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Phytochemical Screening, Antioxidant, Cytotoxic, Analgesic, Antidiarrheal Activity and GC-MS Analysis of the Leaves of *Stachyphrynium Placentarium*

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Abstract

The goal of the present study is to analyze the phytochemicals, antioxidants, cytotoxic, analgesic and anti-diarrheal properties of the leaves of *Stachyphrynium placentarium* methanol extract as well as its dichloromethane soluble fraction using GC-MS technique. The leaves of the *S. placentarium* were extracted with methanol and designated as SPM. Phytochemicals investigation exhibited the presence of several important secondary metabolites. Antioxidants such as total phenolic content, flavonoids content, total antioxidant, reducing power capacity and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay SPM revealed non-significant antioxidant properties. The cytotoxicity study of the extract showed significant cytotoxicity (LC_{50} 35.1093 µg/mL) compared to standard vincristine sulfate (LC_{50} 0.482 µg/mL). Analgesic study of *S. placentarium* by hot plate model indicates both the doses exhibited significant central analgesic activity compared with the control. The maximum analgesic activity was observed at 90 min. SPM (500 mg/kg) is 60.21 %. SPM250 also showed a significant percentage of elongation or latency from 30 to 150 min 57.00, 59.64, 59.88, 58.07 and 53.71 %, respectively 30 min time interval. In the acetic acid writhing test, *S. placentarium* significantly outperformed the control and standard (SPM250 - 52.27 %, SPM500 - 34.09 and STD - 27.27 %), showing a significant increase in peripheral analgesic activity ($p < 0.001$). Antidiarrheal activity of SPM showed that SPM250 and SPM500 substantially decreased the feces in castor oil-induced diarrhea ($p < 0.05$). According to the study, the incidence and severity of diarrhea are significantly reduced by SPM500. Four chemicals are found in the dichloromethane-soluble portion of the methanol extract of *S. placentarium* leaves, according to GC-MS studies such as Octadecane, 6-methyl- (1.326 %), Dodecane (1.02 %), 1-Iodo-2-methylundecane (0.47 %), tetradecanoic acid 12-methyl, methyl ester (72.64 %). Tetradecanoic acid exhibits anti-inflammatory, anticancer, 5-reductase inhibitory, antioxidant, hemolytic and antifibrinolytic properties.

Keywords: *Stachyphrynium placentarium,* Antioxidant, Cytotoxicity, Analgesic, Anti-diarrheal, GC-MS

Introduction

Plants used to treat ailments are known as medicinal plants [1]. We know that plant medicines are safe, easily obtainable and inexpensive [2,3]. The entire plant is sometimes used, but more commonly, various plants' leaves, roots, bark, seeds and flowers are used to create herbal treatments. The medicinal plants can

be inhaled, ingested, or used topically for management of diseases [4]. The value of medicinal plants for individual and community health has increased. The bioactive phytochemical components of these plants give them their therapeutic value; these components have been shown to have specific physiological effects in the human body [2,5-11]. These include alkaloids (inflammation, allergies, cancer, diabetes and many others) [5], essential oils (antimicrobial, antiviral, antibiotic, anti-inflammatory and antioxidant, etc.) [6], flavonoids (anticancer, antioxidant, anti-inflammatory, antiviral properties, neuroprotective and cardio-protective effects [7], tannins (anticancer, virucides, antioxidants, antimicrobial and anti-inflammatory agents, anti-diabetic, wound healing, cardiovascular protection, etc. [8], terpenoids (cancer, antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, anti-inflammatory and immunomodulatory properties [9], saponins (fish and snail poisoning, anti-cancer, ion channel-blocking properties, anti-inflammatory, hypocholesterolemic, immune-stimulating and flavor-modifying substances [10] and phenolic compounds (antioxidant activity, anti-inflammatory properties, antifungal activity, antimicrobial activity, antibacterial properties, anti-coronavirus activities, neuroprotective potential, appropriate for skin health, suitable for wound healing and anticancer activities [11]. There is an increase in the usage of plant-based medications worldwide [12]. There have been significant advancements in the pharmacological assessment of many plants utilized in conventional medical systems thanks to recent studies on herbal plants or medicines. The therapeutic effectiveness of plants, notably their antibacterial properties, is dictated by their chemicals, like flavonoids, terpenoids, alkaloid and tannins [13]. The use of plant-derived substances as an indigenous remedy in ethnomedicine has been related to the advent of pharmaceuticals in modern medicine [14]. Since 3,000 years ago, several plants have been utilized in medical procedures, such as Traditional Medicine in China, India and Africa. The majority of these plants have medicinal properties that have been verified as such by Western standards. The World Health Organization (WHO) estimates that traditional medicine is the primary source of care for 80 % of the world's population. Most of this therapy uses plant extracts and their active ingredients [15]. Numerous studies on various Stachyphrynium species have already been done, revealing a variety of biological activities, including antioxidants, cytotoxicity, antidiabetic, antidiarrheal and antibacterial activity. The literature research also revealed that numerous secondary active metabolites isolated from the various Stachyphrynium species have various pharmacological effects [16-18]. The Marantaceae family of shrubs and trees includes *Stachyphrynium placentarium*. The literature review by searching in google scholar, PubMed and other sources exhibited one new flavonoid glucoside (5,2′-dihydroxy-3′,4′-dimethoxyflavone-7-O-β-D-glucopyranoside) isolated from the aerial parts, no biological function of the leaves of the species *S. placentarium* were documented in any publication [19]. This piqued our interest and we conducted phytochemical analysis, GC-MS analysis and biological activity testing on the methanol extract of *S. placentarium* leaves.

Materials and methods

Collecting and identifying the plant

S. placentarium leaves were selected for phytochemical study and pharmacological investigations. *S. placentarium* leaves were taken from the Bandarban hill tract in Chattogram. The plant *S. placentarium*, formerly *Phrynium placentarium*, was identified in the Bangladesh National Herbarium in Mirpur, Dhaka, Bangladesh, using conventional taxonomical techniques. 63761 is the DACB accession number. India, Myanmar, Bangladesh, Bhutan, China and Thailand are all home to the plant.

Extraction of the powder materials

The plant's leaves were initially dried in the shade for a few weeks before being crushed with hands and dried once more. Then, using a mechanical grinder, the crushed plant components were ground into a coarse powder. The extraction procedure employed the Soxhlet extractors. A permeable bag containing 500 g of the dry powder was transferred to the Soxhlet chamber. The materials were adequately cleaned and dried before being put into the extractors. Methanol was used to extract the powder for 36 h. Methanol was used as extraction solvent due to its high polarity, exhibit the properties of good extraction ability as compared to other solvents and could produce high extraction yields [20,21]. The resulting extracts were collected and filtered through Wattman filter paper to get rid of the fine powder. The produced filtrates were evaporated by using a rotary evaporator connected to a water bath and keeping the temperature below 40 - 45 °C. After complete evaporation, the undiluted extracts were recovered. Then, at 4 °C, the refrigerators were used to store the dried crude extract. After evaporation, a dark green viscous concentrate was discovered in the instance of *S. placentarium* and this concentrate was created as SPM (methanol extract of the leaves of *S. placentarium*).

