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Analgesic and Antioxidant Activities of the Methanolic Extract of *Spilanthes calva* (D.C) Leaves in Male Rats

[1]M.D. Sekendar Ali, [2]M.D. Razibul Habib, [1]M.D. Rafikul Islam,
[1]M.D. Saiful Islam, [1]M.D. Mominur Rahman, [3]Nahid Hasan

[1]Department of Pharmacy, International Islamic University, Chittagong, Bangladesh
[2]Department of Pharmacy, East West University, Dhaka, Bangladesh
[3]Departement of Pharmacy, Jahangirnagar University, Dhaka, Bangladesh

Abstract: *Spilanthes calva* (D.C) is used as folk medicinal plant for the treatment of various complications. So, our present study was designed to evaluate the analgesic and antioxidant activities of methanolic extract of plant leaves in male rats. The phytochemical analysis of *Spilanthes calva* has indicated the presence of alkaloids, glycosides, saponins, steroids and terpenoids. The extract (100 and 200 mg/kg) and indomethacin (10 mg/kg) produced a significant ($P<0.01$) inhibition of the first phase in the formalin pain model, while the analgesic effect was moderately produced in the second phase. The extract also showed a dose dependent inhibition of acetic acid induced abdominal writhing. A dose dependent scavenging of DPPH radical was observed with good reducing power with the extract. In DPPH radical scavenging assay, the IC_{50} value of the extract was 419.59 µg/ml while the IC_{50} value for the reference ascorbic acid was 52.45 µg/ml and a moderate to good reducing power activity showed by the extract while increasing the dose and a total phenol content in the plant was 23.91mg/gm of gallic acid and was found to contain 73.67mg/gm of quercetin in flavonoid assay.

Key words: Analgesic · Antioxidant · *S. calva* and writhing

INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [1]. It is the most common reason for physician consultation in the United States [2]. It is a major symptom in many medical conditions and can significantly interfere with a person's quality of life and general functioning [3]. Psychological factors such as social support, hypnotic suggestion, excitement in sport or war and distraction can significantly modulate pain's intensity or unpleasantness [4-5]. Analgesic therapy is dominated by two major classes of analgesic drugs; namely opioids and non steroidal anti-inflammatory drugs (NSAIDs). Both classes of analgesic drugs produce serious side effects, such as gastrointestinal disturbances, renal damages (with NSAIDs drugs), respiratory depression and possibly dependence (with opioids) [6-7]. In this respect new compounds with improved pain management capacity and fewer side effects are being sought with urgency.

Moreover, although oxidation reactions are crucial for life, they can also be damaging. Due to oxidation the reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and free radicals such as the hydroxyl radical (•OH) and the superoxide anion (O_2^-) [8]. The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biologi-cal molecules. These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, by oxidizing DNA or proteins [9]. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms [10-11] while damage to proteins causes enzyme inhibition, denaturation and protein degradation [12].

In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell [13].

The antioxidants may also act by raising the levels of endogenous defenses by up-regulating the expression of genes encoding the enzymes such as superoxide

Corresponding Author: M.D. Sekendar Ali, Department of Pharmacy, International Islamic University, Chittagong, Bangladesh. Tel: +8801717927804.

dismutase (SOD), catalase, or glutathione peroxidase [14-15]. Currently, the possible toxicity of synthetic antioxidants has been criticized. Thus, interest in natural antioxidant, especially of plant origin, has greatly increased in recent years [16].

Spilanthes calva (D.C) belongs to the family Asteraceae, locally known as Marhatitiga is an annual herb having 60cm height; spreading plant with bicoloured, red/gold flower buds is an important medicinal plant with rich source of therapeutic constituents. It is distributed all over the Bangladesh. The roots, flower heads and whole aerial part yield a compound known as spilanthol which is a powerful stimulant, sialogogue and local anesthetic. Tincture of flowers relieves toothache and is useful in infections of throat and paralysis of the tongue [17]. Besides, oil of *S. calva* has been reported in folk medicine in the treatment of inflammation, toothache, skin diseases, purgative, diuretic, lithotriptic and dysentery [17, 18]. The plant shows potential antioxidant activity [19], antimycotic activity and anti-microbial properties [20].

