INVESTIGATION OF ANALGESIC, ANTIOXIDANT, AND CYTOTOXIC PROPERTIES OF THE ETHANOLIC EXTRACT OF *STREBLUS ASPER* (ROOTS)

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ABSTRACT

Objective: This study evaluated the analgesic, antioxidant, and cytotoxic effects of ethanolic extract of *Streblus asper* roots.

Methods: Analgesic activities were carried out using paw licking and writhing tests. The extract was used at doses of 250 and 500 mg/kg per orally in Swiss albino mice of body weight 25–30 g. Antioxidant properties were investigated using 1, 1-diphenyl-1-picrylhydrazyl free-radical scavenging assay, total phenol content, total flavonoid content as well as reducing power capacity. The cytotoxic activity was determined by the Brine Shrimp lethality bioassay.

Results: The ethanolic extract of *S. asper* roots showed a significant effect in the formalin test (p<0.01). They also exhibit the highest analgesia (p<0.01) in the acetic acid-induced writhing model as well. The extract possessed antioxidant effects in all the models. It possessed a good cytotoxic activity.

Conclusion: The results suggested that *S. asper* root extract has a suitable analgesic, antioxidant effect as well as significant cytotoxic potential.

Keywords: *Streblus asper,* Analgesic, Antioxidant, Cytotoxicity, 1, 1-diphenyl-1-picrylhydrazyl free-radical scavenging assay.

INTRODUCTION

Pain is a sensorial modality, primarily protective, but often causes discomfort. It is the most important symptom that brings the patient to the physician. Conventional medicine, including treatments with steroids and nonsteroidal anti-inflammatory drugs, has shown only limited success against all forms of inflammatory conditions and pains associated with unpleasant side effects such as gastrointestinal disturbances [1].

Free radicals are chemical species possessing an unpaired electron and are implicated in the progression of a variety of disorders in humans including central nervous system injury, arthritis, atherosclerosis, ischemic heart diseases, gastritis, and cancer and reperfusion injury of many tissues [2-5]. Oxidative stress results when the generation of reactive oxygen species (ROS) supersedes incompetence with cellular antioxidant defenses [6]. Mainly, the existence of phenolic cancer prohibition factors is acknowledged to have the covering mechanisms thus these phenolic compounds have antioxidative properties that useful as cancer chemo-preventive agents and at most inhibit carcinogenesis during the initiation phase, since they act as radical scavengers, for example, ROS [7]. The antioxidant present in the markets is mostly synthetic. Such synthetic antioxidants are known to have potential side effects and toxicity. Hence, their use is being restricted nowadays and there is increasing interest in finding out safer and bioactive natural antioxidants present in plant species [8].

Toxicology is just pharmacology at a higher dose. Thus, if we find toxic compounds, a lower, non-toxic, dose might elicit a useful, pharmacological, and perturbation on a physiologic system [9].

Since a very ancient time, herbal medications have been used for the relief of symptoms of disease [10]. The secondary metabolites found in plants and exhibit health beneficial activities such as antioxidant, anti-inflammatory, antihepatotoxic, antitumor, and antimicrobial [11].

Despite the great advances observed in modern medicine in recent decades, much interest, in medicinal plants, however, emanates from their long use in folk medicines in developing countries [9]. The biodiversity of the flora of Bangladesh is very broad, and several native Bangladeshi medicinal plant species have a long history of use with great phyto therapeutic potential [12].

Streblus asper (known as Sheora in Bengali), of the Moraceae family, is indigenous to Bangladesh, India, Sri Lanka, Malaysia, the Philippines, Thailand, and Vietnam [13-16]. It is an evergreen, dioecious, or rarely monoecious tree, to 10 m high, barks 10–20 mm thick, fibrous; exudation milky white latex; leaves simple, alternate, spiral; and stipules 2–5 mm long [17]. *S. asper* is a well-known ethnomedicinal plant that is used as an anti-inflammatory, anticancer, an antidote to snakebite, gingivitis, to treat wounds, skin diseases, filariasis, leprosy, toothache, fever, diarrhea, and dysentery and is especially effective in the oral cavity, also used in menorrhagia, epilepsy, stomachache, and urinary complaints in folk medicines [15,18-24]. The literature review showed that the methanol extract of *S. asper* (roots) has better activity than other extracts. However, no study appears about analgesic, antioxidant, and cytotoxicity of the ethanolic extract as analgesic, antioxidant, and cytotoxic effect of *S. asper* (roots). Therefore, the present study aimed to evaluate the analgesic, antioxidant, and cytotoxic effect of *S. asper* (roots).