Phytochemical screening

S. placentarium extract was subjected to phytochemical analysis. The freshly created crude extracts' chemical components, such as alkaloids, carbohydrates, steroids, amino acids, phenolic compounds, fats and oils, phytosterols, flavonoids, glycosides, saponins, gums and tannins, were qualitatively examined. The tests were conducted using various standard techniques and a 10 % (w/v) solution was used in each test unless specifically stated in the individual test. Alkaloids [22]; Carbohydrates [23]; Glycosides [24]; Saponins [25]; Protein [26]; Amino Acids [27]; Phytosterols [28]; Fixed Oils and Fats [27]; Phenolic Compounds [29].

Tests for antioxidant activity

Total phenolic content

The colorimetric reaction involving the Folin-Ciocalteu reagent (FCR) was employed to quantify the total phenol contents (TPC) of the extract. The test tubes were filled with 1 mL of plant extract and standards at various concentrations, with each concentration containing 200 µg/mL. Subsequently, the mixture was thoroughly combined with 4 mL of a 7.5 % sodium carbonate (Na_2CO_3) solution and 5 mL of a Folin-Ciocalteu reagent (FCR). The standard and extract solutions were incubated in test tubes for 30 min and 1 h, respectively, at room temperature. The measurement of absorbance at a wavelength of 765 nm was conducted using a UV-visible spectrophotometer for both the extract and the standard [29,30]. Gallic acid was employed as the reference standard for the generation of a calibration curve. The phenol content of the extract is quantified in terms of milligrams equivalents of gallic acid (GAE) per gram as follows;

$$C = (c \times V)/m \quad (1)$$

Total flavonoid content (TFC)

The TFC of the plant extract was ascertained by a colorimetric process employing aluminum chloride. In this approach, 1 mL of plant extract (200 µg/mL), at varied concentration standards, was added to test tubes along with 200 µL of an aluminum chloride (10 %) solution, 200 µL of a potassium acetate (1 M) solution and 3 mL of methanol included in the test tubes. The mixes were then incorporated with 5.6 mL of distilled water. The mixture was then incubated for 30 min at the specified temperature. Using an ultraviolet-

visible spectrophotometer, we measured that the absorbance of the combined solutions was 415 nm [31]. The TFC of the extract was determined as mg of quercetin equivalent (QE)/g using quercetin as the reference.

$$C = (c \times V)/m \quad (2)$$

DPPH free radical scavenging assay

A free radical scavenging experiment using DPPH was carried out to assess the extract antioxidant properties. The oxidation of other substances is sparked by DPPH, an oxidizing agent and a reactive free radical. Conversely, antioxidants serve as electron suppliers (reducing agents). Antioxidants counteract DPPH by becoming oxidized. DPPH is a stable free-radical-molecule-based powder with a deep violet tint in its solid state. The deep violet tint altering to a pale yellow or colorless state (neutralization) indicates that the DPPH free radical has been scavenged [32]. The proportion of inhibition is calculated as follows:

$$\% \text{ Inhibition} = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ Control}\right) \times 100 \quad (3)$$

Total antioxidant capacity (TAC)

With the help of the phosphomolybdenum technique, the extract's overall antioxidant activity was assessed. The technique is based on the antioxidant components reducing Mo (VI)-Mo (V). The development of a green PO_4/Mo (V) complex serves as evidence for this. A UV-visible spectrometer measures the complex's production through absorbance at 695 nm [33]. The TAC of the extract was determined using the ascorbic acid equivalent (AAE) mg/g value as;

$$C = (c \times V)/m \quad (4)$$

Reducing power capacity

The ability of the *S. placentarium* leaf extract to reduce was measured using the Oyaizu technique. According to the reduction capacity of the antioxidant samples in this study, yellow solution turns green and blue. A significant predictor of a compound's potential antioxidant action is its ability to reduce other substances. The presence of antioxidants and other reductants in the samples leads to the reduction of the Fe3+ ferricyanide complex to its ferrous form, resulting in the donation of electrons. Then, monitoring the development at 700 nm may determine the amount of $Fe3^+$ complex [34].

$$C = (c \times V)/m \quad (5)$$

Cytotoxicity

The brine shrimp lethality bioassay technique was used to assess the extract's potential for cytotoxicity. The procedure is a sophisticated bioassay that is both quick and retarded for bioactive substances of natural origin. This technique allows for the bioactivity testing of extracts, fractions and pure compounds derived from natural products. In these tests, different concentrations of the standard and extracts were tested with a defined number of brine shrimps. Following a period of 24 h, an examination was conducted on the test tubes, wherein the number of viable nauplii present in each tube was quantified [35]. These data were used to calculate each concentration's lethality/mortality %. The formula was used to calculate the mortality.

$$\% \, Mortality = \frac{No.of \ nauplii \ (taken - alive \ )}{No.of \ nauplii \ alive} \times 100 \quad (6)$$

A median lethal concentration is typically used to represent how well plant products follow the concentration-mortality relationship (LC_{50}). By plotting the equivalent log concentration versus the percentage of death, linear regression reveals the chemical dose that kills 50 % of test volunteers after a certain exposure duration.

In-vivo pharmacological activity

Experimental animals

Swiss Albino mice of either sex that were healthy (20 - 30 g, 5 - 7 weeks old) purchase from department of pharmacy Jahangirnagar University. Each mouse had a supply of commercial pellets to eat and unrestricted access to water. Before the trial began, the mice were used to all the procedures for a week to reduce stress. Regarding all the mice used in this investigation, the generally established international standards for the care of laboratory animals were adhered to [36].

Acute toxicity study

Studies on acute oral toxicity utilized 25 - 35 g Swiss albino mice of either sex. Acute toxicity studies were determined using Lorke's technique [37]. The Gono Bishwabidyalay Centre for Multidisciplinary Research Ethical Committee approved the project with registration number CMR/EC/007. Each animal group in this experiment receives multiple dosages of the plant extract: 10, 100, 500 and 1,000 mg/kg. The animals are kept under observation for 24 h to track their behavior and determine whether any of them will die. Up to 1,000 mg/kg, p.o., the mice showed no side effects or death over the 24-hour observation period. The analgesic and activity dosages for the dose-dependent trial were fixed at 250 and 500 mg/kg body weight, respectively, based on the results of this investigation.

Animal grouping and dosing

Four groups of 5 Swiss albino mice weighing 20 to 30 g were randomly divided. Group I was given vehicles and 10 mL/kg of negative control as part of the group. As a positive control, Group II received regular medication, including Aspirin (100 mg/kg) for the hot plate test and the writing test. Groups III and IV in the tests were given samples or extracts at 250 and 500 mg/kg, respectively. An earlier study on acute toxicity was used to determine the doses. Each therapy was given orally at a 10 mL/kg rate.

