

## Original Article

**Biological investigation of ethanolic extract of *Spilanthes calva* DC**

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**Abstract**

Phytochemical evaluation of the ethanolic extracts of *Spilanthes calva* DC indicated the presence of alkaloids, steroids, tannins, and gums. The pharmacological interests of these compounds, coupled with the use of this plant in traditional medicine tended to investigate the plant extracts for possible analgesic, antidiarrheal and anti-oxidant activities. The plant extracts exhibited significant inhibition of writhing reflex 59.32% ( $P < 0.001$ ) at a dose of  $500 \text{ mg kg}^{-1}$  bdwt while the standard drug diclofenac sodium showed 75.52% ( $P < 0.0005$ ) at a dose of  $25 \text{ mg kg}^{-1}$  bdwt. In the castor oil-induced diarrhoeal mice, the plant extracts significantly increased the latent period ( $P < 0.0001$ ) at a dose of  $500 \text{ mg kg}^{-1}$  bdwt compared to standard drug Loperamide ( $P < 0.0001$ ). It also significantly reduced the number of stools at the doses of 250 ( $P < 0.01$ ) and  $500 \text{ mg kg}^{-1}$  bdwt ( $P < 0.001$ ) comparing with standard drug Loperamide ( $P < 0.0001$ ). By spraying 0.02% DPPH solution of ethanol on TLC plate which developed in non-polar, medium polar and polar medium, the extracts showed yellow spot that indicated the presence of anti-oxidant components. The  $\text{IC}_{50}$  for ascorbic acid was approximately  $1 \mu\text{g/ml}$  whereas the plant extracts showed  $500 \mu\text{g/ml}$ . Our results show that the plant extract has potent analgesic, antidiarrheal and anti-oxidant activities.

**Keywords:** *Spilanthes calva*, antioxidant, antidiarrheal, analgesic.

**1. Introduction**

*Spilanthes calva* DC is an annual herb, stem usually glabrous and commonly named as tooth paste plant. It belongs to the Asteraceae family, usually grows in open sunny places in jhums, sandstones, dry soil, riversides, waste places and widely found in Bangladesh, Brazil, West Indies, China, India, South Asia, Thailand, Malaysia, New Caledonia and tropical Africa. In recent years, *S. calva* has been implicated as a potential medicinal herb used for oral health care, non-toxic to human beings and enhances the immune system.

*S. calva* is a rich source of bioactive compounds. The most active biomolecule is spilanthol (Yasuda *et al.* 1980).

Several reports have suggested that aerial parts of the plants contain palmitic and stearic acids, tetra-triacontanoic acid, sitosterol, stigmasterol and sitosterol glucoside (Jeripa and Chowdhury 2008). In addition, heads of flower contain a local anaesthetic amide, spilanthol, sterol and non-reducing polysaccharide. It has been reported that the plant is used in the treatment of allergy, foot mud sore, hysteria, insanity, stomachache, toothache, traumatic paralysis, rheumatism and scabies. However, one study showed that it can possess  $\alpha$ - and  $\beta$ -amyryn esters and sitosterol glucoside. The roots, flower heads and whole aerial part of *S. calva* produces spilanthol which can be used for the local anesthetic properties (Ghani 2003). The essential oil of fresh plant of *S. calva* displayed more than 45 components including (E)-2-hexenol, 2-tridecanone, germacrene D, hexanol,  $\beta$ -caryophyllene

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and (Z)-3-hexenol as significantly dominating compounds. A system for biocontrol of malaria and filarial mosquito vectors has been developed using crude hexane extract obtained from flowering heads of *Spilanthes* spp. Cent percent mortalities was achieved against the late third/early fourth instar larvae of *Anopheles stephensi* Liston, *A. culicifacies* species and *C. quinquefasciatus* using minimal doses of the plant extract (Pandey *et al.* 2007).