METHODS

Plant collection and identification

The roots of *S. asper* were collected from Dhamrai under the Dhaka district. The plant species were taxonomically identified and authenticated by the Bangladesh National Herbarium, Mirpur, Dhaka.

Preparation of the extract

The collected materials were shed dry at below 40°C and pulverized in an electric grinder. The powdered crude drug of *S. asper* roots (500 g)

was subjected to a Soxhlet extraction process with 96% ethanol. The extract was filtered and evaporated to dryness in a rotary evaporator under reduced pressure to a solid mass and denoted as SA (*Streblus asper* ethanolic extract).

Experimental animals

Male Swiss Albino Mice (25–30 g) were used and collected from the animal research lab of the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh. The animals were randomly distributed into groups, housed in cages and maintained under standard environmental conditions and had free access to feed and water *ad libitum*. All protocols for animal experiments were subject to approval by the institutional animal ethics committee.

Acute toxicity studies

Swiss albino mice of either sex (25–30 g weight) were used for acute oral toxicity studies. No adverse effects or mortality were detected in the mice up to 4.0 g/kg, p.o., during the 24 h observation period. Based on the results obtained from this study, the dose for analgesic and activity was fixed to be 250 and 500 mg/kg B.W. for dose-dependent study.

Analgesic activity

Acetic acid-induced writhing test

The method, according to Koster *et al.*, 1959 was employed for this test. Forty-five minutes after administration time each mouse was injected with 0.7% acetic acid at a dose of 10 ml/kg intraperitoneal body weight. The number of writhing responses was recorded for each animal during a subsequent 5 min period after 15 min of the intraperitoneal administration of acetic acid and the mean abdominal writhes for each group were obtained [25].

Formalin-induced paw licking test

The method used by Hunskaar and Hole 1987 was used for the study. After 1 h of drug administration, 2.7% formalin was injected into the dorsal surface of the left hind paw. Time spent licking the injecting paw was recorded. The animals were found for the 5 min post formalin (acute phase) and for 5 min starting at 20th min post formalin (delayed phase) [26].

Antioxidant activity evaluation

1, 1-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay

The free-radical scavenging activity of the crude ethanolic extract was identified using the DPPH free radical method Silva IK, Soysa P, 2011. Various concentrations of the samples were an addition to 3 ml of methanol DPPH solution that was prepared daily. The mixture was shaken and left to stand at room temperature in the dark. After 30 min, the absorbance was measured at 517 nm against a blank containing all of the reagents except for the test samples. Ascorbic acid was served as a positive control. The IC_{50} (the concentration needed for 50% inhibition of DPPH) was calculated using the plot of percentage inhibition versus the extract concentration in μg/ml [27]. The percentage of DPPH inhibition was calculated using the following equation:

% inhibition=A_0-A/A_0)×100

Where,

A_0 is the absorbance of DPPH (control), and A is the absorbance of the sample with DPPH.

Determination of total flavonoid content

The total flavonoid content was calculated using the method described by Ordonez *et al.,* 2006 with slight modification. To 1.0 ml of samples/standard, 3.0 ml of methanol, 200 μl of 10% aluminum chloride, 200 μl of 1M potassium acetate solution, and 5.6 ml of distilled water were added. After 30 min of incubation at room temperature, the absorbance was measured at 415 nm. The total flavonoid content was reflected in terms of quercetin equivalent, QE, mg/g of the dry extract [28].

Determination of total phenol content

The total phenol content of SA extract/standard was determined as described using Folin-Ciocalteu reagent and Gallic acid as a standard [29,30]. 1 ml of SA in methanol (200 μg/ml) was mixed with 5 ml of Folin-Ciocaleu's (1:10 v/v) reagent. After 3 min, 4 ml of a saturated solution of Na_2CO_3 (7.5%) was inserted. After 1 h (but 30 min for the standard), the absorbance was measured spectrophotometrically at 765 nm, and the results were expressed as mg Gallic acid equivalent (GAE) per g of extract (mg GAE/g). The methanol solution was used as a blank.