Analgesic activities of SPM

Hot plate test

A mouse was placed in a cylindrical chamber with a metal plate heated by a thermos at the bottom to assess the extracts' ability to reduce central discomfort. The plate's temperature was continued at 55 ± 1 °C, which caused the behavioral components- paw licking, paw withdrawal and jumping- to be quantified in terms of their response times. All of the reactions were regarded as supraspinally integrated. Each mouse was put on a warm plate with a 15-second cutoff time in order to protect the paws from damage. Reaction time was calculated by measuring the time needed to jump or lick the paw off the hot plate [38]. In this study, Group I received distilled water orally at a dose of 10 mL/kg of body weight. Group II received Aspirin orally at a dose of 100 mg/kg of body weight. Group III and Group IV were administered SPM at doses of 250 and 500 mg/kg of body weight, respectively. The reaction durations were assessed at 0, 30,

60, 90, 120 and 150 min. The equation presented below is the method used to compute the percentage increase in response time.

$$\% \text{ elongation} = \frac{latency\ (Test) - latency\ (control)}{latency\ control} \times 100 \quad (7)$$

Writhing test induced by acetic acid

This study employed overnight-deprived mice with unrestricted water access to evaluate the potential of SPM extract for peripheral analgesia. One hour following the feeding of the extract, medium and standard, depending on the group, mice received an intraperitoneal (ip.) injection of "acetic acid (0.6 %v/v, 10 mL/kg)" in all mouse groups. The number of animals writhing for 15 min after a 5-minute latency period, which involves contracting their abdominal muscles and stretching their rear limbs, was used to quantity the extract's analgesic activity compared to the control [39]. The difference was shown as a percentage inhibition of writhing as follows:

$$\% \text{ Analgesic activity} = \frac{mean\ writhing\ (control\ -\ treated)}{mean\ writhing\ control} \times 100 \quad (8)$$

Test for anti-diarrhea using mice with diarrhea induced by castor oil

With a few minor adjustments, this study used the Uddin *et al.* [40] approach for measuring the anti-diarrheal activity of plant extracts. Four groups of 6 mice each were formed using mice of either sex that had been fasting for 12 h. Group I acted as the control group and was given 10 mL/kg of water orally. Group III and IV were the methanol extracts of *S. placentarium* at 250 and 500 mg/kg b.w. PO, respectively, whereas Group II served as the standard group. Mice were given 0.5 mL of castor oil orally 1 h after injection to induce diarrhea. Every hour for 4 h, the number of both hard and soft pellets was counted for each rat. The presence of feces containing a fluid substance that stained the paper used to line the cages was the definition of diarrhea [41]. Following is a calculation of percent inhibition:

$$\text{Percent Inhibition} = \frac{\text{Mean defecation (control} - \text{test)}}{\text{Mean defecation of control}} \times 100 \quad (9)$$

Equipment and methodology for GC-MS analysis

GC-MS, a specialist analysis method for identification and quantification, is only permitted for analytes that can tolerate the stringent partitioning conditions of the gas chromatograph and are volatile and thermally labile. Each component receives a unique spectral pick, which is electronically recorded on a paper chart. The SPM dichloromethane soluble component was examined in the current investigation using the GC-17A gas chromatograph with an MS-2010 plus spectrometer attached, using the electron impact ionization (EI) technique. Helium was employed as the carrier gas and its constant pressure of 49.5 kPa was used to maintain the fused silica capillary column's temperature at 40 °C. A split ratio of 50 was used to inject the sample. The operating circumstances can be described as follows: The column is named SH-Rxi-5Sil and possesses the subsequent specifications. The experimental parameters used in this study were as follows: The column length was 30 M, with a diameter of 0.25 mm. The flow rate of the column was set at 1 mL/min. The injector temperature was maintained at 250 °C and a holding duration of 10 min was applied. The samples were injected using a split ratio of 50. The carrier gas used was helium and the sample was liquefied in dichloromethane. A linear temperature rise of 10 °C (The analysis of the mass spectrum obtained from the gas chromatography-mass spectrometry (GC-MS) instrument was conducted using the database provided by the

National Institute of Standards and Technology (NIST). A comparison was made between the spectra of the unidentified molecule and the known component stored in the NIST collection [42,43].

Statistical analysis

The data analysis was conducted with the Microsoft Excel 2019 and IBM-SPSS (Statistical Package for Social Sciences) version 26. The results were reported by presenting the mean ± SEM (standard errors of the means) and mean ± SD (standard deviation) for each variable. Upon doing a one-way analysis of variance (ANOVA) and a Dunnett post hoc test, it was established that the observed differences across the groups were statistically significant at a significance level of $p < 0.05$. Tables and graphs were utilized to present the results following the investigation.

Results and discussion

Phytochemical studies

Methanolic extracts of *S. placentarium* are subjected to phytochemical analysis, identifying the presence of various bioactive components. **Table 1** contains the phytochemical test results. According to the findings of the phytochemical analysis, there are no proteins or amino acids present, but there are alkaloids, phytosterols, saponins, carbohydrates, tannins, flavonoids, steroids, glycosides and phenolic compounds. These phytochemicals are responsible for several medicinal activities [5-11]. Thus, these phytochemicals indicate the plant leaves have good medicinal properties.

Table 1 Analysis of Phytochemicals of SPM.

Investigated phytochemicals	SPM
Alkaloid	+
Carbohydrate	+
Saponin	+
Flavonoid	+
Tannins	+
Steroids	+
Glycosides	+
Fat and Oil	+
Proteins	−
Amino Acid	−
Phytosterols	+
Phenolic compounds	+

Note: Symbol (+) indicates the presence and (–) indicates the absence of the phytochemical.

Total phenol content (TPC)

The TPC of the SPM is evaluated using the gallic acid standard curve, which has an equation of $y = 0.0108x + 0.0561$ and R^2 value of 0.9999. The findings are presented in **Table 2** and **Figure S(1)**. Polyphenols like phenolic acid and diterpenes have a significant role in the antioxidant activity. Medicinal herbs possess antioxidant capabilities that extend beyond phenolic compounds. In addition to those as mentioned earlier beneficial volatile oils, carotenes and vitamins are examples of secondary metabolites, other active compounds may exist with similar properties. Phenolics' redox characteristics make them

effective antioxidants by acting as reductants, hydrogen donors and oxygen species suppressors [44]. Total Phenol Content 59.83 ± 3.937 mg/g as GAE in the methanol extract of *S. placentarium*, indicating the presence of lower phenolic content as well as lower antioxidant properties.

Table 2 *S. placentarium* methanol extracts' total phenolic content.

Sample name	Extract's dry weight (g/mL)	Absorbance of sample	Conc. (c) GAE (µg/mL)	Conc. (c) GAE (mg/mL)	TPC (mg/g) as GAE, A = (c×V)/m	Mean ± SD
SPM	0.0002	0.195	12.861	0.01286	64.305	59.83 ± 3.937
	0.0002	0.182	11.657	0.01165	58.287	
	0.0002	0.179	11.379	0.075	56.898	

Total flavonoid content (TFC)

The TFC of the methanol extracts of the leaves of *S. placentarium* was measured using the aluminum chloride colorimetric technique. According to the quercetin standard curve ($y = 0.0065x + 0.0923$; $R^2 = 0.999$; **Figure S(2)**), Quercetin equivalents (QE) mg/g were used to indicate the TFC. **Table 3** presents the outcomes of the total flavonoids study. The flavonoid content test of *S. placentarium* methanol extract was found 204.384 ± 11.487 mg/g as QE of flavonoids. Flavanols, flavones and iso-flavonoids, the largest class of phenolic chemicals found in plants, have potent antioxidant and anticancer properties [45]. The TFC of the SPM is of importance for assessing its antioxidant activities because flavonoids are renowned for their antioxidant qualities. The TFC test of *S. placentarium* methanol extract was found to be 204.384 ± 11.487 mg/g as QE of flavonoids. The higher flavonoid content correlated to good antioxidant activity. The flavonoid content study designates the good antioxidant action of the *S. placentarium* leaves.

