IJPSR (2018), Volume 9, Issue 10 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 15 January, 2018; received in revised form, 01 June, 2018; accepted, 08 June, 2018; published 01 October, 2018

PHARMACOLOGICAL INVESTIGATIONS OF CHLOROFORM EXTRACT OF *STEREOSPERMUM CHELONOIDES* LEAVES

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Keywords:

Stereospermum chelonoides, Antioxidants, DPPH, MTT, Anti-bacterial activity, Anti-inflammatory

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ABSTRACT: Oxidative stress is involved in the pathogenesis of various degenerative diseases like cancer, inflammation *etc*. Thus, the study of antioxidant activity of natural molecules has remained a fertile ground for the researchers. In continuation of that effort, the present study was designed to evaluate the antioxidant, cytotoxic, antibacterial and anti-inflammatory potential of chloroform extract of *Stereospermum chelonoides* leaves (SCLC). Preliminary phytochemical analysis followed by total phenol and total flavonoid determination assays were carried out. Antioxidant potential was evaluated using DPPH radical scavenging assay, total antioxidant capacity, ferric reducing antioxidant power and cupric reducing antioxidant assays. Brine shrimp lethality bioassay and MTT based cytotoxicity assay was also conducted. Antibacterial assay was carried out using disc diffusion method on both gram-positive and gram-negative bacteria. Croton oil and xylene induced ear oedema models were used to determine anti-inflammatory activity. Phytochemical screening ensured the presence of various secondary metabolites notably polyphenols (38.04 ± 0.77 mg/g, GAE) and flavonoids (130 ± 7.86, mg/g, QE) which explained its dose dependent antioxidant activities. Moreover, cupric ion reducing capacity of SCLC was as similar as standard ascorbic acid indicating strong metal chelating activity. In cytotoxicity assay, SCLC produced significant cell killing effect on hela cells in high doses. Significant anti-inflammatory activity was observed in croton oil induced ear edema. Antioxidant activity of SCLC is suggestive of its ability to halt free radical generation process and its subsequent potential in the physiologic conditions adversely affected by radical generations.

INTRODUCTION: Oxidation is body's natural process of producing energy which generates Reactive Oxygen Species (ROS) thereby. In low concentration, they trigger cell signalling pathways like cell proliferation, apoptosis *etc*.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(10).4256-66

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(10).4256-66

These ROS generated during metabolism get scavenged by an ingenious defence mechanism comprised of antioxidant enzymes (*i.e*. superoxide dismutase, peroxidase, xanthine oxidase *etc*.) and low molecular weight antioxidants (*i.e*. glutathiones, tocopherols, ascorbic acids *etc*.). But when this balance of producing and eliminating ROS is disturbed, ROS get accumulated in body and develop oxidative stress. Oxidative stress has strong links to the processes where ROS damage various cellular macromolecules like DNA, protein and lipids.

These processes remains silent yet can be few of the vital reasons of developing various diseases like Parkinson's diseases, atherosclerosis, aging, immune suppression, ischemic heart disease, diabetes, cancer, inflammation *etc*. To find and characterize potential compounds which can be used in these degenerative diseases safely and efficiently, nature comes with secondary metabolites particularly flavonoids and poly-phenolic compounds. Another pivotal feature of these natural metabolites is their structural diversities which also allow them to render versatile biological functions like antioxidant, antimicrobial, cytotoxic, anti-inflammatory properties *etc*. as well [1, 2, 3].

Being a member of Bignoniaceae family, *Stereospermum chelonoides* (locally known as 'Paruli') has traditionally been used in various diseases like bilious diarrhoea, burning sensation, sprain, rheumatism, leukemia, tuberculosis etc. This plant has also been reported to contain lapachol, dinatin, dinatin-7-glucuroniside, beta-sitosterol, stereochenol A, sterechenol B along with naphthoquinones, sterekunthal B and sterequinone C [4]. Methanol extract of the bark and leaves also possesses good antioxidant property [5]. However, there is no scientific evidence regarding the chloroform extract of *S. chelonoides* leaves. Keeping the potential antioxidant activity of methanol extract in mind, the present study was designed to evaluate the antioxidant, cytotoxic, antibacterial and anti-inflammatory potential of SCLC.

MATERIALS AND METHODS:

Chemicals and Spectral Measurements: DPPH (1,1-diphenyl,2-picrylhydrazyl) was purchased from Sigma Chemical Co. (USA). Potassium ferricyanide [$K_3Fe(CN)_6$] and ascorbic acid were bought from Loba Chemie Pvt., Ltd., (Mumbai, India) and SD Fine Chem. Ltd. (Biosar, India) respectively. Neocuproine ($C_{14}H_{12}N_2$), ammonium molybdate, Folin-Ciocalteu reagent, gallic acid ($C_7H_6O_5.H_2O$), quercetin were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents used for extractions and assays were of analytical grade. All UV-vis measurements in antioxidant activity tests were recorded on a Shimadzu UV-1601 (Kyoto, Japan) spectro-photometer. UV absorbance in MTT cell viability assay were recorded on VERSA max (California, USA) micro plate reader.

