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ANTI-INFLAMMATORY ACTIVITY, TOTAL FLAVONOIDS AND TANNINS CONTENT FROM THE ETHANOLIC EXTRACT OF *SPILANTHES PANICULATA* LEAF GROWING IN BANGLADESH

Hemayet Hossain[1*], A.F.M Shahid-Ud-Daula[2], Kamrul Hasan[2], Abdullah-Al-Mansur[1] and Muhammad Mahbubul Haq[3]

[1]BCSIR Laboratories, Dhaka, Bangladesh Council of Scientific and Industrial Research, Dr. Qudrat-E-Khuda Road, Dhaka-1205, Bangladesh
2Dept. of Pharmacy, Noakhali Science and Technology University, Noakhali-3814, Bangladesh
[3]Dept. of Pharmacy, University of Asia Pacific, Dhaka-1209, Bangladesh

***Corresponding author e-mail:** hemayethossain02@yahoo.com

ABSTRACT

The crude ethanolic extract of the leaves of *Spilanthes paniculata* Wall.ex DC (Family: Asteraceae) was evaluated for its possible anti-inflammatory activity as well as total flavonoids and tannins content growing in northeast part of Bangladesh. The anti-inflammatory activity was studied using carrageenan and histamine-induced rat paw edema test at different doses (200 and 400 mg/kg body weight) of the ethanol extract. At the dose of 400 mg/kg body weight, the extract showed a significant anti-inflammatory activity both in the carrageenan and histamine-induced oedema test models in rats showing 55.63% and 56.52% reduction in the paw volume ($P<0.01$) comparable to that produced by the standard drug indomethacin (61.27% and 63.35%) at 4h respectively. The percentage inhibition of the oedema paw volume by the 400 mg/kg body weight of the extract was also statistically significant ($P<0.05$; $P<0.01$) compared favorably with the indomethacin treated animals at 1, 2 and 3 h in both models. The total flavonoids and tannins content were calculated as quite high in ethanolic extract (112.98 mg/g of quercetin equivalent and 187.27 mg of gallic acid equivalent respectively). Acute toxicity test showed that the plant might be safe for pharmacological uses. Therefore, the obtained results tend to suggest the acute anti-inflammatory activity as well as total flavonoids and tannins content from the ethanolic extract of the leaves of *Spilanthes paniculata* and thus provide the scientific basis for the traditional uses of this plant as a remedy for toothache, pain and inflammations.

Key words: *Spilanthes paniculata*, anti-inflammatory, carrageenan, histamine, total flavonoids, total tannins

INTRODUCTION

Spilanthes paniculata (*S. paniculata*) is an important medicinal plant with rich source of therapeutic and medicinal constituents. The genus *Spilanthes* (Asteraceae) comprises 30 species and 9 additional intraspecific taxa that are mainly distributed in the tropical and subtropical regions around the world. [1] This species is famous as a folklore remedy for toothache and for throat and gum infections, earning it the English nickname, the "toothache plant". *S. paniculata* all showed larvicidal activity against Anopheles mosquitoes suggesting a possible role for *Spilanthes* in not just the treatment but also prevention of malaria. [2] *Spilanthes* contains a number of biologically active compounds, [3] of which the most studied have been the alkylamides. [4] Isolated alkylamides from *Spilanthes* have demonstrated activity against mosquito larvae. Although there are no published reports of antiplasmodial activity of isolated *Spilanthes* alkylamides, alkylamides from other plants have shown such activity. [5] Roots of *S. paniculata* release more than 90% of N, P and K within 150 day. *S. paniculata* can play a significant role in soil nutrient enrichment in poorly managed shifting cultivation systems. [6]

Chronic inflammatory diseases remain one of the world's major health problems. [7, 8] Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release, and extravasations of fluid, cell migration, tissue breakdown and repair. [9, 10] Non-steroidal anti-inflammatory drugs (NSAID) are among the most commonly prescribed drugs due to their consistent effectiveness in the treatment of pain, fever, inflammation and rheumatic disorders. However, their use is associated with adverse effects at the level of digestive tract, ranging from dyspeptic symptoms, gastrointestinal erosions and peptic ulcers to more serious complications, such as over bleeding or perforation. [11]