Table 3 *S. placentarium* methanol extracts' total flavonoid concentrations.

Sample Name	Extract's Dry Weight (g/mL)	Absorbance of sample	Conc. (c) QE (µg/mL)	Conc. (c) QE (mg/mL)	TFC (mg/g) as QE, A = (c×V)/m	Mean ± SD
SPM	0.0002	0.347	39.185	0.039	195.9231	204.384 ± 11.487
	0.0002	0.375	43.492	0.043	217.4615	
	0.0002	0.352	39.954	0.040	199.7692	

***In-vitro* antioxidant assays**

The antioxidative properties of polyphenols arise from their notable reactivity as donors of hydrogen or electrons, enabling them to stabilize and distribute unpaired electrons and form complexes with metal ions. Numerous studies have provided evidence of a correlation between cardiovascular diseases and oxidation processes, which have been proposed to have a significant impact on the development of atherosclerosis. The reduction of reactive intermediates can be achieved through the inhibition of the Cytochrome P450 superfamily of enzymes. These enzymes break down various pro-carcinogens into reactive compounds, which can then interact with DNA and lead to malignant transformation.

DPPH free radical scavenging assay

The quantification of decolorization resulting from the transfer of an electron from an antioxidant chemical to DPPH can be determined by analyzing alterations in absorbance. **Table 4** and **S(1)** and **Figures**

S3(a) - S3(b) illustrate the findings and IC_{50} values for the SPM and ascorbic acid (AA). The DPPH radical scavenging method is a widely employed methodology for evaluating the antioxidant capacity of extracts or the efficacy of compounds in combating free radicals. The alteration in colour of DPPH, a chemically stable nitrogen-centered radical, is observed as a transition from a violet hue to a yellow hue, resulting from the reduction process facilitated by electron donation. Lower IC_{50} values are indicative of better antioxidant effects and more decolorizing impacts. Antioxidants are substances that can carry out this reaction. The DPPH free radical scavenging activity showed that SPM has less scavenging than ascorbic acid, with IC_{50} 215.04 µg/mL for SPM and 9.63 µg/mL for ascorbic acid.

Table 4 IC_{50} values of the Ascorbic acid and SPM.

Sample name	IC_{50}
Ascorbic acid	9.63 µg/mL
SPM	215.04 µg/mL

Total antioxidant capacity (TAC)

The antioxidant action of these substances is mostly attributable to the redox properties of phenolic substances, which can be beneficial in trapping and deactivating radicals as well as lowering reactive oxygen. The TAC of plant extract (SPM) is expressed as the gram equivalent of ascorbic acid standard ($y = 0.0128x + 0.1413$; $R^2 = 0.9788$). The finding is presented in **Table 5** and **Figure S(4)**, the study of total antioxidant capacity, methanol extract of *S. placentarium* leaves 76.97 ± 4.14 mg/g ascorbic acid equivalent indicates mild antioxidant properties.

Table 5 Total antioxidant capacity of SPM.

Sample name	Extract's dry weight (g/mL)	Absorbance of sample	Conc. (c) AAE (µg/mL)	Conc. (c) AAE (mg/mL)	TAC (mg/g) as AAE, (A) = (c×V)/m	Mean ± SD
SPM	0.0002	0.327	14.508	0.015	72.539	76.97 ± 4.14
	0.0002	0.348	16.148	0.016	80.742	
	0.0002	0.34	15.523	0.016	77.617	

Reducing power capacity (RPC)

The Oyaizu technique (1986) calculates the extract's Fe^{3+} reduction power with a little modification [34]. The number of grams equivalent to ascorbic acid is used to express the reducing power capability of plant extracts, which is determined using the ascorbic acid calibration ($y = 0.0045x + 0.6177$; $R^2 = 0.994$). The results are presented in **Table 6** and **Figure S(5)**. Ferric-reducing power activity methanol extracts of *S. placentarium* leaves 72.56 ± 5.09 mg/g reduction power as ascorbic acid equivalent, indicating non-significant antioxidant properties.

Table 6 Reducing power capacity of the SPM.

Sample name	Extract's dry weight (g/mL)	Absorbance of sample	Conc. (c) AAE (µg/mL)	Conc. (c) AAE (mg/mL)	RPC (mg/g) as AAE, A = (c×V)/m	Mean ± SD
SPM	0.0002	0.682	14.289	0.014	71.444	72.56 ± 5.09
	0.0002	0.688	15.622	0.016	78.111	
	0.0002	0.679	13.622	0.014	68.111	

In-vitro antioxidant study using different methods such as total antioxidant capacity, DPPH free radical scavenging assay, ferric reducing power exhibited that methanol extract of *Stachyphrynium placentarium* have moderate antioxidant activity.

Cytotoxicity study

The Brine Shrimp Lethality Test (BSLT) is a wide-ranging experiment that capable of identifying cytotoxicity found in crude extracts. The use of BSLT in bioassays for bioactive substances suggests that it is a reliable predictor of cytotoxic and insecticide action [46,47]. The extracts' percent mortality and LC_{50} values are given below in **Tables S(2) - S(3)** and **Figures S(6) - S(7)**. For each concentration, the lethality percentage was determined. The cytotoxicity investigation demonstrated the significant cytotoxicity of the extract. Specifically, the methanolic extract exhibited notable cytotoxicity with an LC_{50} value of 35.109 µg/mL, compared to the standard vincristine sulfate with an LC_{50} value of 0.418 µg/mL.

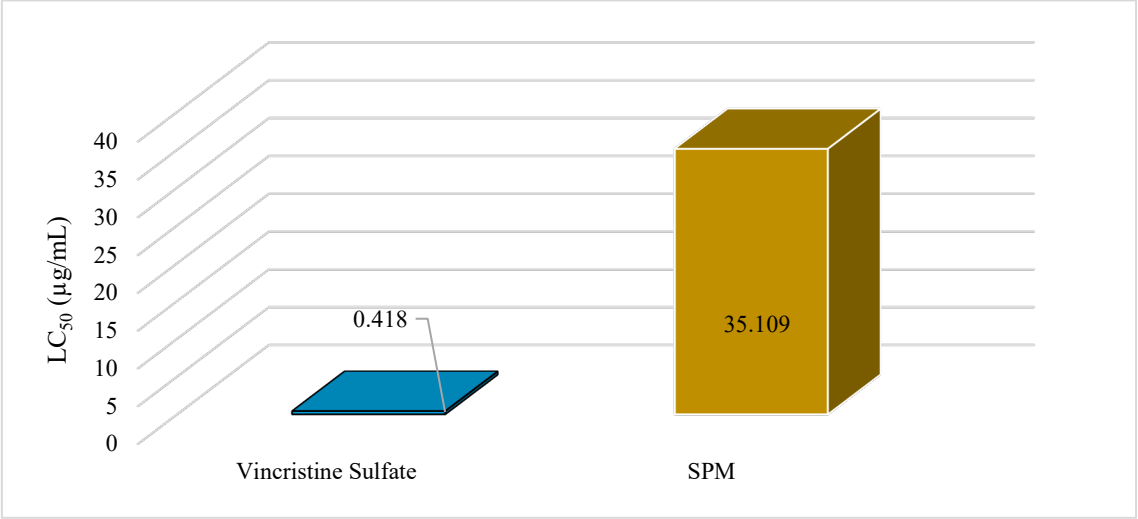

Figure 1 LC_{50} values of vincristine sulfate and *S. placentarium* methanol extract (SPM).