Plant Material: Leaves of the plant were collected from the campus of Jahangirnagar University, Savar, Dhakain the month of June, 2016. The plant was identified by Technical Officer, Abdur Rahim, Department of Botany, Jahangirnagar University. A voucher specimen of the plant is retained in herbarium (Accession no: DACB 43467) for further reference.

Preparation of Plant Extract: After collection, the leaves were thoroughly washed with water and air dried. Powdered dried leaves (300 gm) were macerated with chloroform with occasional stirring at 25 ± 2 °C for 7 days. Then the extract was filtered using a Buchner funnel and sterilized cotton filter. It was further filtered using Whatman Grade 1 qualitative filter papers. Rotary evaporator was used to remove the solvent. The dried extract (9 gm, yield 3%) obtained, were kept in air tight container in 4 °C.

Preliminary Phytochemical Screening of SCLC: The freshly prepared crude extract was tested for the presence of chemical constituents. The presences of phytochemicals were detected using the following reagents and identified by characteristic color changes as described by Ghani[6]: alkaloids using Dragendroff's reagents, flavonoids with the use of Mg and HCl; tannins with ferric chloride, carbohydrates using Molisch reagent and concentrated sulphuric acid; reducing sugars with Benedict's reagent and steroids with Liebermann - Burchard reagent. The presence of saponins was confirmed by its ability to produce stable foams.

Quantitative Phytochemical Analysis:

Determination of Total Phenol Contents of SCLC: The total phenol contents of plant extract was determined using Folin-Ciocalteu reagent following the method described by Yu *et al.* [7]. Specific amount of SCLC was dissolved in ethanol to get the final concentration 200 µg/mL to prepare the stock. Then, 100 µL of SCLC was withdrawn from the stock and mixed with 500 µL of Folin-Ciocalteu reagent and 1.5 mL of 20% w/v sodium carbonate. After shaking thoroughly, distilled water was added to the reaction mixer to make the final

volume 10 mL. Then it was allowed to stand for 2 h at room temperature. Then the absorbances were measured at 765 nm using a spectrophotometer against blank (1 mL ethanol). The total phenol contents of SCLC were determined using a standard curve obtained from various concentration of gallic acid and expressed as gallic acid equivalent (GAE). The experiments were carried out in duplicates.

Determination of Total Flavonoid Contents of SCLC: The flavonoid contents of the extract were determined by using the aluminium chloride colorimetric method [8]. Stock solution of SCLC was prepared by dissolving appropriate amount of SCLC in methanol to get final concentration 200 µg/mL. Then, 1 mL from the stock was taken and mixed with methanol (3 mL), aluminium chloride (0.2 mL, 10% w/v), potassium acetate (0.2 mL, 1 M). Final volume 10 mL was made using distilled water and allowed the mixture at rest for 30 min at room temperature. Absorbance at 415 nm was taken against blank (1 mL methanol). The flavonoid contents were obtained from the standard curve of quercetin and expressed as quercetin equivalent (QE). The experiments were carried out in duplicates.

Determination of *In-vitro* Antioxidant Potential of SCLC:

DPPH Free Radical Scavenging Activity of SCLC: The free radical scavenging activity of SCLC was measured by scavenging the stable DPPH free radical as the method described earlier [9]. Specific amount of SCLC was dissolved in absolute ethanol to get the multiple concentrations of SCLC (1-200 µg/mL). Briefly, 0.1 mL from the stock of SCLC was added to 3 mL of a 0.004 % ethanol solution of DPPH for each concentration. The mixtures were allowed to rest for 30 min at dark place. Absorbances were taken at 517 nm after 30 min against blank (absolute ethanol). The percentage inhibition activity was calculated using the following formula:

$$[(A_0–A_1)/A_0] \times 100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard. The experiments were carried out in duplicates and IC_{50} values were calculated from the inhibition curve.

Determination of Total Antioxidant Capacity of SCLC: The total antioxidant activity of the extract was evaluated by the phosphomolybdenum method [10]. Stock of SCLC was prepared using methanol. Here, 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added with 0.3 mL extract (200 µg/mL) taken from the stock.

Then the reaction mixture was incubated at 95 ºC for 90 min. After that, the reaction mixer was cooled at room temperature and absorbance was measured at 695 nm using a spectrophotometer against blank (0.3 mL methanol). The total antioxidant activity was expressed as the number of equivalents of ascorbic acid (AAE) and calculated from the standard curve of ascorbic acid. The experiments were carried out in duplicates.

Ferric Reducing Antioxidant Power (FRAP) of SCLC: The ferric reducing antioxidant power was determined by its ability to reduce Fe^{3+} to Fe^{2+} following the method described previously [11]. Stock solution (5 mg/mL) was prepared by dissolving appropriate amount of SCLC in ethanol. Experimental concentrations (5-200 µg/mL) were made using distilled water to get final volume of 5 mL. Then 2 mL solution containing extract for each concentration were taken and mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1% w/v).