Therefore to overcome the toxicity of NSAID, the development of new anti-inflammatory drugs is still necessary and the natural product such as medicinal plants could lead in discovering new anti-inflammatory drugs with less undesirable effects. [12] Now-a-days attention is being focused on the investigation of the efficacy of plant-based drugs used in the traditional medicine because they are cheap, have little side effects and according to WHO, about 80% of the world population still rely mainly on herbal remedies. [13]

Since no literature is currently available to substantiate anti-inflammatory activity as well as flavonoids and tannins content from the ethanolic extract of the leaves of *Spilanthes paniculata* growing in Bangladesh, the present study was designed to provide scientific evidence for its use as a traditional folk remedy by investigating the anti-inflammatory activity as well as total flavonoids and tannins content from the ethanol extract that also confirm its use as a remedy for toothache, pain and inflammations.

MATERIALS AND METHODS

Collection and identification of plant materials: The plant (leaves) *S. paniculata* was collected at December, 2010 from Noakhali, northeast distrct of Bangladesh and was identified by Bangladesh National Herbarium, Mirpur, Dhaka (Accession number-DACB-39538).

Preparation of ethanolic extract: The leaves of *S. paniculata* were freed from any of the foreign materials. Then the plant materials were chopped and air-dried under shed temperature followed by drying in an electric oven at 40º C. The dried plant materials were then ground into powder. About 600g of powdered material was taken in a clean, flat-bottomed glass container and soaked in 1.5 liters of 80% ethanol. The container with its contents was sealed and kept for a period of 4 days accompanying occasional shaking and stirring. The ethanolic extract was filtered by Buchner funnel and the filtrate was concentrated with rotary evaporator at bath temperature not exceeding 40˚ to have gummy concentrate of greenish black extract (yield approx. 16.55%).

Test for different chemical groups: The crude ethanolic extract was tested for its different chemical groups as alkaloids, flavonoids, gums, reducing sugars, saponins, steroids and tannins. [14] In each test 10% (w/v) solution of the extract in ethanol was taken.

Experimental animals and Drug: For the screening of in vivo anti-inflammatory activity male rats of Wister strain weighing 175-202 g were used. The animals were housed under standard Laboratory (at Pharmacology Laboratory of BCSIR, Chittagong) conditions maintained at 25±1°C and under 12/12 h light/double cycle and feed with Balanced Trusty Chunts and water ad libitumn. All experimental protocols were in compliance with BCSIR Ethics Committee on Research in Animals as well as internationally accepted principles for laboratory animal use and care. The standard drug Indomethacin was used for this study and purchased from Square Pharmaceuticals Ltd, Bangladesh.

Chemicals: Tannic acid, quercetin, carrageenan, folin-ciocalteu phenol reagent and histamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tween 80, aluminium chloride, potassium acetate and sodium carbonate were of analytical grade and purchased from Merck (Darmstat, Germany).

Acute toxicity test: The acute toxicity of *S. paniculata* ethanolic extract was determined in rats according to the method of Hilaly *et al* [15] with slight modifications. Rats fasted for 16h were randomly divided into groups of five rats per group. Graded doses of the extract (200, 400, 800, 1600 and 3200 mg/kg p.o.) were separately administered to the rats in each of the groups by means of bulbed steel needle. All the animals were then allowed free access to food and water and observed over a period of 48h for signs of acute toxicity. The number of deaths within this period was recorded.

Anti-inflammatory activity

Carrageenan-induced oedema test: Carrageenan induced rat hind paw edema was used as the animal

model of acute inflammation according to the method of Lanhers *et al.* [16] In this experiment, the rats were divided into four groups of five animals each. Group I (control) received 2% Tween 80 in normal saline (2 ml/kg). Group II (Positive control) received 10 mg/kg body wt. of Indomethacin orally. Group III and IV received 200 and 400 mg/kg body wt. of the extract orally respectively. Acute inflammation was induced in all the four groups by sub plantar injection of 0.05 ml of its suspension of Carrageenan with 2% Tween 80 in normal saline in the right Paw of the rats 30 minutes after the oral administration of the tested materials. The paw volume was measured with a micrometer screw gause at 1, 2, 3 and 4h after the administration of the drug and the extract. The percentage inhibition of inflammatory effect of the extract was calculated using the following expression:

Percentage inhibition of inflammation =

$$[(V_c-V_t)/V_c] \times 100$$

Where V_c is the average degree of inflammation by the control group and V_t is the average degree of inflammation by the test group (Table-2).