Analgesic study

Hot plate analgesic test

The hot plate test revealed the presence of central antinociceptive pathways and supraspinal nociception. The central analgesic efficacy of *S. placentarium* methanol extract (SPM) in mice was assessed using the hot plate method. The results are presented in **Table 7**. The peak analgesic effect is seen after 90 min SPM (500 mg/kg) is 60.21 %. But the standard drug (Aspirin-100 mg/kg) produced 61.84 % at 120 min. The study also showed that the percent of elongation of the reaction latency decreased from 90 min to

150 min. The % of elongation or latency on SPM from 30 to 150 min 57.00, 59.64, 59.88, 58.07 and 53.71 % respectively 30 min time interval.

Table 7 Effects of administration of aspirin and SPM on latency to hot plate test in mice.

Treatment groups	0 min	30 min	60 min	90 min	120 min	150 min
Control	5.52 ± 0.35	5.12 ± 0.40	5.97 ± 0.36	5.78 ± 0.22	5.8 ± 0.85	5.62 ± 0.23
STD	6.82 ± 0.47	7.5 ± 0.51	13.52 ± 3.31	13.12 ± 1.33**	15.2 ± 1.6***	12.45 ± 1.02***
SPM 250	6.32 ± 0.65	11.9 ± 1.87**	14.78 ± 2.14**	14.42 ± 1.32***	13.83 ± 1.93**	12.13 ± 1.4***
SPM 500	4.33 ± 0.1	8.28 ± 0.74	12.67 ± 1.75	14.53 ± 1.54***	10.20 ± 0.9	8.58 ± 0.57
Percent of elongation (inhibition of pain threshold or reaction time)						
STD	-	31.78 %	55.86 %	55.90 %	61.84 %	54.89 %
SPM 250	-	57.00 %	59.64 %	59.88 %	58.07 %	53.71 %
SPM 500	-	38.23 %	52.89 %	60.21 %	43.14 %	34.56 %

Note: The results are given as Mean ± SEM. One-way ANOVA was used in the statistical analysis to compare the 2 groups, along with the post hoc Dunnett test. Control = Water Control; STD = Aspirin treated group with a dose 100mg; SPM with dose 250 and 500 mg. $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

Test for acetic-induced writhing

The extract's effectiveness as a peripheral analgesic was evaluated using test writhing induced by acetic acid. The plant's analgesic activity is exhibited due to several bioactive components such as flavonoids, glycosides, terpenoids, etc. The results of analgesic activity showed that the S. *placentarium* extract at dosages of 250 mg and 500 mg demonstrated statistically significant peripheral analgesic action compared to the control ($p < 0.001$) **Table 8**. The results of a one-way ANOVA test analysis revealed considerably lower writhing. The greatest percentage of writhing numbers that the standard medication could suppress and the corresponding percentages of S. *placentarium* extract. In comparison to the control SPM 250 - 52.27 %, SPM 500 - 34.09 and STD 27.27 %, the percent of inhibition of SPM 250 and SPM 500 demonstrated a considerable suppression of the writhing generated by acetic acid. SPM 250 showed highly significant ($p < 0.001$) inhibition compared to standard Aspirin.

The analgesic study exhibited that, lower doses of *Stachyphrynium placentarium* methanol extract (SPM 250 mg) showed superior analgesic effect than higher concentrations (SPM 500 mg) in hot plate and acetic acid writhing tests.

Table 8 Effects of Aspirin and SPM on mice's writhing after exposure to acetic acid.

Treatment groups	No. of writhing	Percent inhibition	*p*-value
Control	14.67 ± 0.49	-	-
STD	10.68 ± 0.49***	27.27 %	0.000
SPM250	7.00 ± 0.68^{***a}	52.27 %	0.000
SPM500	10.50 ± 0.63***	34.09 %	0.000

Note: The results are given as Mean±SEM. One-way ANOVA was used in the statistical analysis to compare the 2 groups, along with the post hoc Dunnett test. Control = Water Control; STD = Aspirin treated group with a dose of 100 mg; SPM with doses 250 and 500 mg. $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, Compare to STD $p < 0.001$ = ^{***a}.

Antidiarrheal test

Castor oil-induced diarrhea in mice

The antidiarrheal activity of the SPM was assessed by measuring the percentage inhibition of defecation and the percentage inhibition of diarrhea. The findings of frequency and severity of diarrhea are presented in **Table 9**. The SPM 500 showed an excellent capacity to defend against diarrheal episodes at 88 % compared to the SPM 250 at 21 % and standard at 81 %.

Table 9 Effect of STD and extracts *S. placentarium.* on castor oil-induced anti-diarrheal test in mice.

Groups	Mean of total feces ± SEM	% Inhibition of defecation	Mean of diarrheal feces	% Inhibition of defecation
Control	16.5 ± 1.94	-	10.75 ± 3.2	-
STD	$5 \pm 0.82^{**}$	70	$2 \pm 0.71^{*}$	81
SPM 250	$7.8 \pm 2.58^{*}$	53	8.5 ± 2.9	21
SPM 500	$7.5 \pm 1.04^{*}$	55	$1.25 \pm 0.48^{*}$	88

Note: The results are given as Mean ± SEM. One-way ANOVA was used in the statistical analysis to compare the 2 groups, along with the post hoc Dunnett test. Control = Water Control; STD = Loperamide treated group with a dose of 10mg; SPM with 250 and 500 mg. $p < 0.05 = {}^{*}$, $p < 0.01 = {}^{**}$, $p < 0.001 = {}^{***}$.

GC-MS analysis of dichloromethane soluble part of methanol extract

GC-MS Analysis of the dichloromethane soluble part of methanol extract (SPM) showed 4 distinct phytochemical substances were found in the dichloromethane soluble portion of the methanol extract of *Stachyphrynium placentarium* leaves, according to the GC-MS analyses. The National Institute of Standards and Technology (NIST) library was used to match the phytoconstituents compounds discovered in the mass spectra. The presence of tetradecanoic acid 12-methyl, methyl ester (72.64 %), dodecane (1.02 %), 1-iodo-2-methylundecane (0.47 %) and octadecane,6-methyl- (1.326 %) was found to be the predominant phytoconstituents in the dichloromethane soluble portion of the methanol extract of leaves **Figure 2** and **Table 10**. The compound 1-Iodo-2-methylundecane is antimicrobial, improves polycystic ovarian syndrome and acts as an estrogen [48,49]. Tetradecanoic acid exhibits anti-inflammatory, anticancer, 5-reductase inhibitory, antioxidant, hemolytic and antifibrinolytic properties [50].

