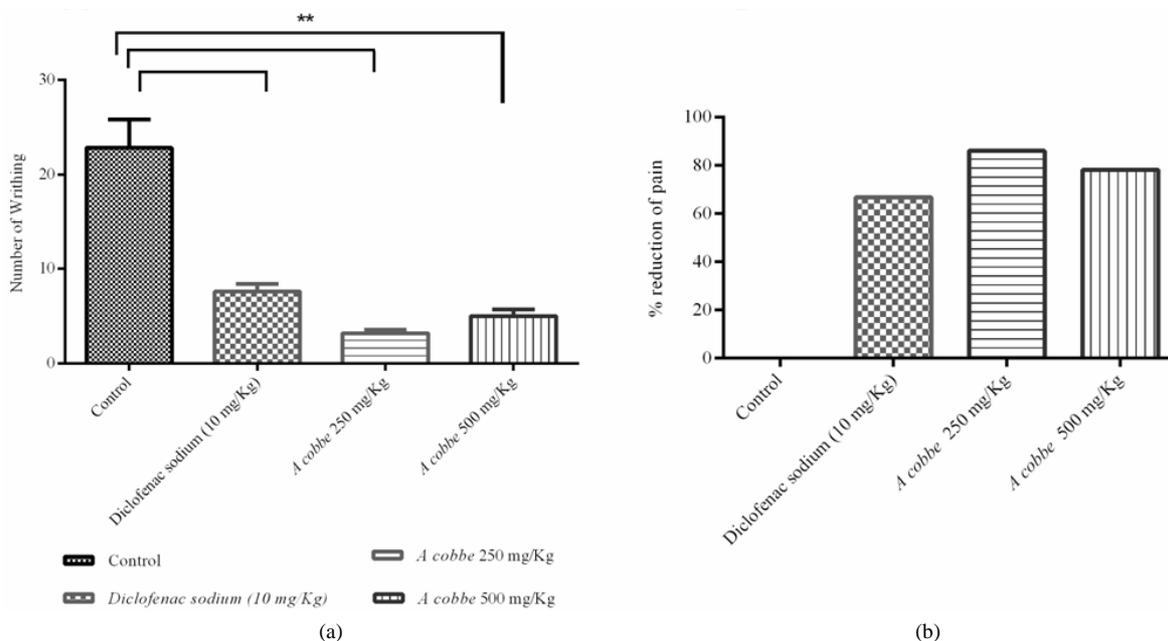


Fig. 2. Effect of the ethanol extract of *Allophylus cobbe* on acetic acid-induced writhing in mice. A) Number of writhing, B) % inhibition of pain. Data are represented as the mean ± SEM, (n = 5); **p<0.001 was considered statistically significant as compared to control