Reducing power capacity assessment

The ferrous reduction antioxidant capacity of samples/standard (Ascorbic acid) was evaluated by the method described by Islam *et al.*, with slight modification. 2 ml samples/standard solutions at different concentrations and 2.5 ml of 1% potassium ferricyanide solution were added into the test tubes. The reaction mixture was incubated for 10 min at 50°C to complete the reaction. Then, a 2.5 ml of 10% TCA (Trichloro Acetic Acid) solution was in addition to the test tubes. The total mixture was centrifuged at 3000 rpm for 10 min. After this, 2.5 ml supernatant was withdrawn from the test tubes and was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ solution. The absorbance of the solution was measured at 700 nm using a spectrophotometer against a typical blank [31].

Cytotoxicity test (brine shrimp lethality bioassay [BSLT])

BSLT is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin [32]. *Artemia salina* leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in a 1000 ml beaker and shrimp eggs were added to one side of the beaker and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Ten nauplii were taken carefully by micropipette. Thirty-two milligrams of the test sample were taken and dissolved in 200 μl of pure dimethyl sulfoxide and the final volume was made to 20 ml with seawater. Thus, the concentration of the stock solution was 1600 μg/ml. Then, the solution was serial diluted to 800, 400, 200, 100, 50, 25, 12.5, and 6.25 μg/ml with seawater. Then, 2.5 ml of plant extract solution was an addition to 2.5 ml of seawater containing 10 nauplii. After 24 h, the test tubes were inspected using a magnifying glass against a black background and the number of surviving nauplii in each tube was counted. From this data, the percentage of the lethality of the brine shrimp nauplii was calculated for each concentration. The effectiveness of the concentration-mortality relationship of plant products is usually expressed as a median lethal concentration (LC_{50}) value.

Statistical analysis

Correlation/Regression analysis by Microsoft Office Excel was used as a statistical tool for analgesia inhibition assay data, inhibitory concentration, and cytotoxicity assay data. Statistical analysis for animal experiments was carried out by independent-sample t-test using SPSS. The data were presented as mean ±SEM/SD. The results obtained were compared with the vehicle control group where $p < 0.05$, < 0.01, and < 0.001 were considered to be statistically significant, highly significant, and very highly significant, respectively.

RESULTS

Analgesic activity

The result of the present study of *S. asper* ethanolic extract is exhibited in Tables 1 and 2.

Antioxidant effect

The IC_{50} values of DPPH radical scavenging activity are shown in Table 3 and Fig. 1.

The total content of phenolic compounds of ethanolic extract was determined from the regression equation of the calibration curve ($y=0.014x+0.219$, $R^2=0.996$) and expressed in GAE shown in Table 4 and Fig. 2.
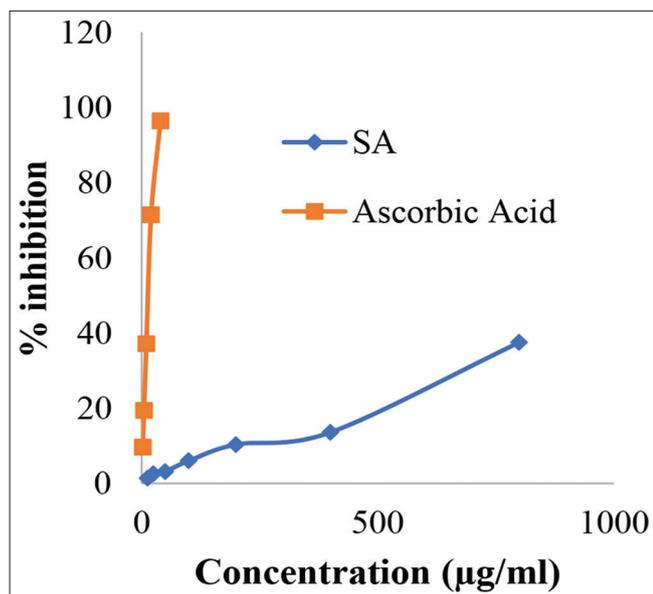
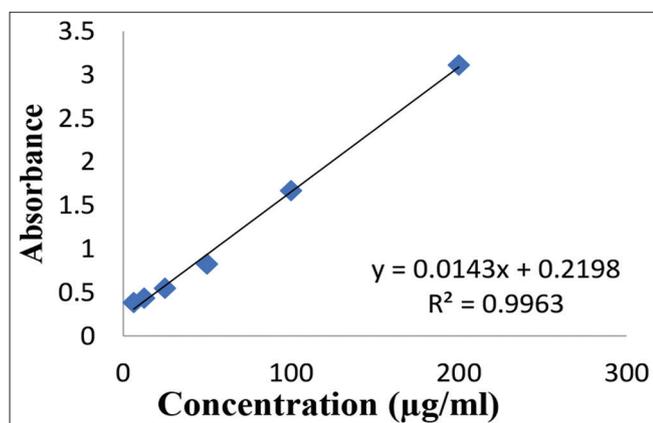**Fig. 1: 1, 1-diphenyl-1-picrylhydrazyl radical scavenging activity of ascorbic acid and SA ethanolic extract**