Another literature review suggests that the whole plant of *S. calva* possesses antioxidant activity [21]. But there are no any scientific detailed reports of leaves as antioxidant or analgesic activity. So, our present investigation was to evaluate the antioxidant and analgesic activity of methanolic extract of leaves of *S. calva.*

MATERIAL AND METHODS

Chemicals and Drugs: DPPH (1, 1-diphenyl, 2-picryl hydrazyl), was obtained from Sigma chemical Co. USA. Ascorbic acid was obtained from SD Fine chem. Ltd. Biosar, India and potassium ferricyanide from May and Backer, Dagenham, UK. Acetic acid was obtained from Merck, Germany. Formalin was purchased from CDH, India, Tween-80 was obtained from BDH Chemicals, UK. Normal saline solution was purchased from Beximco Infusion Ltd. and indomethacin were obtained from Square Pharmaceuticals Ltd, Bangladesh. All chemicals used were of analytical reagent grade.

Plant Material: For the present investigation, the fresh leaves of *S. calva* were collected from local area of Chittagong, Bangladesh in November, 2010 and were authenticated by Dr. Sheikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Bangladesh. The leaves were dried at room temperature in shade and away from direct sunlight for 5 days and in hot air oven for 2 days.

Extraction: The dried leaves were coarsely powdered and extracted keeping 7 days with methanol. The sediments were filtered and filtrates were dried at 40ºC in an oven. The solvent was completely removed by filtering with Hartman filter paper and obtained dried crude extract which was used for investigation.

Animals: Male Wister rats (70-90gm) were collected from International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B) and were kept at the Laboratory Animal Centre, Department of Pharmacy, International Islamic University Chittagong. The animals were housed in standard cages with free access to food (standard laboratory rodent's chow) and water. The animal house temperature was maintained at 23±3°C with a 12 hr light/dark cycle. The ethical guidelines for the investigation of experimental animals were followed in all tests [22].

Phytochemical Screening: A phytochemical analysis of leaves of *S. calva* was conducted to detect alkaloids, carbohydrate glycosides, saponins, steroids and terpenoids and tannins according to the method described by Harborne (1998) [23].

Analgesic Screening: Acetic Acid Induced Writhing Assay: The analgesic activity of the samples was also studied using acetic acid-induced writhing model in rats [24]. Plant leaves extract (100 and 200 mg/kg body weight), vehicle (1% tween -80 in water) 0.1 ml/10gm body weight (b. w.) and indomethacin (10mg/kg b. w.) were administered orally 30 min. before intraperitoneal administration of 0.7% acetic acid. Then the rats were observed for specific contraction of body referred to as 'writhing' for the next 20 min. full writhing was not always accomplished by the animal, because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhes in each treated group was compared to that of a control group while indomethacin (10mg/kg b. w.) was used as a reference substance (positive control). The percent inhibition (% analgesic activity) was calculated by

$$\% \text{ inhibition} = \{(A-B)/A\} \times 100$$

Where, A= Average number of writhing of the control group; B= Average number of writhing of the test group.

Formalin Test: The analgesic activity of the drugs was determined using the formalin test described by Sharma *et al*. [24]. Control group received 5% formalin. 20 µl of 5% formalin was injected into the dorsal surface of the right hind paw 60 min after administration of extract (100 and 200 mg/kg b. w. p.o.) and indomethacin (10 mg/kg b. w. p.o.). The rats were observed for 30 min. after the injection of formalin and the amount of time spent licking the injected hind paw was recorded. The first 5 min. post formalin injection is referred to as the early phase and the period between 15 and 30 min. as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch.

Tests for Antioxidant Activity

DPPH Radical Scavenging Activity: The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca *et al*. [25]. Plant extract (0.1 ml) was added to 3 ml of a 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min. and the percentage inhibition activity was calculated from $[(A_0-A_1)/A_0]$ X100, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. The inhibition curves were prepared and IC_{50} values were obtained by probit analysis [26].

Reducing Power: The reducing power of *S. calva* was determined according to method followed by Srinivas *et al*. [27]. Different concentrations of extract (50-250 µg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide-$K_3Fe(CN)_6$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) 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 was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference.