In recent years, antifungal activity has been investigated using aqueous and petroleum ether extracts of *S. calva* against *Fusarium oxysporum* and *Trichophyton mentagrophytes*. However, the petroleum ether extract of *S. calva* was found more effective than the aqueous extract in inoculated as well as un-inoculated plants. Moreover, it suggested that the antifungal activity was enhanced due to the increase in spilanthol content after inoculation of *P. indica* (Rai *et al.* 2004).

From the literature survey, we found that no scientifically elegant study supporting traditional uses of the plant has yet been reported. Therefore, to judge the traditional use of this medicinal plant, the present study was undertaken to evaluate the anti-oxidant, analgesic, anti-diarrhoeal effects of *S. calva*.

## 2. Materials and methods

**2.1. Collection and Identification:** The plant *S. calva* was collected from Khulna University, Bangladesh, during the month of 14th December, 2009 on the day time. The plant was identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka (Accession no: DACB - 34992) and a voucher specimen was also deposited there.

**2.2. Preparation of plant extract:** The collected plant parts were separated from undesirable materials or plants and air dried for three weeks. The plant parts were ground into a fine powder by using grinder (Capacitor start motor, Wuhu motor factory, China). The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced. A glass jar with plastic cover was taken and washed thoroughly. The jar was rinsed with ethanol and dried. Then, 150gm of the dried powder was taken in the jar. After that 95% ethanol (750 ml) was poured into the jar up to 1 inch height above the sample surface as it can sufficiently cover the sample surface. Aluminum foil was used to resist the entrance of air into the jar. This process was performed for 8 days. The jar was shaken and stirred several times during the process to get better extraction. After the extraction process, the extract was filtered by a piece of clean, white cotton material. Then, it was filtered through Whatman filter paper. The filtrate was collected in a beaker. The filtrate (ethanolic extracts) obtained was evaporated by rotary evaporator

and after that, it was kept under ceiling fan to evaporate the ethanol completely. It rendered a gummy concentrate (5.2 gm) of greenish black color. The gummy concentrate was designated as crude extract of ethanol. The yield value was almost 3.46%.

**2.3. Antidiarrheal activity test:** To prepare suspension of the test samples at the doses of 250 and 500 mg/kg per body weight, 250 and 500 mg of ethanolic extract were measured respectively. The extract was triturated in unidirectional manner by the addition of small amount of tween-80. After proper mixing of extract and tween-80, the distilled water was slowly added. The final volume of the suspensions was made 10.0 ml. For the preparation of standard drug, 3 mg of Loperamide was taken and triturated in unidirectional manner by the addition of small amount of tween-80. After proper mixing, distilled water was slowly added up to the final volume 10ml. The amount to be administered to get desired concentration = (Body weight of mice  $\times$  0.01) ml. For the test, fresh mice were randomly chosen from the animal laboratory of Khulna University, Khulna, Bangladesh and divided into four groups having five mice in each. Of the experimental groups, group-I or the control received only distilled water. Group-II or the positive control received standard anti-motility drug, Loperamide at a dose of 3 mg/kg-body weight as oral suspension. The test groups were treated with suspension of ethanolic extracts of *S. calva* DC at the oral dose of 250 mg/kg-body weight and 500 mg/kg-body weight. The mice were fed with the samples for 40 minutes (for proper absorption) prior to the oral administration of castor oil. After that, the mice were fed castor oil at a dose of 0.5 per ml. Individual animal of each group were placed in separate cages having adsorbent paper beneath and examined for the latent period and after that, diarrheal symptom was observed for every four hours. Number of stools or any fluid material that stained the adsorbent paper were counted at each successive hour during the 4-hour period and were noted for each mouse. At the beginning of each hour, new papers were placed for the old ones.