The mixture was incubated for 20 min at 50°C. Then 2.5 mL trichloroacetic acid (10% w/v) was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL, 0.1% w/v $FeCl_3$. The absorbance was measured at 700 nm against blank. A typical blank solution contained the reagent mixture without extract or standard and treated as same. Ascorbic acid was used as the reference.

Cupric Reducing Antioxidant Capacity (CUPRAC) of SCLC: The cupric reducing antioxidant activity of the plant extract was determined by its ability to reduce Cu^{2+} to Cu^{1+}. This assay had been carried out following the method described by Apak *et al.*[12]. Appropriate amount of SCLC was dissolved in ethanol to get final concentration 5 mg/mL.

Multiple concentrations (5-200 µg/mL) of SCLC were made by mixing with distilled water. Then, 500 µL of plant extract for each concentration was mixed with cupric chloride (1 mL, 0.01 M), ammonium acetate buffer (1 mL, pH 7.0), neocuproine (1 mL, 0.0075 M) and distilled water (0.6 mL). The mixtures were allowed to rest for 1 h at room temperature. Then the absorbance was measured at 450 nm against blank (blank solution contained the reagent mixture without extract or standard and treated as same). Ascorbic acid was utilized as the standard solution.

Cytotoxicity Test:

Brine Shrimp Lethality Bioassay: This assay is used as a preliminary tool to determine the preliminary cytotoxic potential for the bioactive compounds of natural and synthetic origin [13]. *Artemia salina* leach was used as test organism. The eggs of the brine shrimp was collected from the local pet shops and hatched for 2 days in sea water to allow the nauplii to get matured. During the hatching period continuous oxygen supply was maintained and temperature was kept at 37 °C. Stock solution of SCLC was prepared by dissolving specific volume of plant extract in 0.5% DMSO and sea water to get the concentration 1600 µg/mL.

Then specific volumes of plant extract were withdrawn from the stock and were taken into the vials containing sea water for serial dilution to get the final concentrations (6.25-800 µg/mL). With the help of Pasteur pipette, 2.5 mL of sea water containing 10 living nauplii was added to the 2.5 mL of plant extract taken from the sample vials. Same volume of DMSO as in sample vials was used as negative control. As positive control, multiple concentration (0.06 - 10 µg/mL) of vincristine sulphate was used. After 24 h, the numbers of living naupliiin the vials were counted and the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

Cell Culture and MTT Based Cytotoxicity Assay of SCLC: To determine the cytotoxicity, hela cells were used and this experiment was carried out following the method described by Mosman [14]. Hela cells were maintained in Dulbecco's Modified Eagles medium supplemented with 10% v/v fetal bovine serum, 4.5 g/L D-glucose, 100 mg/L sodium pyruvate and L-glutamine, 1% penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. 100 µL of the culture medium containing hela cells (1×10^4 cells/well) were seeded in 96-well plates. After 24 h, multiple concentrations of SCLC (3.125-200 µg/mL) solubilized in DMSO, were added to 96-well plates and incubated for 24 h. Later on, 20 µL of the 5 mg/mL MTT solution in PBS (Phosphate-buffered saline) was added to each well and incubated further for 2 h.

Later on, 100 µL of solubilization buffer [20% SDS (Sodium dodecyl sulphate) solution in 50% (v/v) DMF (dimethyl formamide) (pH 4.7)] was added to each well and incubated for another 12-16 h. At 570 nm wavelengths, absorbance was taken using micro plate reader. The experiment was carried out in triplicates. The % cell viability was the ratio of cell viability of treated groups to that of control group. The inhibition curves were prepared and IC_{50} values were calculated.

Antibacterial Activity by Disc Diffusion Method: The antibacterial activity of the plant extract was performed by the well accepted Bauer-Kirby disc diffusion method against three Gram-positive (*Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*) and five gram-negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Serratiama rcescens* and *Proteus mirrabillis*) [15, 16].

At first 100 µL of suspension of each microorganism containing approximately 100-150 CFU/mL, was spread over the nutrient agar media (Himedia, India). Then, 20 mg and 30 mg SCLC was dissolved in 1 mL methanol to get final concentration 20 µg/µL and 30µg/µL in aseptic condition. Sterilized and dried metrical Whatman no. 1 filter paper discs having diameter 6 mm, were impregnated with 10 µL of solutions of test samples containing 200 µg and 300 µg of SCLC and gently placed on agar plates pre-inoculated with test bacteria. Amoxicillin (10 µg/disc) standard disc was used as the positive control and blank disc (soaked with solvent and evaporated) were used as negative control. All the plates were incubated at 37 °C for 24 h. After incubation, the antibacterial activities of SCLC were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

Determination of Anti-Inflammatory Activity of SCLC: Experimental Animals: For the experiments, Swiss Albino mice of either sex, 6-7 weeks of age, weighing between 25-30 g, were collected from the Animal Research Laboratory, Department of Pharmacy Jahangirnagar University, Savar, Dhaka.