Fig. 2: Calibration curve of gallic acid

Table 1: The effect of control, STD, SA 250 mg, and SA 500 mg in the acetic acid-induced writhing test

Group	No. of writhing (Mean ±SEM)	T/p value	95% confidence interval.	% inhibition of pain
Control	11.17±0.65			00
Std (Diclofenac-Na)	1.17±0.48***	12.35/ 0.000	8.20 to 11.81	89.53
SA 250 mg	10.50±0.69*	0.41/ 0.022	-3.16 to 4.50	6.00
SA 500 mg	7.83±0.83*	3.15/ 0.010	0.97 to 5.69	29.90

N.B: *(<0.05)=Significant, ** (<0.01)=Highly significant, *** (<0.001)=Very Highly Significant (n=6)

For flavonoid determination, the regression equation of the calibration curve was (y=0.000x+0.021, R^2=0.992) and expressed as quercetin equivalent (QE) exposed in Table 5 and Fig. 3.

In the reducing power assay, SA was assayed for the reducing power activity where ascorbic acid was used as a standard. It has shown *in vitro* ferric reducing potential given in Table 6 and Fig. 4.

The cytotoxic activity of the extract was determined in accordance with the BSLT and expressed as the LC_{50} value (Table 7 and Fig. 5 and 6).

DISCUSSION

In the traditional systems of medicine, certain plants are claimed to provide relief from pain and inflammation. In the current study, plant *S. asper* roots were taken for the study. *S. asper* is a well-known ethnomedicinal plant. It is also used in Ayurveda [15,22]. Analgesic activities were evaluated by two animal models, which could respond to two different grades of noxious stimuli [33].

Acetic acid causes an increase in peritoneal fluids of Prostaglandin E_2 PGE_2 and Prostaglandin $F_2{}^\alpha$ $PGF_2{}^\alpha$, serotonin, and histamine, which are a model commonly used for screening peripheral analgesics [34,35]. With respect to the acetic acid-induced abdominal writhing which is the visceral pain model [36]. The results are presented in Table 1 showed that the ethanolic extract of the roots of *S. asper* induced a significant decrease in the number of writhes when compared to the control. The SA extracts of 250 mg/kg, 500 mg/kg thus possessed significant (p<0.05) analgesic effect where reference drug diclofenac at 100 mg/kg exhibited the highest analgesic power. Both the doses inhibited pain at 6% and 29%, respectively, indicating that the extract had an analgesic effect than the reference drug employed in this study. In general, acetic acid causes pain by liberating endogenous substances such as serotonin, histamine, PGs, bradykinins, and substance P [37]. The method has also been associated with prostanoids in general, that is, increased levels of PGE_2 and $PGF_2{}^\alpha$ in peritoneal fluids, as well as lipoxygenase products [34,38]. The reduction in acetic acid-induced writhes of *S. asper* suggests that the analgesic effect may be peripherally mediated through the inhibition of synthesis and release of PGs and other endogenous substances.

The formalin test is believed to be a more valid analgesic model which is better correlated with clinical pain [39,40]. The formalin test is biphasic, and measures pain in both neurogenic (first phase) and inflammatory origin (second phase). The first phase (0–5 min) being a result of direct stimulation of nociceptors measures centrally mediated effects and insensitive to anti-inflammatory agents while the second phase (15–30 min) which is qualitatively different from the first phase is dependent on peripheral inflammation and changes in central procession due to chemical mediators released from damaged cells that stimulate nociception and thus induced pain [26]. The results of this study, presented in Table 2, indicate that the ethanolic extract of *S. asper* caused a dose-dependent decrease in licking time and licking frequency in mice injected with formalin. This inhibitory effect is more pronounced in the later phase. The ability of SA ethanolic extract to inhibit late phases of the formalin test more prominently indicates its involvement in the peripherally mediated activity, probably by prostaglandin synthesis inhibition. The results of the analgesic study suggested that the ethanolic extract of SA process good analgesic activity by inhibition of peripheral mediators.