Determination of Total Phenol Content: The total phenol content of extracts was determined using Folin-Ciocalteu method [28]. The extracts were oxidized with Folin-Ciocalteu reagent and were neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 760 nm after 60 min. using gallic acid as standard total phenol content was expressed as mg gallic acid equivalent/gm of extract.

Determination of Total Flavonoids Content: The flavonoids content was determined using a method as described by Kumaran and Karunakaran using quercetin as a reference compound [29]. 1 mg of plant extract in methanol was mixed with 1ml aluminium trichloride in ethanol (20 mg/ml) and a drop of acetic acid and then diluted with Ethanol to 25 ml. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 1 mg of plant extract and a drop of acetic acid and then diluted to 25 ml with ethanol. The absorption of standard quercetin solution (0.5 mg/ml) in methanol was measured under the same conditions.

Statistical Analysis: Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnet's multiple comparisons. The results obtained were compared with the vehicle control group. $P^*<0.01$ were considered to be statistically significant.

RESULTS

Phytochemical Screening: Phytochemical screening of the crude extract revealed the absence of carbohydrate, tannins and presence of glycosides, steroids, saponins, terpenoids and also mixed results for alkaloids which have been shown in Table 1.

Analgesic Screening:

Acetic Acid Induced Writhing Test: Table 2 shows the effect of the methanolic extract of leaves of *S. calva* on acetic acid-induced writhing in rats. Both doses of extract of *S. calva* showed significant reduction ($p<0.01$) of writhing induced by the acetic acid after oral administration in a dose dependant manner.

After oral administration of two different doses (100 and 200 mg/kg body weight) of the extract and standard drug indomethacin (10 mg/kg body weight), the percent inhibitions were 58.87%, 30.12% and 55.18%, respectively. The plant extract at the dose 200 mg/kg body weight was found more potent.

Formalin test: The methanol extract of leaves of *S. calva* (100 and 200 mg/kg, p.o.) significantly suppressed the licking activity in either phase of the formalin-induced pain in rats (Table 3) in a dose dependant manner. The reference analgesic drug indomethacin (10 mg/kg) also significantly inhibited the licking activity against both phases of formalin-induced nociception. But the extract of *S. calva* at the dose of 200 mg/kg body weight showed the more licking activity against late phase of formalin-induced pain.

Table 1: Phytochemical screening of the crude methanol leaves extract of *S. calva*

Extract	Carbohydrate	Terpenoids	Glycosides	Saponins	Tannins	Alkaloids	Steroids
S. calva	-	+	+	+	-	±	+

+ indicates presence, - indicates absence and ± indicates mixed result.

Table 2: Analgesic activity of the extract of *S. calva* on acetic acid induced writhing method in rats

Groups	No. of writhing	Percent inhibition
Group-I (Control)	26.33±1.366	-
Group-II (Standard)	10.83±2.994*	58.87
Group-III(Extract-100)	18.4± 2.07*	30.12
Group-IV(Extract-200)	11.8±3.493*	55.18

All values are expressed as mean ± SD (n=6); One way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.01, significant compared to control.

Table 3: Effect of the extract of leaves of *S. calva* on hind paw licking in the formalin test in rats

Groups	Early phase (Sec)	Late phase (Sec)
Group-I(Control)	35.67±3.386	46±2.53
Group-II (Standard)	16.83±2.229*	21.833±1.722*
Group-III (Extract-100)	18.2±1.92*	28.2±4.82*
Group-IV (Extract-200)	14.6±3.21*	22.6±2.88*

All values are expressed as mean ± STD (n=6); One way Analysis of Variance (ANOVA) followed by Dunnet's test. *P<0.01, significant compared to control.

Table 4: Total amount of phenol, flavonoid content of methanolic extract of *S. calva* leaves

Extract	Total phenol(mg/gm, Gallic acid equivalent)	Total flavonoid (mg/gm, quercitn equivalent)
S. calva	23.91	73.67

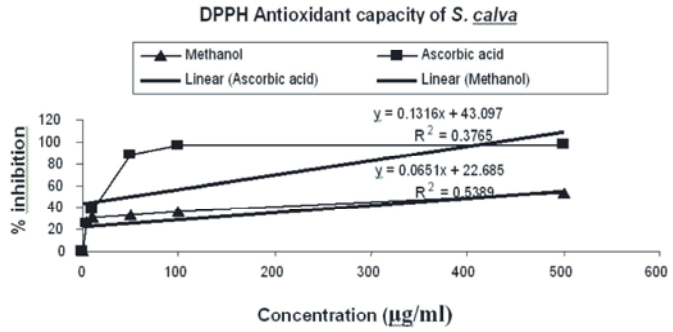

Fig. 1: DPPH radical scavenging activity of the methanol extract of S. calva. Values are the average of duplicate experiments and represented as mean ± SD.