Table 10 Phytocomponents identified in dichloromethane soluble part of SPM.

SI. No.	Retention time	Name of the compounds	Molecular weight	Molecular formula	Conc.
1	10.303	Octadecane 6-methyl-	268.5209	$C_{19}H_{40}$	1.326 %
2	11.958	Dodecane	170.3348	$C_{12}H_{26}$	1.022 %
3	13.513	1-Iodo-2-methylundecane	296.231	$C_{12}H_{25}I$	0.477 %
4	21.547	Tetradecanoic acid 12-methyl, methyl ester	256.4241	$C_{16}H_{32}O_2$	72.643

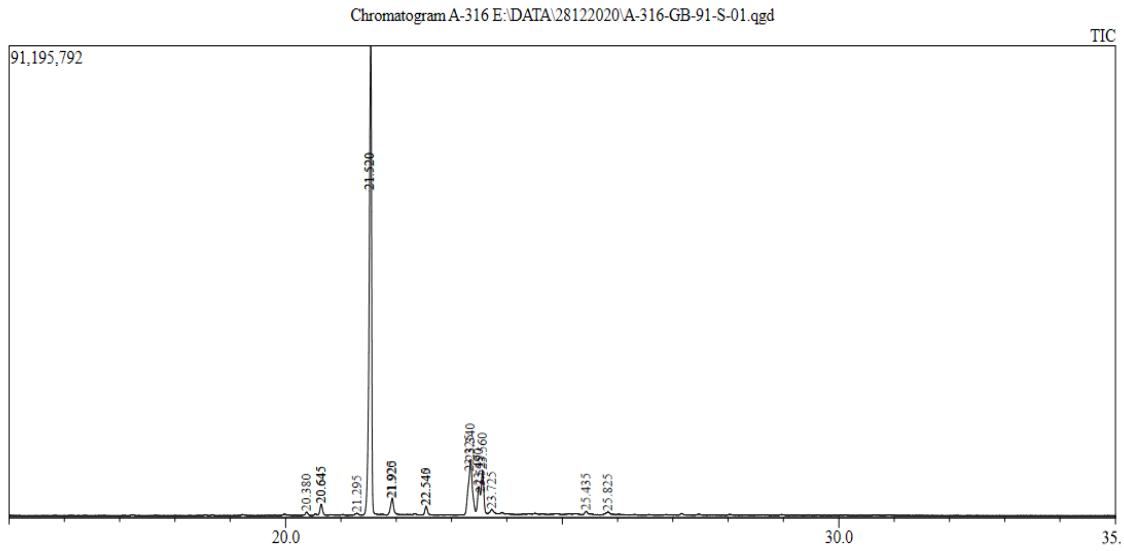

Figure 2 The total ion chromatogram of dichloromethane soluble part of SPM.

Conclusions

In the present study, phytochemical analysis exhibits the presence of biologically important secondary metabolites such as alkaloids, phytosterols, saponins, flavonoids, steroids, glycosides, phenolic compounds, etc. responsible for different therapeutic activities. Different antioxidant study indicates that methanol extract showed mild antioxidant. Brine shrimp cytotoxicity bioassay found that SPM has significant cytotoxicity compare to standard vincristine sulfate. The *in-vivo* assessment also revealed that methanol extracts of *Stachyphrynium placentarium* has significant analgesic and antidiarrheal activity. In the dichloromethane-soluble fraction of the methanol extract of *S. placentarium* leaves, GC-MS tests identified Octadecane, 6-methyl-, Dodecane, 1-Iodo-2-methylundecane and tetradecanoic acid 12-methyl, methyl ester. Further advances and more studies are required to isolate and identify the biologically active components from the leaves of the plant *Stachyphrynium placentarium to develop new therapeutic agents.*