Table 2: The effect of control, STD (Diclofenac-Na), SA 250 mg, and SA 500 mg in the formalin-induced paw licking test (1st and 2nd 5 min)

Group	1st 5 min		2nd 5 min	
	Licking Time (mean±SEM)	%Inhibition of Inflammation	Licking Time (mean±SEM)	% Inhibition of pain
Control	36.67±1.76	00	31±2.79	00
Std (Diclofenac-Na)	8.67±2.03***	75.92	6.00±1.51***	80.65
ESA 250 mg	26.67±3.24*	25.92	13.67±3.85**	39.78
ESA 500 mg	26.0±2.22**	27.78	18.67±0.67**	55.93

N.B: *(<0.05)=Significant, ** (<0.01)=Highly Significant, *** (<0.001)=Very Highly Significant (n=6)

Table 3: IC$_{50}$ values of the STD (ascorbic acid) and SA extract

Plant sample/STD	IC$_{50}$ (µg/ml)
Ascorbic acid (STD)	16.88
(*SA* ethanolic extract)	1143.16

Values expressed as a means of duplicating experiments and represented as mean±SD

Table 4: Total phenol contents of the ethanolic extract of SA

Plants/Samples	Total flavonoid content (mg/g, QE)
(SA extract)	134.67 ± 2.00

Value is expressed as a means of duplicating experiments and represented as mean ± SD

Table 5: Total flavonoid contents of the ethanol extract of SA

Plants/Samples	Total phenol content (mg/g, gallic acid equivalents)
(SA extract)	118.33 ± 14.14

Value is expressed as a means of duplicating experiments and represented as mean ± SD

Table 6: Reducing power capacity of the ethanolic extract of SA

Plants/Samples	Reducing power capacity of SA (mg/g, ascorbic acid)
(SA ethanolic extract)	805.5±38.90

Value is expressed as a means of duplicating experiments and represented as mean±SD

Antioxidants are substances that stabilize the molecule, preventing damage to other cells caused by free radicals by supplying electrons [41]. Natural antioxidants are either in the form of raw extracts or their chemical constituents are very effective to prevent the destructive processes caused by oxidative stress [12]. The secondary metabolites such as phenolics and flavonoids from plants have been reported to be potent free-radical scavengers [42]. Antioxidant molecules can quench DPPH free radicals either by electron donation or by providing hydrogen atoms resulting in a decrease in absorbance at 517 nm. This test is a commonly employed assay in antioxidant studies of specific compounds or extracts across a short time scale [43]. The IC$_{50}$ values of DPPH radical scavenging activity are shown in Table 3 and Fig. 1. IC$_{50}$ values of the sample and ascorbic acids were 1143.16 µg/ml and 16.88 µg/ml, respectively. Although our results indicate that the DPPH radical scavenging activity of SA extract is lower than ascorbic acid; it can serve as a free radical scavenger, acting possibly as an antioxidant.