**2.4. In vitro Anti-oxidant Activity test (Qualitative and Quantitative analysis):** **Qualitative test:** A small amount of ethanolic extract of the plant was dissolved in ethanol and diluted suitably and was applied on the two different TLC plate by spotter. A small amount of ascorbic acid was also dissolved in ethanol and diluted suitably and was applied on the former TLC plate by spotter at the same way. The plate was then kept in a TLC jar containing a solvent system. About 5 minutes later, spotted TLC plate was kept into the jar dipping into the solvent system. When the solvent system reached at the desired level then, it was removed from TLC jar and was subjected for air drying. At first, two

chromatograms were developed into the solvent system of n-hexane and Acetone (2:1). This is a non-polar solvent system. After drying, chromatograms were observed under ultraviolet (UV) detector at longer wavelength (306 nm) and shorter wavelength (256 nm) and colored spots were marked by pencil. Spot which was observed into longer wavelength was manifested by the symbol ( ) and spot which was observed into shorter wavelength was evident by the symbol < >. Then, one of the chromatograms were taken and 0.02 % DPPH solution of ethanol was sprayed on it by a spray gun. Yellow color was formed on the chromatogram. Other chromatogram was treated with 10% H<sub>2</sub>SO<sub>4</sub> at the same way. Then, the plate was heated on hot plate and was developed. Then, the sample was compared with standard. Another two chromatograms were developed into the solvent system of Chloroform, Methanol and Water (40:10:1) and also two chromatogram were developed using the solvent system Chloroform and Methanol (5:1). This is polar solvent system. Then, by the similar procedure described above was applied to compare the sample with standard.

## 2.5. Quantitative analysis

**2.5.1 DPPH radical scavenging assay:** At first, 6 test tubes were taken to make aliquots of 6 conc. (1, 5, 10, 50, 100, 500 µg/ml). Plant extract and ascorbic acid were weighed 2 times and dissolved in ethanol to make the required concentrations by dilution technique. Here ascorbic acid was taken as '+ve' control. DPPH was weighed and dissolved in ethanol to make 0.004% (w/v) solution. To dissolve homogeneously, magnetic stirrer was used. After making the desired concentrations, 1 ml plant extracts or standard of different concentration solution were taken into different test tubes and 3 ml of reagent solution was added into each of the test tubes. The test tubes were kept for 30 minutes in at room temperature in the light to complete the reactions. DPPH was also taken as blank in a test tube. After 30 minutes, absorbances of each test tube were determined by UV spectrophotometer at 517 nm. IC<sub>50</sub> was determined from % inhibition vs concentration graph. The formula used for % inhibition ratio was % inhibition = (Blank OD - Sample OD / Blank OD) × 100.

**2.5.2 Posphomolybdenum method:** 0.6 ml plant extracts or standard of different concentration solution were taken into different test tubes and 6 ml of reagent solution was added into each of the test tubes. The test tubes were incubated at 95°C for 90 minutes to complete the reaction. The absorbance of the solutions was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. A typical blank solution contained 6 ml of reagent solution and the appropriate volume (0.6 ml) of the same solvent for the sample was incubated under the same condition.

The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

**2.6. Analgesic activity test:** Young Swiss-albino mice aged 4-5 weeks; average weight ranging from 20-28 gm were used for the experiment. The mice were purchased from the Animal Research Branch of the International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR). They were kept in standard environmental condition for one week in the animal house of Pharmacy Discipline, Khulna University, Bangladesh for adaptation after their purchase. Experimental animals were randomly selected and divided into four groups denoted as group-I, group-II, group-III, group-IV consisting of 5 mice in each group. Each group received a particular treatment i.e. control, positive control and the two doses of the extract. Each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. Test samples, control and Diclofenac sodium were given orally by means of a feeding needle. A thirty minutes interval was given to ensure proper absorption of the administered substances. Next, the writhing inducing chemical, acetic acid solution (0.7%, 10ml/kg) was administered intraperitoneally to each of the animals of a group. After an interval of five minutes which was given for absorption of acetic acid, number of squirms (writhing) was counted for 15 minutes.