Animals were maintained under standard environmental conditions [temperature: (27.0 ± 1.0°), relative humidity: 55-65% and 12h light/12h dark cycle] and had free access to feed and water *ad libitum*. The animals were acclimatized to laboratory condition for one week prior to experiments. All the experimental procedures were approved by the Bio-safety and Ethical Clearance Committee, Jahangirnagar University, Savar, Dhaka.

Acute Toxicity Study: Mice were divided into control and test groups having 6 mice in each groups. Multiple doses of SCLC (1000, 1500 and 2000 mg/kg, bw) was administered to the test groups by oral gavage. The control group received distilled water. The test animals were then kept in separate cages for 72 h and observed for allergic response, behavioural change and mortality. During this experiment, mice were allowed to free access to water and food *ad libitum*.

Preparation of Extract and Standard for *in-vivo* Studies: Mice were divided into four groups for each study paradigm. Two groups received test extracts at indicated dose (250 mg/kg and 500 mg/kg, bw). Tween 80 (1-2 drops) was used to improve the solubility of the extracts. Control group received distilled water containing 1-2 drops of 1% tween 80 at a dose of 10 mL/kg. The last group received standard. In croton oil induced ear oedema model, ibuprofen was used as standard at dose 500 mg/kg and diclofenac sodium was used in xylene induced ear oedema at a dose of 100 mg/kg. All the treatments were administered to mice using oral gavage.

Xylene Induced Ear Oedema: The xylene-induced ear oedema test was performed following the method described earlier [17]. Appropriate amount of SCLC was dissolved in distilled water with the help of 1-2 drops of tween 80 (1%) to prepare the doses. Different doses of SCLC (250 mg/kg & 500 mg/kg, body weight) and diclofenac (100 mg/kg, body weight) were given orally to the mice. One h later, each animal received 20 µL of xylene on the anterior and posterior surfaces of the right ear lobe. The left ear was considered as control. Mice were sacrificed one hour after xylene application and circular sections were taken, using a cork borer with a diameter of 3 mm and weighed. The weight of oedema was considered as the difference between weight of ear treated with xylene (right ear) and the weight of ear without xylene treatment (left ear). The percentage inhibition of ear oedema was calculated by the following formula:

$$\% \text{ inhibition} = \frac{\text{1-(Weight of oedema (drug/standard)}}{\text{Weight of oedema control}} \times 100$$

Croton Oil Induced Ear Oedema: The croton oil induced ear oedema test was performed as described earlier [18]. Doses of SCLC were made by dissolving specific amount of SCLC in distilled water. For dissolution purpose 1-2 drops of tween 80 (1%) was also added. Indicated doses of SCLC (250 mg/kg and 500 mg/kg, body weight) and a dose of ibuprofen (100 mg/kg, body weight) as a positive- control were administered to the mice orally. One h later, each animal received 15 µVL of croton oil on the inner surfaces of the right ear lobe and 15 µL acetone on the inner surface of left ear lobe. Mice were sacrificed one hour after croton oil application and circular sections were taken, using a cork borer with a diameter of 3 mm, and weighed. Oedematous response was measured as the weight difference between the two plugs. The anti-inflammatory activity was expressed as percentage reduction of oedema in treated mice compared with the control mice.

$$\% \text{ inhibition} = \frac{\text{1- Weight of oedema (drug/standard)}}{\text{Weight of oedema control}} \times 100$$

Statistical Analysis: Microsoft excel (version 2007) was used for calculating antioxidant data. Data were statistically analyzed using one way analysis of variance (ANOVA) followed by Dunnett's test (for MTT based cytotoxicity assay) and independent t-test (for animal experiments) using SPSS 17 for windows. The results obtained were compared with the control group. p values < 0.05, 0.01 and 0.001 were considered to be statistically significant.

RESULTS:

Preliminary Phytochemical Screening of SCLC: Preliminary phytochemical screening of the SCLC revealed the presence of alkaloids, carbohydrates, saponins, flavonoids and tannins **Table 1**.

TABLE 1: PHYTOCHEMICAL SCREENING OF SCLC

Alkaloids	Carbohydrates	Glycosides	Saponins	Flavonoids	Steroids	Tannins	Glucosides
+	+	-	+	+	-	+	-

SCLC- Chloroform extract of leaf of *S. Chelonoides*; '+' denotes presence and '-' denotes absence.

Quantitative Phytochemical Analysis:

Determination of Total Phenol Contents and Flavonoid Contents: The total phenol and flavonoid contents of SCLC were determined from the standard curve plotted for gallic acid and quercetin respectively shown in **Fig. 1**. SCLC seemed to contain a good amount of phenolic compounds (38.04 ± 0.77 mg/g, GAE) and flavonoids (130. ± 7.86 mg/g, QE). The results of these tests are enlisted in **Table 2**.