Histamine-induced oedema test: Using the method of Perianayagam *et al*, [17] the paw oedema was produced by sub-plantar administration of 0.1% freshly prepared solution of histamine into the right hind paw of the rats. In this experiment, twenty rats were divided into four groups of five animals each. Group I (control) received 2% Tween 80 in normal saline (2 ml/kg). Group II (Positive control) received 10 mg/kg body wt. of Indomethacin orally. Group III and IV received 200 and 400 mg/kg body wt. of the extract orally respectively. Acute inflammation was induced in all the four groups by sub plantar injection of 0.1 ml of Histamine with 2% Tween 80 in normal saline in the right hind paw of the rats 1h after the oral administration of the tested materials. The paw volume was measured with a micrometer screw gause at 1, 2, 3 and 4h after the administration of the drug and the extract. The percentage inhibition of inflammatory effect of the extract was calculated using the same formula for carrageenan-induced paw oedema.

Total flavonoids content determination: Aluminium chloride colorimetric method was used for determination of total flavonoids concentration of the ethanol extract. [18] The extract (0.5 ml, 1:10 g ml^{-1}) in ethanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 415 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Jena, Germany). Total flavonoids content was determined as mg of Quercetin equivalent per gram using the equation obtained from a standard Quercetin calibration curve $y=4.7385x + 0.0355$; $R^2 = 0.9993$.

Total tannins content determination: The tannins were determined using the Folin-ciocalteu Phenol reagent as reported by Amorim *et al.* [19] Briefly, 0.1 ml of the sample extract was added with 7.5 ml of distilled water and added 0.5 ml of Folin-ciocalteu Phenol reagent, 1 ml of 35% sodium carbonate solution and dilute to 10 ml with distilled water. The mixture was shaken well, kept at room temperature for 30 min and absorbance was measured at 725 nm. Blank was prepared with water instead of the sample. A set of standard solutions of tannic acid is read against a blank. The results of tannins were expressed in terms of tannic acid in mg/g of dry extract. Total tannin content was determined as mg of tannic acid equivalent per gram using the equation obtained from a standard tannic acid calibration curve $y=4.5692x-0.2538$, $R^2=0.9953$.

Statistical Analysis: Data were presented as mean ± Standard deviation (S.D). Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnet's multiple comparisons using SPSS Data Editor for Windows, Version 11.5.0 (SPSS Inc., U.S.A.). The results obtained were compared with the control group. p values < 0.05 were considered to be statistically significant (p denotes probability).

RESULTS

Phytochemical screening*:* Results of different chemical tests on the ethanolic extract of *S. paniculata* leaves showed the presence of saponins, gums, tannins and significantly presence of flavonoids (Table 1).

Acute toxicity test: In acute toxicity study, oral administration of graded doses (200, 400, 800, 1600 and 3200 mg/kg p.o.) of the ethanol extract of *S. paniculata* to rats did not produce any significant changes in behaviour, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects during the observation period. No mortality or any toxic reaction was recorded in any group after 48h of administering the extract to the animals. *S. paniculata* was safe upto a dose level of 3200 mg/kg of body weight.

Anti-inflammatory activity

Carrageenan-induced paw oedema: The anti inflammation effect of the ethanolic extract of the leaves of *S. paniculata* using carrageenan induced oedema tests is expressed in (table-2). In this test, the positive control (Indomethacin) significantly ($p<0.05$; $p<0.01$) decreased the paw edema at 1h to 4h after carrageenan injection compared to saline with inhibition 55.44% to 61.27% (table-2).

A maximum oedema paw volume of 1.42±0.07 mm was observed in the control rats, 4 h after the carrageenan injection. Rats with the extract at 400 mg/kg body weight significantly decreased ($p<0.05$; $P<0.01$) the carrageenan-induced oedema paw volume from 1h to 4h compared to the standard drug indomethacin at a dose of 10 mg/kg body weight.

The inhibition percentage of the oedema paw volume by the 400 mg/kg body weight of the extract was found statistically significant when it was compared with the indomethacin treated animals at 1, 2, 3 and 4 h. The highest reduction in the paw volume by the 400 mg/kg body weight was 55.63% was comparable to that of the indomethacin (61.27%) at 4 h.