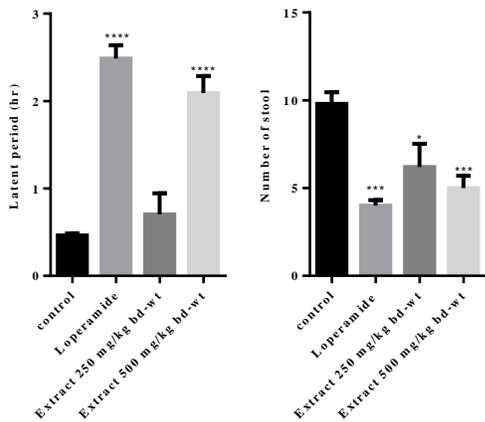
## 3. Results

**3.1. Phytochemical test:** Ethanolic extracts of *S. calva* showed the presence of the functional group tannin, alkaloid, steroid, gum and showed the absence of reducing sugar, flavonoid and saponin group.

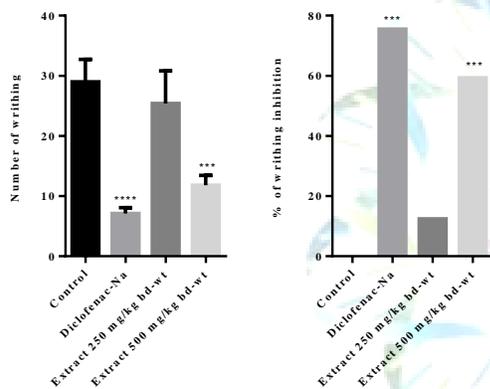
**3.2. Antidiarrheal activity test :** In the castor oil-induced diarrhoeal mice, the plant extracts significantly increased the latent period ( $P < 0.0001$ ) at a dose of 500 mgkg<sup>-1</sup> bdwt compared to standard drug Loperamide ( $P < 0.0001$ ).

It also significantly reduced the number of stools at the doses of 250 ( $P < 0.01$ ) and 500 mgkg<sup>-1</sup> bdwt ( $P < 0.001$ ) comparing with standard drug Loperamide ( $P < 0.0001$ ) (Fig 1).

**3.3. Analgesic activity test:** Ethanolic extracts of *S calva* exhibited significant inhibition of writhing reflex 59.32% ( $P < 0.001$ ) at a dose of 500 mgkg<sup>-1</sup>-bdwt while the standard drug diclofenac inhibition was found to be 75.52% ( $P < 0.0005$ ) at a dose of 25 mgkg<sup>-1</sup>-bdwt (Fig 2).



**Fig 1** Effects of ethanolic extracts of *S. calva* DC on the latent period and number of stool in castor oil-induced diarrheal mice model. N=5; \*p< 0.01, \*\*p<0.001, \*\*\*\* P<0.0001.



**Fig 2** Effects of ethanolic extracts of *S. Calva* DC on acetic acid induced writhing in mice. n=5; \*\*p<0.001, \*\*\* P<0.0005.

**Table 1** Total antioxidant capacity of *Spilanthes calva* DC Vs Ascorbic acid.

Concentration (µg/ml)	Mean absorbance of Extract	Mean absorbance of Ascorbic acid	Number of Equivalent
5	0.0595	0.110	0.540
25	0.0600	0.143	0.419
50	0.0685	0.236	0.290
100	0.0850	0.444	0.191
200	0.1145	0.730	0.156
500	0.2210	1.5895	0.139