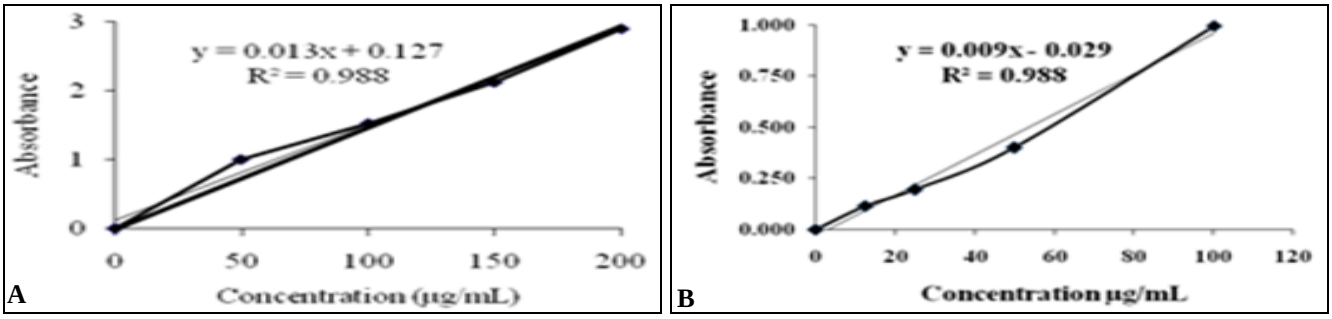

FIG. 1: DETERMINATION OF TOTAL PHENOL AND FLAVONOID CONTENTS

(A) Calibration curve of gallic acid to determine total phenol contents. (B) Calibration curve of quercetin to determine flavonoid contents

TABLE 2: TOTAL PHENOL, FLAVONOID AND TOTAL ANTIOXIDANT CAPACITY OF SCLC

Sample	Total phenol content (mg/g, GAE)	Total flavonoid content (mg/g, QE)	Total antioxidant capacity (mg/g, AAE)
SCLC	38.04 ± 0.77	130 ± 7.86	332.5 ± 5.3

SCLC: Chloroform extract of leaf of *S. chelonoides*. GAE- Gallic acid equivalents; QE- Quercetin equivalents; AAE- Ascorbic acid equivalents

Results of *In-vitro* Antioxidant Assays:

DPPH Radical Scavenging Assay of SCLC: To determine the ability of SCLC to neutralize free radical, DPPH radical scavenging assay was carried out where the % scavenging of DPPH radical was found to rise in a dose dependent manner upto concentration of 50 µg/mL **Fig. 2A**. At 100 µg/mL concentration, the % scavenging activity of SCLC greatly reduced but at 200 µg/mL concentration, it restored its radical neutralizing capacity.

The IC_{50} value of SCLC and ascorbic acid were calculated 707.34 µg/mL and 48.91 µg/mL respectively **Table 3**.

TABLE 3: IC_{50} VALUES OF SCLC IN DPPH SCAVENGING ASSAY

Sample/Standard	IC_{50} (µg/mL)
SCLC	707.34
Ascorbic Acid	48.91

SCLC- Chloroform extract of leaf of *S. Chelonoides*

Determination of Total Antioxidant Capacity of SCLC: The total antioxidant capacity of SCLC was estimated from the standard curve of ascorbic acid shown in **Fig. 2B**. The total antioxidant capacity of SCLC was found 332.5 ± 5.3 mg/g, AAE. The result of this study is enlisted in **Table 2**.

Ferric Reducing Antioxidant Power of SCLC: Reducing capacity of a compound is a significant indicator of antioxidant potential of that particular compound. That's why, FRAP assay had been carried out where SCLC was been able to reduce Fe^{3+} to Fe^{2+} in a dose dependant manner. From **Fig. 2C**, it can be implicated that, SCLC possessed very moderate iron chelating property.

Cupric Reducing Antioxidant Capacity of SCLC: Another very important reducing capacity measurement method is CUPRAC where the reduction of Cu^{2+} to Cu^{1+} ions was found to rise with increasing concentrations of SCLC.

From **Fig. 2D**, it can be inferred that, SCLC possessed better reducing activity at low doses (upto 50 µg/mL) than standard. At high doses (100 µg/mL & 200 µg/mL), its reducing capacity was as similar as ascorbic acid indicating strong copper ion chelating property.