Histamine-induced paw oedema: Table 3 showed the anti-inflammation effect of the ethanolic extract of *S. paniculata* leaves using histamine-induced paw oedema tests. In the histamine-induced oedema test, a maximum oedema paw volume of 1.61 ± 0.08 mm was observed in the control rats, 4 h after the histamine injection. Rats pre-treated with the extract at 400 mg/kg body weight significantly decreased ($p<0.05$; $P<0.01$)

The histamine-induced oedema paw volume from 1h to 4 h compared to the standard drug indomethacin at a dose of 10 mg/kg body weight. The percentage inhibition of the oedema paw volume by the 400 mg/kg body weight of the extract was statistically significant ($p<0.05$; $P<0.01$) compared favorably with the indomethacin treated animals at 1, 2, 3 and 4 h. The maximum reduction in the paw volume by the 400 mg/kg body weight was 56.52% compared to the indomethacin (63.35%) at 4 h.

Total flavonoids content: The total flavonoids content was calculated as significant in ethanolic extract of *S. paniculata* 112.98 mg/g of quercetin equivalent per gm of dry extract that are shown at table 4.

Total tannin content: The total tannin content was calculated as quite high in ethanolic extract 187.27 mg/g of tannic acid equivalent that are shown at table 5.

DISCUSSION

The anti-inflammatory activity was studied using two established method namely carrageenan and histamine-induced rat paw edema test at different doses (200 and 400 mg/kg body weight) of the ethanol extract of *S. paniculata* leaf. Carrageenan-induced oedema involves the synthesis or release of mediators at the injured site. These mediators include prostaglandins, especially the E series, histamine, bradykinins, leucotrienes and serotonin, all of which also cause pain and fever. [20] Inhibitions of these mediators from reaching the injured site or from bringing out their pharmacological effects normally ameliorate the inflammation and other symptoms.

Development of oedema induced by carrageenan is commonly correlated with early exudative stage of inflammation. [21] Carrageenan oedema is a multimediated phenomenon that liberates diversity of mediators. It is believed to be biphasic; the first phase (1h) involves the release of serotonin and histamine while the second phase (over 1h) is mediated by prostaglandins, the cyclooxygenase products, and the continuity between the two phases is provided by kinins. [17] Since carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation, [22, 23] the results of this study are an indication that *S. paniculata* can be effective in acute inflammatory disorders. The extract also exibited pronounced reduction in the oedema produced by histamine. This result tends to suggest that the anti-inflammatory activity of the extract is possibly backed by its anti-histamine activity.

The antihistaminic effect of the extract increased with increase in the dose of the extract. Histamine is an important inflammation mediator, potent vasodilator substance and also increases the vascular permeability. [24, 25, 26] Since the extract effectively suppressed the oedema produced by histamine, it showed that the extract exhibited anti-inflammatory actions by inhibiting the synthesis, release or action of inflammatory mediators such as histamine, serotonin and prostaglandins. . This study has shown that the ethanol extract of the leaves of *S. paniculata* possessed a significant anti-oedematogenic effect ($P<0.01$) on paw oedema induced by carrageenan and histamine compared favorably with the standard drug (indomethacin) in treated rats.

The anti-inflammatory activity of the ethanolic extract of *S. paniculata* may also be proved due to the presence of flavonoids in a significant amount (112.98±0.08 mg quercetin equivalent per g of dry extract). Flavonoids (or bioflavonoids) are naturally occurring compounds, containing in vascular plants. These compounds have been considered to possess anti inflammatory properties, both in vitro and in vivo. [27] Numerous studies have proposed that flavonoids act through a variety mechanisms to prevent and attenuate inflammatory responses and serve as possible cardioprotective, neuroprotective and chemopreventive agents. [28]

Phytochemically, the leaves of *S. paniculata* have been also reported to yield tannins. The total tannin amount was calculated as quite high in ethanolic crude extract (187.27±0.09 mg/g of tannic acid equivalent) (table 5). Tannins are important compounds known to be potent cyclooxygenase-1 inhibitors and with anti-phlogistic activity. [29] The mechanisms of anti-inflammatory activity may be related to the anti-phlogistic action of the tannins.

Non-steroidal anti-inflammatory drugs (NSAID) such as indomethacin used in this study are known to inhibit cyclooxygenase enzymes I and II which are implicated in the production of inflammation-mediating agent prostaglandin E_2 (PGE_2) from arachidonic acid. [30, 31] Therefore, the pattern of anti-inflammatory activity exhibited by this extract was similar to that of indomethacin.