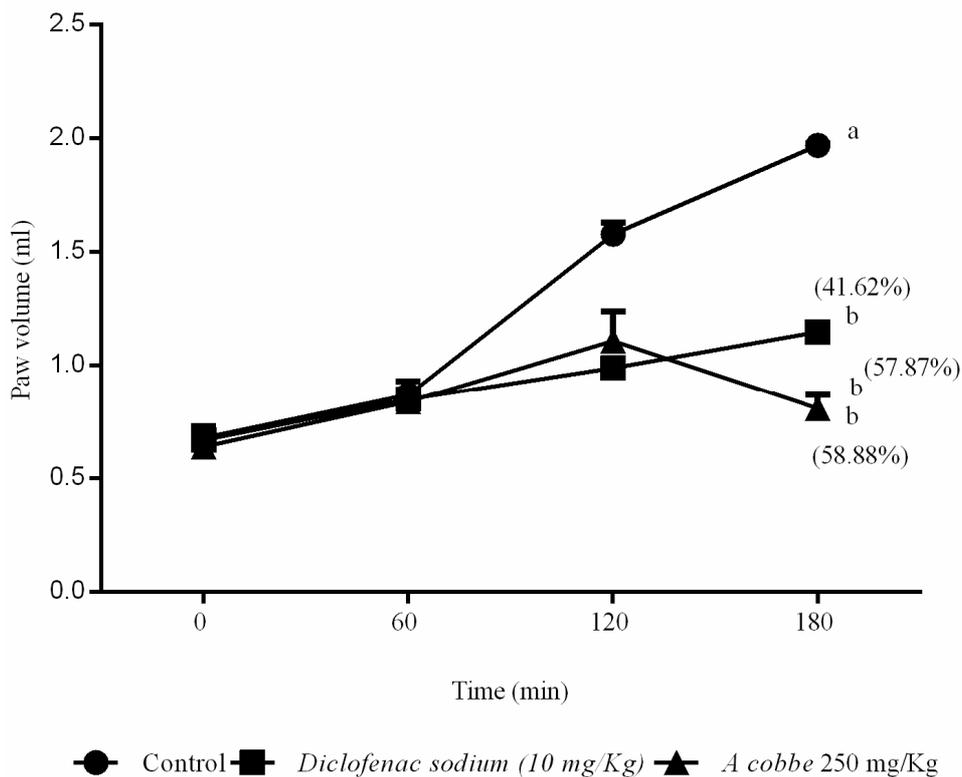


Fig. 3. Anti-inflammatory activity of ethanol extract of *Allophylus cobbe* using carrageenan-induced rat paw edema method. Data are represented as the mean \pm SEM, (n = 5); a vs b, carrageenan vs treatment, $p < 0.05$ was considered significantly different in comparison with control

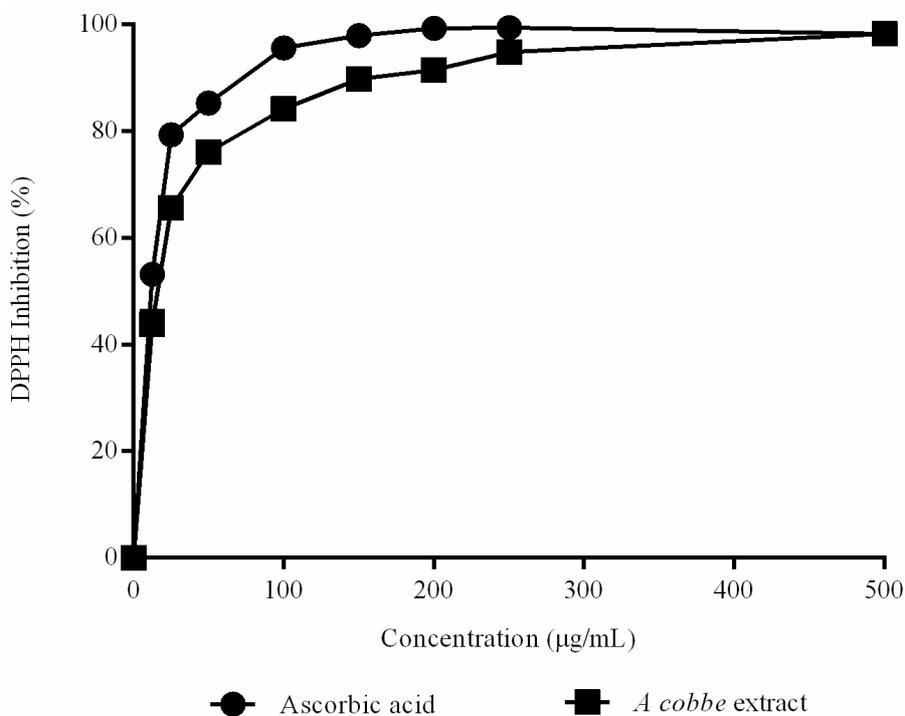


Fig. 4. DPPH radical scavenging activity of the ethanolic extract of *A. cobbe* leaf in comparison to ascorbic acid

Discussion

In this study, we reported the effect of ethanol extract of *Allophylus cobbe* on various experimental animal models of pain and inflammation. Antioxidant effect of the extract was also evaluated using DPPH method. The extract significantly inhibited the nociception produced by hot plate and writhing induced by acetic acid. The extract also significantly attenuated carrageenan-induced rat right hind paw edema. Moreover, HPLC-DAD analysis of *Allophylus cobbe* extract confirmed the presence of anti-inflammatory phenolic compounds such as catechin and epicatechin.