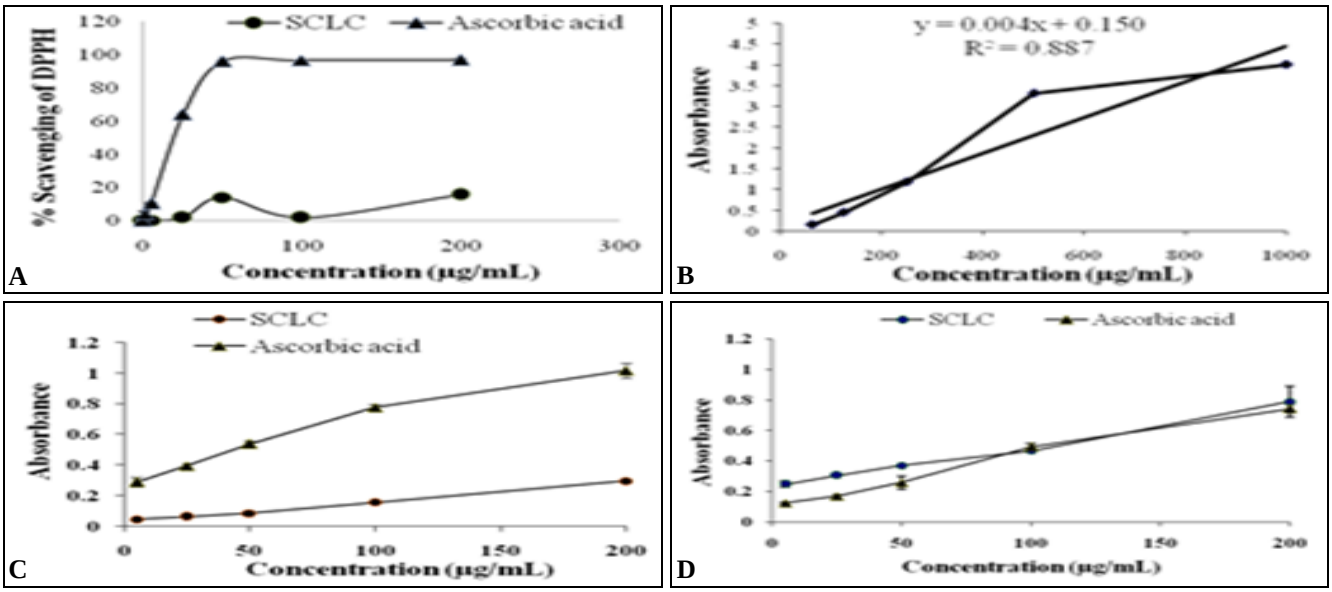

FIG. 2: DETERMINATION OF ANTIOXIDANT PROPERTY OF SCLC

(A) The DPPH scavenging activity of chloroform extract of leaf of *S. chelonoides* and ascorbic acid. (B) Calibration curve of ascorbic acid to determine total antioxidant capacity. (C) Ferric reducing antioxidant power activity of chloroform extract of leaf of *S. chelonoides* and ascorbic acid. (d) Cupric reducing antioxidant capacity of chloroform extract of leaf of *S. chelonoides* and ascorbic acid

Determination of Cytotoxic Potential of SCLC:

Brine Shrimp Lethality Bioassay: Having found the antioxidant potential of SCLC, we conducted brine shrimp lethality bioassay to determine the cytotoxic potential of SCLC. The result of this assay is given in **Table 4**. At high doses, SCLC was been able to produce significant brine nauplii killing effect. The LC_{50} value of SCLC was calculated 42.35 µg/mL whereas, for standard, it was 0.66 µg/mL. The LC_{90} value for SCLC was estimated 874.15 µg/mL and for vincristine sulphate, it was 7.94 µg/mL.

TABLE 4: RESULT OF BRINE SHRIMP LETHALITY BIOASSAY OF SCLC

	Conc. (µg/ml)	Log conc.	% mortality	LC_{50} (µg/mL)	LC_{90} (µg/mL)
SCLC	1	0	20	42.35	874.15
	5	0.69897	20		
	10	1	20		
	20	1.30103	30		
	50	1.69897	40		
	100	2	50		
	200	2.30103	80		
	500	2.69897	100		
Vincristine sulphate	0.078125	-1.10721	20	0.66	7.94
	0.15625	-0.80618	30		
	0.3125	-0.50515	40		
	0.625	-0.20412	50		
	1.25	0.09691	60		
	2.5	0.39794	80		
	5	0.69897	90		
	10	1	100		

SCLC: Chloroform extract of leaf of *S. chelonoides*.

MTT Based Cytotoxicity Assay of SCLC: The results of brine shrimp lethality bioassay encouraged us to conduct MTT based cytotoxicity assay on hela cell. Significant dose dependant cytotoxicity was observed in all doses of SCLC **Table 5**.

The percentage inhibition of cell viability was increased from 3.43% to 67.57% with increasing concentration (3.125-200 µg/mL) of SCLC. The IC_{50} value of SCLC was calculated 119.05 µg/mL.

TABLE 5: RESULT OF MTT BASED CYTOTOXICITY ASSAY OF SCLC ON HELA CELL

Concentration	% Cytotoxicity	IC_{50} (µg/mL)
3.125	3.43 ± 0.11	
6.25	17.83 ± 0.16*	
12.5	23.79 ± 0.04**	
25	33.20 ± 0.07***	119.06
50	40.69 ± 0.03***	
100	48.58 ± 0.04***	
200	67.57 ± 0.04***	

SCLC: Chloroform extract of leaf of *S. chelonoides*. Data are the mean ± standard deviation of three independent experiments. Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnett's test. *** p<0.001, **p<0.01 and *p<0.05 were considered significant.