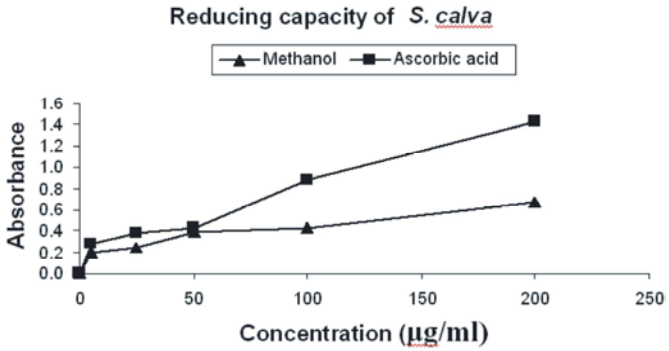

Fig. 2: Reducing power of the methanol extract S. calva Values are the average of duplicate experiments and represented as mean ± SD

Antioxidant Screening

Dpph Radical Scavenging Activity: The DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *S. calva is* shown in Fig. 1. This activity was found to increase with increasing concentration of the extract. DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quanti-tatively measured from the changes in absorbance. The IC_{50} value of the extract was 419.59µg/ml as opposed to that of ascorbic acid (IC_{50} 52.45 µg/ml) which is a well known antioxidant.

Reducing Power: The methanol extract displayed remarkable reducing power which was found to rise with increasing concentration as shown in Fig. 2.

DISCUSSION

The two different doses (100 and 200 mg/kg b. w.) of crude extract of the plant showed significant analgesic action compared to the reference drug indomethacin but higher dose (200 mg/kg) was found to exhibit higher analgesic activity against acetic acid induced pain in rats. Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipid [30] via cyclooxygenase (COX) and prostaglandin biosynthesis [31]. In other words, the acetic acid induced writhing has been associated with increased level of PGE-2 and PGF-2α in peritoneal fluids as well as lipoxygenase products [32]. In this study, the extract caused a dose-dependent decrease in licking time (Table 3) by the rats injected with formalin signifying the analgesic effect of the extract. Preliminary phytochemical screening reveals the presence of flavonoid, steroid, alkaloid, terpeniods, glycosides and saponin in the plant extract. So, the observed analgesic activity may be attributed to these compounds. Moreover, it was suggested that the inflammatory tissue damage is due to the liberation of reactive oxygen species form phagocytes invading the inflammation sites [33]. There are also reports on the role of flavonoid, a powerful antioxidant [34]. Again the plant extract demonstrated good antioxidant action in the tested models. So it can be assumed that cyclooxygenase (COX) inhibitory activity together with antioxidant activity may reduce the production of free arachidonic acid from phospholipid or may inhibit the enzyme system responsible for the synthesis of prostaglandins and ultimately relive pain-sensation in analgesic activity primarily by targeting prostaglandins.

Naturally antioxidant present in many plants, foods and beverages offer health benefits in preventing various diseases by fighting cellular damage caused by free radicals in the body [35]. Due to the presence of flavonoids, steroids and alkaloid in the plant extracts, indicates having antioxidant activity. Furthermore, recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species form phagocytes invading the inflammation sites [36]. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom and may have great relevance in the prevention and treatment of diseases associated with oxidants or free radicals.

CONCLUSION

On the basis of the results, we conclude that the methanolic extract of leaves of *S. calva* possesses strong analgesic and antioxidant potential. However, these studies are preliminary in nature and there are plenty of scopes for further studies to examine underlying mechanisms of analgesic and antioxidant effects and to isolate the active compound (s) responsible for these pharmacological activities which can result in development of a novel compound for drug discovery.

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