Plant phenolics constitute one of the major classes of compounds that act as natural antioxidants [44]. The antioxidant activity of phenolic compounds and flavonoids is attributed to the hydroxyl group attached to the aromatic ring, which is capable of donating electrons and stabilizing free radicals [45]. The total phenolic content was found to be 118.33±14.14 mg/g gallic acid equivalents (Table 4 and Fig. 2). Flavonoids are known as an excellent free-radical scavenging agents and management of diabetes and its complications [45-48]. Therefore, it was reasonable to believe the total flavonoid contents of SA extract. The total flavonoid compounds were found to be 134.67±2.0 mg/g quercetin equivalents. In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's blue at 700 nm. Increased absorbance indicates an increase in reductive ability [49]. Reducing the power of a compound serves as a significant indicator of its antioxidant activity [50]. *S. asper* was assayed for reducing power activity where ascorbic acid was used as a standard. The results of reducing power capacity shown
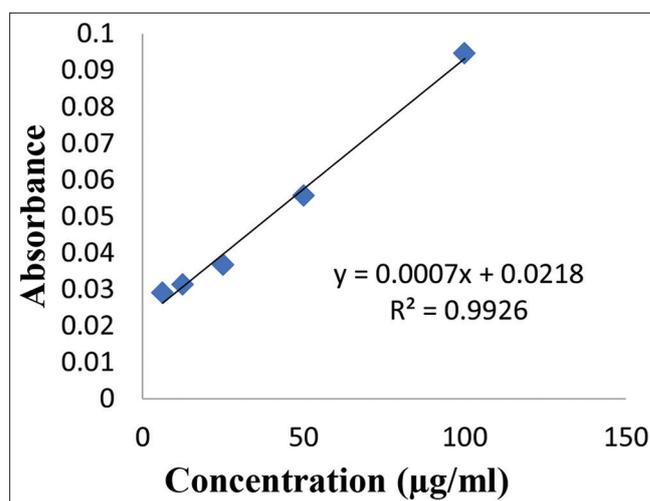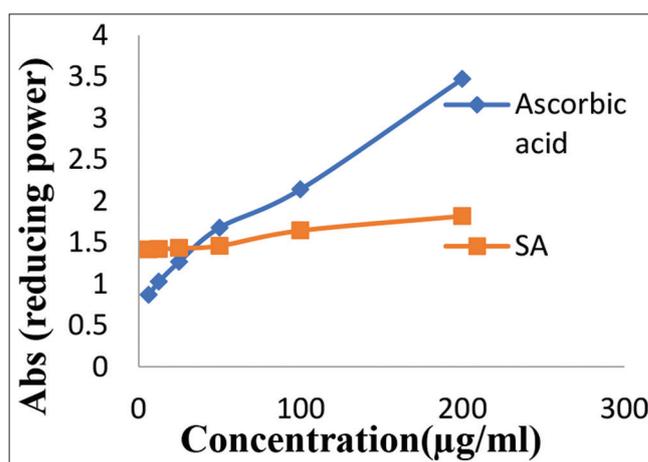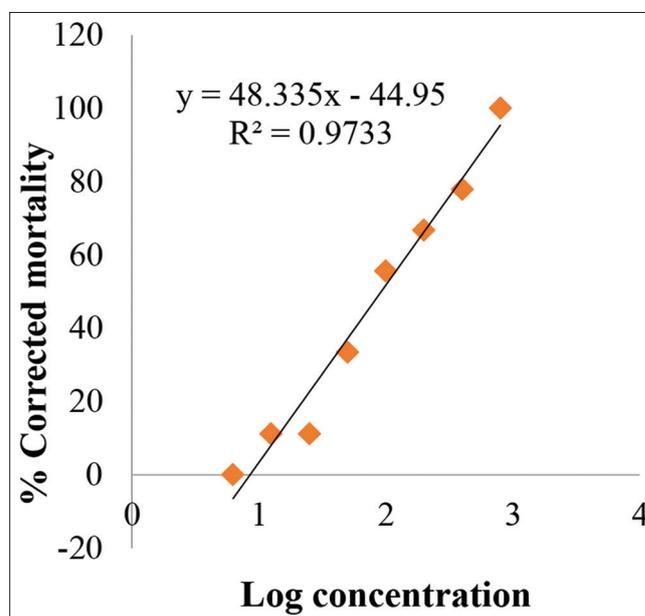

Fig. 3: Calibration curve of quercetin

y = 0.0007x + 0.0218
R² = 0.9926

Fig. 4: Reducing power of ascorbic acid and ethanolic extract of *Streblus asper*

y = 48.335x - 44.95
R² = 0.9733

Fig. 5: Graphical presentation of the cytotoxic potential of SA extract

Table 7: Brine shrimps lethality bioassay of the ethanolic extract of SA roots and vincristine sulfate