Determination of Antibacterial Potential of SCLC: The result of antibacterial activity is listed in **Table 6**.

TABLE 6: RESULT OF ANTIBACTERIAL ASSAY OF SCLC

Test Organism	Diameter of zone of inhibition (mm)		
	SCLC		Amoxicillin
	20 µg/disc	30 µg/disc	10 µg/disc
Gram-positive bacteria			
B. cereus	09	09	9.5
B. subtilis	08	09	28.5
S. aureus	06	08	12.5
Gram-negative bacteria			
E. coli	06	08	18
P. aeruginosa	09	09	20
S. typhi	07	08	12
S. rcescens	07	09	10.5
P. mirrabillis	09	10	9.5

SCLC: Chloroform extract of leaf of *S. chelonoides*.

SCLC exhibited moderate antibacterial activity while compared with standard. The range of zone of inhibition against the all of the organisms range from 6-10 mm. Amoxicillin scored its zone of inhibition ranged from 9.5-28.5 mm. However, the zone of inhibition of SCLC was almost as similar as amoxicillin against *Bacillus cereus, Serratiama rcescens* and *Proteus mirrabillis.*

Anti-inflammatory Potential of SCLC:

Xylene Induced Ear Oedema: It's been scientifically proven that, antioxidants have a pivotal role to play in inflammations also. Keeping that in mind, the anti-inflammatory property of SCLC was tested in xylene induced ear oedema method. In this test, SCLC produced dose dependent anti-inflammatory activity by reducing the ear oedema induced by xylene. The % inhibition of SCLC at dose 500 mg/kg, was 12.16% where for standard diclofenac it was 22.97% (P<0.01). At dose of 250 mg/kg, SCLC reduced 1.35% of ear oedema only **Table 7**. The results were statistically significant.

TABLE 7: ANTI-INFLAMMATORY ACTIVITY OF SCLC INDUCED BY XYLENE

Group	Doses (mg/kg)	Ear weight difference (mg)	Inhibition (%)
Control	1% Tween 80 in Water (10 mL/kg)	12.33 ± 1.05	-
Diclofenac	100	9.5 ± 1.02**	22.97
SCLC	250	12.5 ± 0.92	1.35
	500	10.83 ± 1.11	12.16

SCLC: Chloroform extract of leaf of *S. chelonoides*. (Values are expressed as Mean ± SEM; n=6. Analysis was carried out using independent samples t test. **P <0.01 are considered as significant)

Croton Oil Induced Ear Oedema: In another method of determining anti-inflammatory potential, SCLC was subjected to croton oil induced ear oedema test. Here, the SCLC inhibited inflammation by 12.16% and 33.78% (P<0.05) at dose 250 mg/kg and 500 mg/kg respectively, while the standard, ibuprofen inhibited 78.38% (P<0.001) of ear oedema only at dose of 500 mg/kg **Table 8**. The results were statistically significant.

TABLE 8: ANTI-INFLAMMATORY ACTIVITY OF SCLC INDUCED BY CROTON OIL

Group	Doses (mg/kg)	Ear weight difference (mg)	Inhibition (%)
Control	1% Tween 80 in water (10 mL/kg)	12.33 ± 0.99	
Ibuprofen	500	2.66 ± 0.67***	78.38
SCLC	250	8.17 ± 1.87	12.16
	500	13.83 ± 1.25*	33.78

SCLC: Chloroform extract of leaf of *S. chelonoides*. (Values are expressed as Mean ± SEM; n=6. Analysis was carried out using independent samples t test. * P <0.05 and ***P <0.001 are considered as significant)

DISCUSSION: Antioxidants protects cell from the damaging effects of ROS in mainly three ways: (a) suppressing ROS generation by inhibiting enzymes or chelating metals, (b) scavenging ROS and (c) up regulating body's antioxidant defence system [19, 20]. In the various tests we performed here, it had been observed that, SCLC possessed both radical scavenging and metal chelating properties. Metal chelating properties allowed SCLC to inhibit the

early initiation stage of ROS generation (*e.g*. Fenton reaction and the metal-mediated Haber-Weiss reaction) and radical scavenging ability enabled it to function in chain termination reactions[1]. The antioxidant activity of SCLC might be attributed to its polyphenol and flavonoid contents. The total phenol and flavonoid contents of plants are highly correlated with its antioxidant property [21]. The antioxidant capacities of these compounds are conferred upon by the presence of multiple hydroxyl groups on their structures [22].

There is also a positive correlation between the number of hydroxyl groups and their antioxidant capacities. The hydroxyl groups at B-ring in flavonoids are capable of transferring hydrogen atom or donate electron to the free radical and thereby neutralize them [23]. This structural feature explains the DPPH radical scavenging property of SCLC. Flavonoids play a crucial role in chelating metal as well [19]. The specific position of diphenolic groups at B ring (particularly 3', 4'-o-diphenolic groups as well as hydroxyl-keto groups like 4-keto, 3-hydroxy) or C ring (4-keto, 5-hydroxy groups) potentiate the attachment with the metals and inhibit subsequent metal catalyzed ROS generation [20, 23].