CONCLUSION

Since the plant extract reduced significantly the formation of oedema induced by carrageenan and histamine, the leaves of *S. paniculata* exhibited acute anti-inflammatory activity. The potential of the extract of *S. paniculata* as acute anti-inflammatory agent may be due to the presence of phytoconstituents like flavonoids (high amount), tannins etc and might be responsible for its activity. Again, no mortality was recorded in the acute toxicity test; it showed that the plant might be safe for use. Therefore, it can be revealed that the ethanolic extract of *S. paniculata* leaves possess acute anti-inflammatory activity and justify its use as a traditional folk remedy for toothache, pain etc. However, a more extensive study is necessary to determine the exact mechanism(s) of action of the extract and its active compound(s).

Table 1: Results of different group tests of ethanolic extract of *S. paniculata* leaves.

Plant Extract	Alkaloid	Reducing Sugars	Tannins	Gums	Flavonoids	Saponin	Steroid
EE	-	-	+	+	++	+	-

EE: Ethanol extract of S. paniculata; +: Positive result; - : Negative result; ++: significantly positive

Table 2: Effect of ethanol extract of *S. paniculata* leaves and indomethacin on carrageenan-induced oedema paw volume in male Wistar rats

Treatment Groups	Doses (mg/kg body weight)	Right hind paw volume (mm)			
		1 h	2 h	3 h	4 h
Control	2 ml/kg (2% tween 80 in normal saline)	1.01±0.08	1.25±0.06	1.36±0.09	1.42±0.07
Positive Control (Indomethacin)	10	0.45±0.06* (55.44)	0.52±0.05** (58.40)	0.54±0.08* (60.29)	0.55±0.06** (61.27)
Extract	200	0.90±0.09* (10.89)	0.99±0.07** (20.80)	1.01±0.08** (25.73)	1.05±0.04* (26.06)
Extract	400	0.51 ± 0.09* (49.50)	0.60 ± 0.06** (52.00)	0.61 ± 0.05** (55.14)	0.63 ± 0.08** (55.63)

Values in brackets denote percentage inhibition of the oedema paw volume.

*Values are expressed as mean±SD; Values are calculated as compared to control using one way-ANOVA followed by Dunnet's Test; * indicates P < 0.05; ** indicates P < 0.01 vs. control; n = 5.*

Table 3: Effect of ethanol extract of *S. paniculata* leaf and indomethacin (standard drug) on histamine-induced oedema paw volume in male Wistar rats

Treatment Groups	Doses (mg/kg body weight)	Right hind paw volume (mm)			
		1 h	2 h	3 h	4 h
Control	2 ml/kg (2% tween 80 in normal saline)	1.07±0.07	1.29±0.09	1.38±0.09	1.61±0.08
Positive Control (Indomethacin)	10	0.47±0.05* (56.07)	0.55±0.07* (57.36)	0.56±0.05** (59.42)	0.59±0.08** (63.35)
Extract	200	0.95±0.07* (11.21)	1.01±0.8* (21.71)	1.08±0.06* (21.74)	1.05±0.06* (34.78)
Extract	400	0.52 ± 0.06* (51.40)	0.62 ± 0.09* (51.93)	0.64 ± 0.09** (53.62)	0.70 ± 0.07** (56.52)

Values in brackets denote percentage inhibition of the oedema paw volume.

*Values are expressed as mean±SD; Values are calculated as compared to control using one way-ANOVA followed by Dunnet's Test; * indicates P < 0.05; ** indicates P < 0.01 vs. control; n = 5.*

Table 4: Total flavonoid content determination of ethanol extract of *S. paniculata* leaf.

Extract	Avg. absorbance at 415 nm	Total flavonoid content
		mg of quercetin equivalent (QE) per gm of dry extract
Ethanol extract of *S. paniculata* leaf	0.602±0.09	112.98±0.08

The values are expressed as mean ± standard deviation (n=3).

Table 5: Total tannin content of ethanol extract of *S. paniculata* leaf.

Extract	Avg. absorbance at 725 nm	Total tannin content
		mg of tannic acid equivalent (TAE) per gm of dry extract
Ethanol extract of *S. paniculata* leaf	0.684±0.05	187.27±0.09

The values are expressed as mean ± standard deviation (n=3).

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