Group	Conc. (µg/ml) sample	Log conc.	Number (N)	Dead	Live	% Mortality $=\dfrac{D\,(Dead)}{N\,(Number)}\times100$	% Corrected Mortality $=\dfrac{P0-Pc}{100-Pc}\times100$	LC$_{50}$ value
Ethanolic extract of *Streblus asper* Roots	800	2.90309	10	10	00	100	100	92.18 µg/ml
	400	2.60206	10	8	02	80	77.78	
	200	2.30103	10	07	03	70	66.67	
	100	2	10	06	04	60	55.56	
	50	1.69897	10	04	06	40	33.33	
	25	1.39794	10	02	08	20	11.11	
	12.5	1.09691	10	02	08	20	11.11	
	6.25	0.79588	10	01	09	10	0	
	Control	0	10	01	09	10	00	
Vincristine sulfate	0.06	−1.2218	10	01	09	10	0.00	0.90 µg/ml
	0.125	−0.9031	10	01	09	10	0.00	
	0.25	−0.6021	10	03	07	30	22.22	
	0.5	−0.301	10	04	06	40	33.33	
	1	0	10	06	04	60	55.56	
	5	0.69897	10	09	01	90	88.89	
	10	1	10	10	00	100	100	
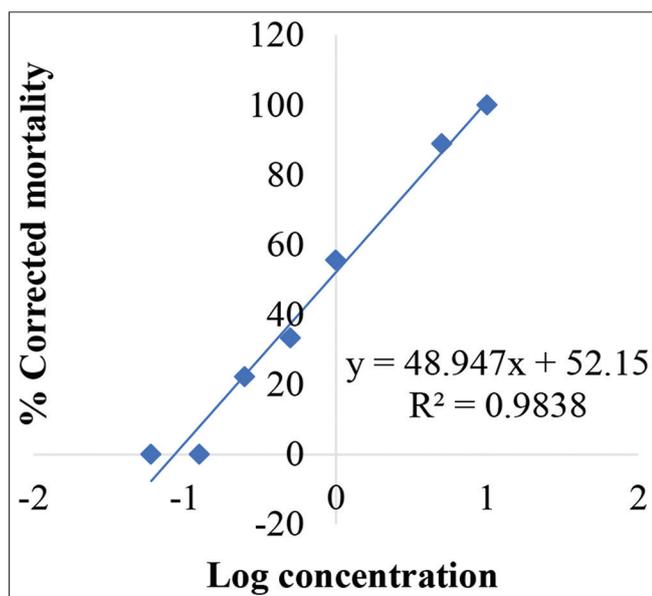
Fig. 6: Graphical presentation of cytotoxicity of vincristine sulfate

$y = 48.947x + 52.15$
$R^2 = 0.9838$

in Table 6 and Fig. 4, which is 805.5±38.90 mg/g of ascorbic acid equivalents. That indicates the good reducing power capacity compared to standard. The antioxidant assays recommend that SA roots ethanolic extract exhibit good antioxidant property due to the presence of a higher amount of phenolic and flavonoid content.

A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in crude extracts is the BSLT. It appears that BSLT is predictive of cytotoxicity and pesticidal activity and is commonly used in the bioassay for the bioactive compounds [51,52]. The cytotoxic activity of the extract was determined by the BSLT and expressed as the LC$_{50}$ value (Table 7 and Fig. 5 and 6). LC$_{50}$ values for SA ethanolic extract and vincristine sulfate were 92.18 µg/ml and 0.90 µg/ml, respectively. The LC$_{50}$ value of the SA extracts is shown that the extract is highly cytotoxic compare to the standard. It may be due to the presence of different phytochemical constituents.

CONCLUSION

The results of the experiments in this study indicate that the ethanolic extract of *S. asper* (roots) possesses good analgesic properties in both models. The extract also showed beneficial antioxidant potential against free radicals. The higher contents of flavonoids and phenolics support the assumption. It is also supported by high reducing power capacity. From the study of cytotoxic activity, it can be asserted that the investigated plant materials *S. asper* is a viable source of natural cytotoxic agents which might be due to the presence of different phytoconstituents. Therefore, it can be demonstrated that the ethanolic extract of *S. asper* (roots) possesses analgesic, antioxidant as well as significant cytotoxic properties. This could provide a rationale for the use of this plant in various disorders in folk medicine. Further sophisticated study is required to isolate the active principle by bioassay-guided fractionation and other methods for the management of diseases.

AUTHOR'S CONTRIBUTIONS

The authors have equally been involved in this research work. All the authors have read and agreed to the final approval of the manuscript.

CONFLICT OF INTEREST

All the authors professed that they do not have any conflicts of interest in publishing this research article.

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