The cytotoxic potential of this plant has not been reported yet. So we conducted brine shrimp lethality bioassay which is a rapid, easy and very useful tool to determine the ED_{50} values for cytotoxicity assay. As a prescreening method, this assay has been used to isolate a number of antitumor and pesticidal natural compounds too [13]. Like brine shrimp lethality bioassay, in MTT based cytotoxicity assay also, SCLC had been able to kill significant amount of cell at high doses. It is scientifically evident that, cytotoxic potential of plants largely attributed to their polyphenol and flavonoid contents. The ability of flavonoids to bind competitively with ATP at enzyme catalytic site renders them with capability of inhibiting protein kinases and thereby halts the signal transduction process.

These enzymes play very crucial role in oncogenesis process by providing signals for growth factors and to override body's regulatory processes. Apart from them, down regulating the expression of mutant p53 gene and R as protein, inhibition of heat shock protein are the other important mechanisms through which these compounds produce cytotoxicity [20].

Additionally, the presence of quinone derivatives like lapachol, stereochenol A and B have an important role to play in its cytotoxic potential. It is been scientifically evident that, quinone derivatives interact with the base pair of DNA double helix and inhibit subsequent DNA replication and RNA synthesis [24]. Extensive use of antibiotics and emergence of resistant bacterial strain necessitates improved antibacterial stewardship. That's why, the antibacterial property of SCLC had been determined where it was most active against *B. cereus* and *P. mirrabillis*. The presence of alkaloids, tannins, saponins and phenols explains the antibacterial property of SCLC, although the mechanisms by which these phytoconstituents exert antibacterial effects are different.

Phenols antibacterial effects are largely due to their ability to inhibit enzymes and bind with various soluble proteins present on the bacterial cell membrane [25]. Tannins can complex with proteins through various nonspecific covalent and non covalent bonds and inactivate the microbial adhesions, enzymes and cell envelop transport proteins while saponins can solubilized membrane cholesterol and enhance membrane permeability [26, 27]. On the other hand, most of the alkaloids are efflux pump inhibitor [28].

Inflammation is body's natural defence system in response to tissue injury caused by either pathogenic infection or chemical irritation. A lot of endogenous chemicals including ROS are capable of inducing inflammation. Aberrant resolution of them leads to chronic inflammation and antioxidants have a vital role to play. The traditional use of this plant in rheumatism and antioxidant property of SCLC encouraged us to test its potential in inflammation. In both xylene and croton oil induced inflammation tests, SCLC produced significant anti-inflammatory effects. 12-o-tetracanoilphorbol-13-acetate (TPA), the main irritating agent of croton oil induces inflammation by activating protein kinase C. On the other hand, xylene induces neurogenous oedema by activating substance P which is widely distributed in central and peripheral nervous system.

The oedematous responses produced by both the croton oil and xylene are associated with the release of inflammatory mediators like 5-hydroxitriptamine, bradykinin, prostaglandins *etc*. These factors ultimately results in vasodilation and plasma extravasations [19-31]. Flavonoids and lapachol are scientifically reported for their capability of inhibiting kinase enzymes and suppressing the expression of nitric oxide synthase, cyclooxygenase and lipoxygenase. This in turn, down regulates the production of NO and other inflammatory mediators and produces anti-inflammatory responses [25, 31].

CONCLUSION: Cancer, inflammation and anti-bacterial resistance are the major reasons of morbidity and mortality now-a-days. In this study, it has been observed that, SCLC possesses antioxidant potential. It is also enriched in various phytochemical constituents who justify its anti-bacterial, cytotoxic anti-inflammatory activities. Although the quantitative analyses of all these phytochemical constituents were not possible. But, our study has validated its traditional use in rheumatism and also enlightens new therapeutic potential.

However, to reach any concrete conclusion, further studies are required along with *in-vivo* studies to isolate compounds responsible for these activities and to understand the mechanisms through which the actions are being produced.

ACKNOWLEDGEMENT: The authors are thankful to Laboratory of Natural Product research and Animal Research Laboratory, Department of Pharmacy Jahangirnagar University, Savar, Dhaka, for all technical supports. We sincerely acknowledge the contribution of Professor IlSeon Park, PhD, Department of Biomaterials, Chosun University, South Korea, for his generous support in cytotoxicity assay. All the supports from Ishtiaque Ahmed and Arindam Saha are gratefully acknowledged.

CONFLICT OF INTEREST: Authors declare no conflict of interest.

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How to cite this article:
Shanta MA, Mondal M, Majumder S, Islam MI, Hoque N, Tithi NS and Rana MS: Pharmacological investigations of chloroform extract of *Stereospermum chelonoides* leaves. Int J Pharm Sci & Res 2018; 9(10): 4256-66. doi: 10.13040/IJPSR.0975-8232.9(10).4256-66.