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Supplementary material

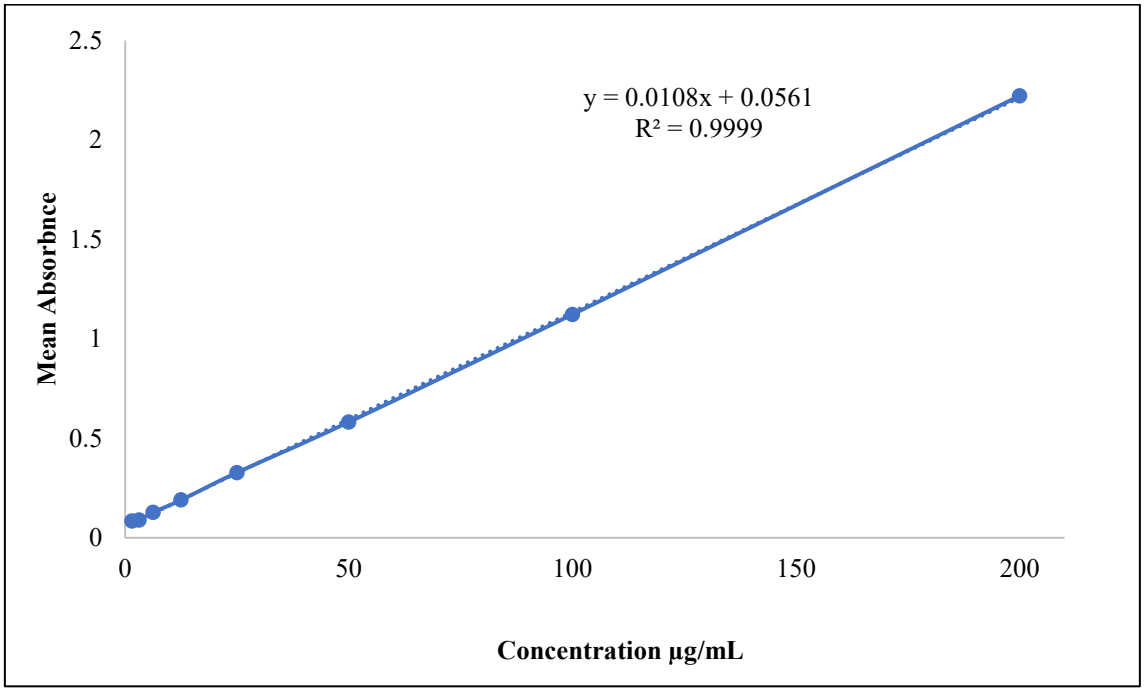

Figure S1 Calibration curve of Gallic Acid

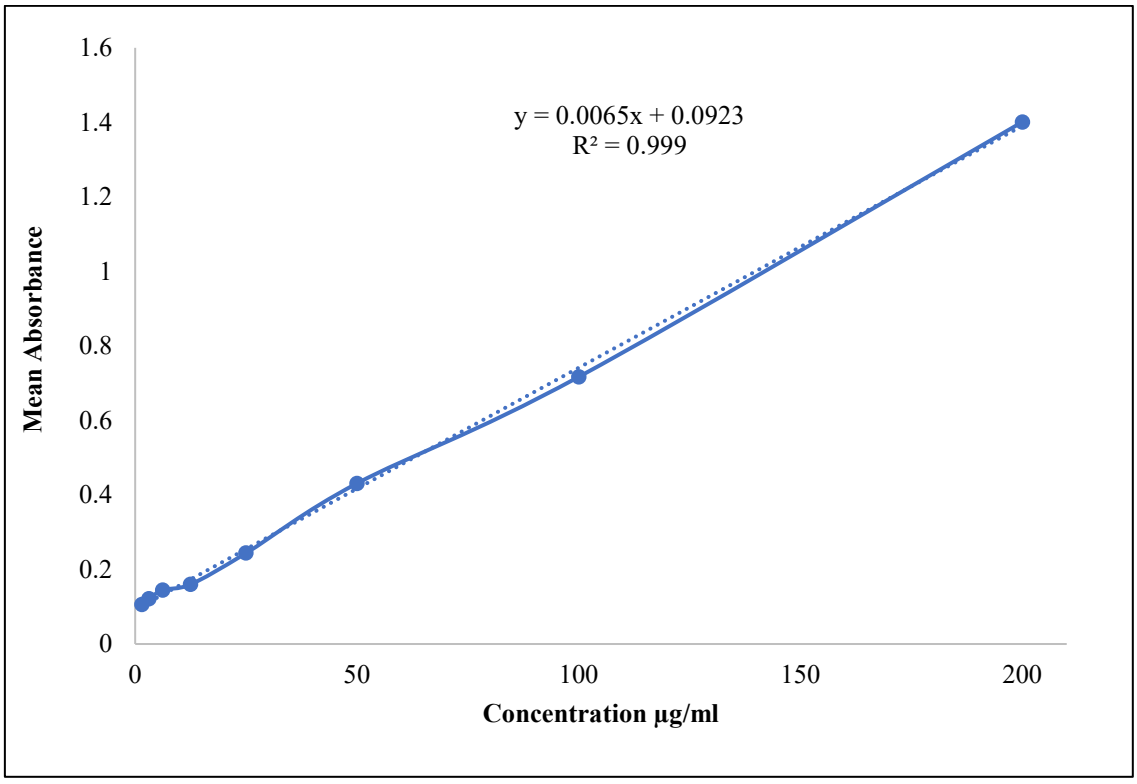

Figure S2 Calibration curve of quercetin.

Table S1 The percentage (%) scavenging activity of ascorbic acid

Concentration (µg/mL)	% Scavenging of AA	% Scavenging of SPM
6.25	34.52	34.31
12.5	48.27	34.94
25	59.18	35.89
50	72.61	37.36
100	81.74	43.55
200	95.90	57.71

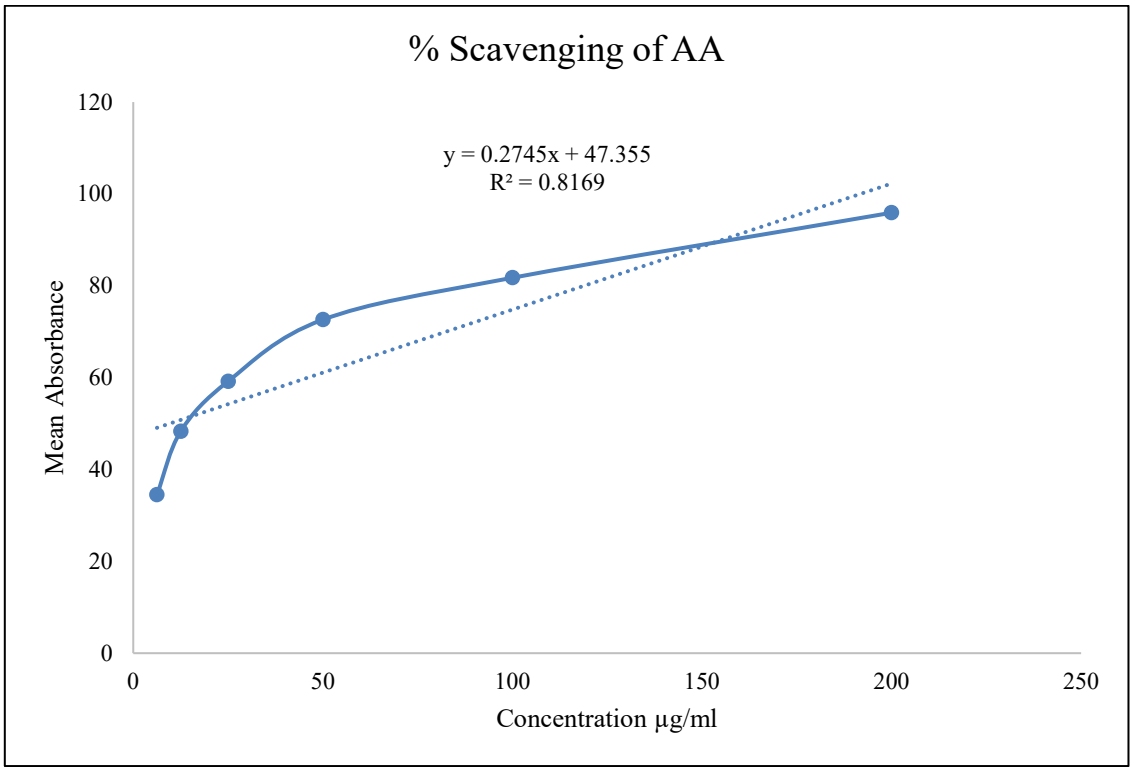

Figure S3a Curve of calibration of ascorbic acid for DPPH free radical scavenging assay.