Earlier investigations suggested that the use of acetic acid induced writhing and hot plate tests for the evaluation of peripherally and centrally acting analgesic drugs respectively (Koster *et al.*, 1959; Williamson *et al.*, 1996). Pain induced by thermal stimulus of the hot plate is specific for centrally mediated nociception (Amabeoku and Kabatende, 2012). The ability of *A. cobbe* extract to prolong the reaction latency to thermally-induced pain in mice as observed in the hot plate test suggests central analgesic activity. The acetic acid-induced abdominal constriction method is widely used for the evaluation of peripheral antinociceptive activity (Koster *et al.*, 1959; Silva *et al.*, 2013). Generally, acetic acid causes writhing or nociception by stimulating the production of prostaglandins (Satyanarayana *et al.*, 2004). Local peritoneal receptors are postulated to be partly involved in the abdominal constriction response (Correa and Calixto, 1993; Mbiantcha *et al.*, 2011). The method has been associated with prostanoids in general, e.g., increased levels of PGE₂ and PGF_{2 α} in peritoneal fluids as well as lipoxygenase products (Duarte *et al.*, 1988; Khan *et al.*, 2010). Therefore, the results of the acetic acid-induced writhing strongly suggest that the mechanism of action of this extract is linked partly to lipoxygenases and/or cyclo-oxygenases pathways. The fact that the extract at the doses tested produced analgesia in both the nociceptive pain models further demonstrates that the extract possesses both central and peripherally mediated analgesic activities.

Carrageenan induced rat paw edema was measured for testing the anti-inflammatory activity of *Allophylus cobbe* extract. Carrageenan is a phlogistic agent which is used for testing anti-inflammatory drugs as it is non-antigenic and is devoid of apparent systemic effect (Lima *et al.*, 2007). Carrageenan model of inflammation is also said to be biphasic. Generally, in the first phase, histamine, serotonin and kinins are released and form nonphagocytic edema in the first hour while the second phase is attributed to the release of prostaglandins and lysosome enzymes in the second to the third hour (Khan *et al.*, 2009; Muhammad *et al.*, 2012). The second phase is sensitive to most clinically effective

anti-inflammatory drugs (Muhammad *et al.*, 2012). The results of present study indicate that the extract significantly inhibited the carrageenan-induced acute inflammation in the 3rd h of study and the finding was comparable to that of the standard diclofenac sodium. So, the anti-inflammatory effect of *A. cobbe* extract may be due to its suppressive action on prostaglandin, protease or lysosome synthesis or activity. The phytochemical screening of the ethanolic extract of *Allophylus cobbe* revealed the presence of alkaloids, carbohydrates, flavonoids, saponins and tannins. HPLC analysis of the extract further confirmed the presence of several flavonoids and tannins. Flavonoids, saponins and tannins have been shown to exert analgesic effect on acetic acid induced writhing test (Calixto *et al.*, 2000). Previous reports also suggest that catechins, quercetin may exhibit anti-nociceptive and anti-inflammatory activity by inhibiting the pronociceptive cytokine production (e.g., TNF- α and IL-1 β) and the oxidative imbalance mediated inflammatory pain (Maroon *et al.*, 2010; Valerio *et al.*, 2009). Hence, the flavonoids and tannins present in the extract might be responsible partly for the observed analgesic and anti-inflammatory effects.

The extract exhibited significant antioxidant activity which was determined by DPPH method. Ascorbic acid was chosen as the reference antioxidant in this study. The results suggest that *A. cobbe* leaf extract *might* serve as a potent therapeutic agent for scavenging of free radicals and the regulation of pathological conditions caused by oxidative stress. The observed antioxidant effect of the plant extract could be attributed to the presence of polyphenolic compounds (Omoruyi *et al.*, 2012). The results are in line with the previous studies which reported that the free radical scavenging activity of plant extracts correlate with the phenolic content (Omoruyi *et al.*, 2012; Jain *et al.*, 2014).

Conclusion

Our current investigation demonstrates the scientific rationale for the folkloric uses of the plant in the management of pain, inflammation and other oxidative stress associated disorders. Nevertheless, further research is needed towards isolation and identification of active principles present in the extracts which could possibly be exploited for pharmaceutical use. This will also help in elucidation of the possible mechanisms of action responsible for various pharmacological activities of this plant extract.

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Declaration of Interest

The authors declare that they do not have any conflict of interest.

Author's Contributions

Preeti Jain: Provided supervision, participated in the research design and drafting of the manuscript.

Md. Selim Hossain, Kaniz Fatema, Md. Ali Akbar, Hossain and Kafil Uddin Mazumder: Carried out the experiments.

Hemayet Hossain: Participated in experiments and analysis of data.

Md. Ashraf Alam: Performed statistical analysis and participated in manuscript preparation.

Hasan Mahmud Reza: Discussed the analyses, presentation of data and critically reviewed the manuscript. All authors read and approved the final manuscript.

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