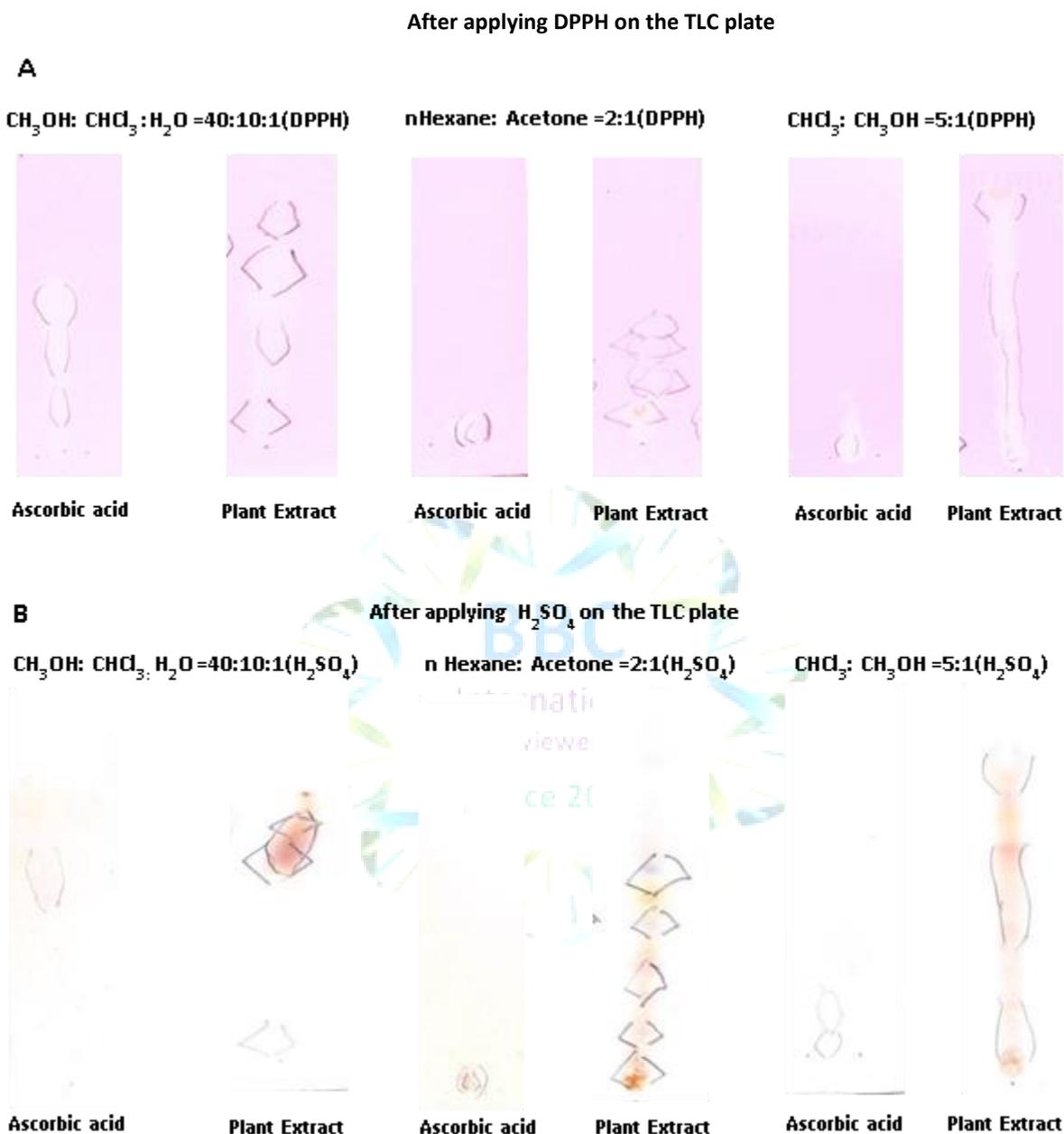
Number of Equivalent = Absorbance of Extract/Absorbance of Ascorbic acid.

**3.4 Anti-oxidant Activity:** Yellow color was formed which indicates the antioxidant property of the ethanolic extracts of *S calva*. The total antioxidant capacity by the extract demonstrated was concentration dependent. The extract was likely to have the capacity reduction of Mo (VI) to Mo (V) by the antioxidant compound (**Fig 3 and Table 1**).

#### 4. Discussion

The phytochemical evaluation showed the presence of alkaloids, steroids, tannin and gums compounds in extract of *S. calva*. Our results also showed that the ethanolic extracts of *S. calva* have potent analgesic activity. Traditionally *S. calva* has been used in the treatment of toothache (Ghani 2003). The plant contains  $\alpha$ - and  $\beta$ -amyrin esters and sitosterol glucoside. The mixture of the two pentacyclic triterpenes,  $\alpha$ -amyrin and  $\beta$ -amyrin given by intraperitoneal (i.p.) or oral (p.o.) routes causes dose-related and significant antinociception against the visceral pain in mice produced by i.p. injection of acetic acid. Moreover, intracerebroventricular (i.c.v.), or intrathecal (i.t.) administration of  $\alpha$ ,  $\beta$ -amyrin inhibited both neurogenic and inflammatory phases of the overt nociception caused by intraplantar (i.p.) injection of formalin. The antinociception caused by the mixture of compounds seems to involve mechanisms independent of opioid,  $\alpha$ -adrenergic, serotonergic, and nitrenergic system mediation since it is not affected by naloxone, prazosin, yohimbine, dl-p-chlorophenylalanine methyl ester or l-arginine (Firoz *et al.* 2007). Interestingly, the i.p. administration of  $\alpha$ ,  $\beta$ -amyrin reduces the mechanical hyperalgesia produced by i.pl injection of carrageenan, capsaicin, bradykinin, substance P, prostaglandin E2, 8-Br-cAMP and TPA in rats. However, the mixture of compounds failed to alter the binding sites of [3H] bradykinin, [3H] resiniferatoxin or [3H] glutamate *in vitro*. It is concluded that the mixture of triterpene  $\alpha$ -amyrin and  $\beta$ -amyrin produced consistent peripheral, spinal and supraspinal antinociception in rodents, especially when assessed in inflammatory models of pain. The mechanisms involved in their action are not completely understood but seem to involve the inhibition of protein kinase A- and protein kinase C-sensitive pathways. Therefore, further investigations are necessary to find out the active constituents responsible for this effect.

Our result also demonstrated that the ethanolic extracts of *S. calva* possess potent anti-diarrheal activity. The antidiarrhoeal property of the ethanolic extract of *S. calva* found in the present study could be owing to the presence of indole alkaloids, secoiridoids, triterpenes and saponins in this plant. Previous studies showed that antidiarrhoeal and antidiarrhoeal properties of medicinal



**Fig 3 Antioxidant effects of ethanolic extracts o *Spilanthes Calva* DC.**

plants were mostly due to tannins, alkaloids, saponins, flavonoids, sterol and triterpenes (Uddin *et al.* 2005, Longanga *et al.* 2000). Further study is needed to isolate the active constituents responsible for this effect.

From the qualitative and quantitative analysis, we demonstrated that the ethanolic extracts of *S. calva* possess significant anti-oxidant activity. The inhibition

(%) of free radicals in DPPH Scavenging method and no of equivalent of ascorbic acid neutralizing effect in Posphomolybdenum method displayed significant antioxidant effect. The IC50 (inhibitory conc. 50%) for ascorbic acid was about 1  $\mu\text{g}/\text{ml}$  and for the sample it was about 500  $\mu\text{g}/\text{ml}$ . It is now well established that free radicals (e.g. super oxide, hydroxyl radical, and

nitric oxide) and other reactive species (e.g. hydrogen peroxide, single oxygen, hypochlorous acid) contribute to the pathology of many disorders including atherogenesis, neurodegeneration, chronic inflammation and function of the immune system. Recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species from phagocytes invading the inflammation site (Alam *et al.* 2008, Badmus *et al.* 2010). Therefore, the substance which relieves pain or inflammation may act by neutralizing (anti-oxidant) the oxidative group present in the inflammatory site.

## 5. Conclusion

Our observation indicates that the ethanolic extracts of *S. calva* possess potent analgesic, anti-diarrheal and anti-oxidant activities. Therefore, *S. calva* could be a potential source of compounds for analgesic, anti-oxidant and antidiarrhoeal activities.

## 6. Acknowledgement

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## 7. Conflict of interest statement

We declare that we have no conflict of interest.

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