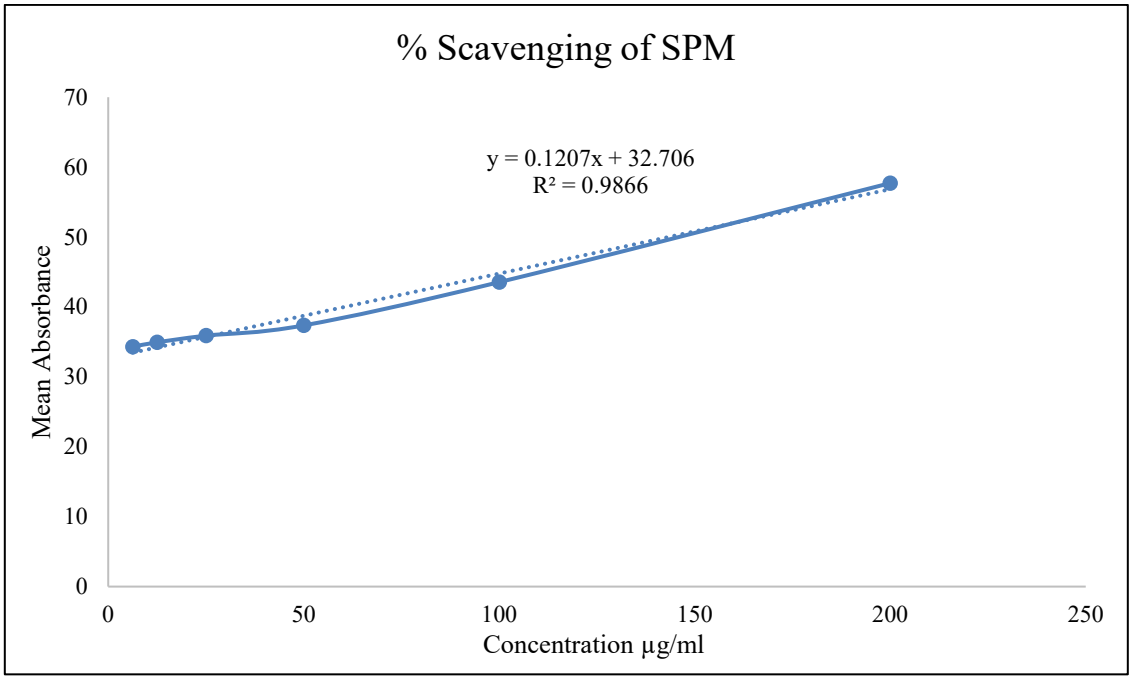

Figure S3b Curve of calibration of SPM for DPPH free radical scavenging assay.

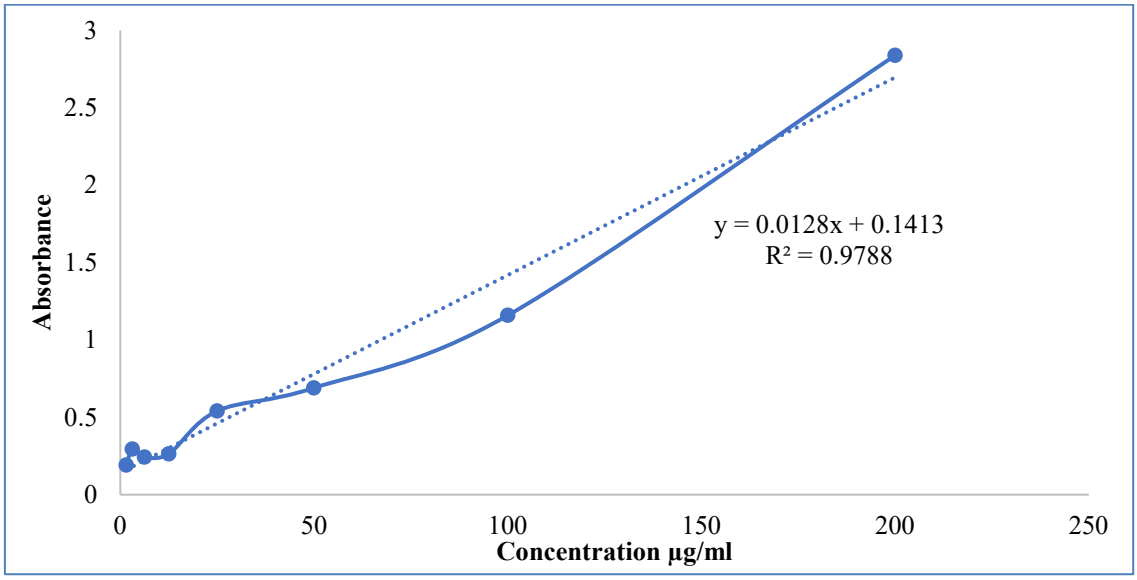

Figure S4 Calibration curve for ascorbic acid for determination of antioxidant capacity.

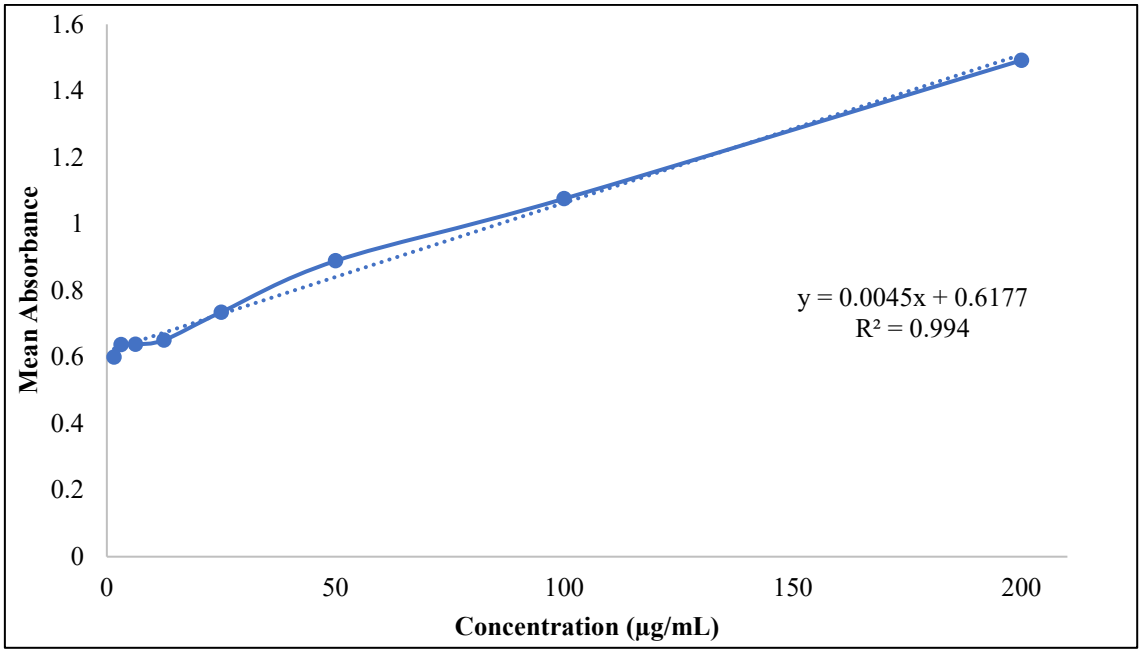

Figure S5 Calibration curve for ascorbic acid for reducing power assessment.

Table S2 Vincristine sulfate lethality bioassay using brine shrimp.

Conc. (µg/mL)	Log conc.	No. of nauplii taken (N_0)	No. of nauplii dead	No. of nauplii alive (N_1)	Mortality, $M=\frac{N_0-N_1}{N_0}\times100$	LC_{50} (µg/mL)
0.313	-0.50446	10	1	9	10	
0.625	-0.20412	11	2	9	18.18	
1.25	0.09691	11	4	7	36.36	0.418
2.5	0.39794	11	4	7	36.36	
5	0.69897	10	7	3	70	
10	1	10	8	2	80	

Table S3 Data for Brine Shrimp lethality bioassay for methanolic extract.

Conc. (µg/mL)	Log conc.	No. of nauplii taken (N_0)	No. of nauplii dead	No. of nauplii alive (N_1)	Mortality, $M=\frac{N_0-N_1}{N_0}\times100$	LC_{50} (µg/mL)
6.25	0.79588	10	1	9	10	
12.5	1.09691	10	1	9	10	
25	1.39794	10	4	6	40	35.109
50	1.69897	12	8	4	66.66	
100	2	15	13	2	86.66	
200	2.30103	12	12	0	100	

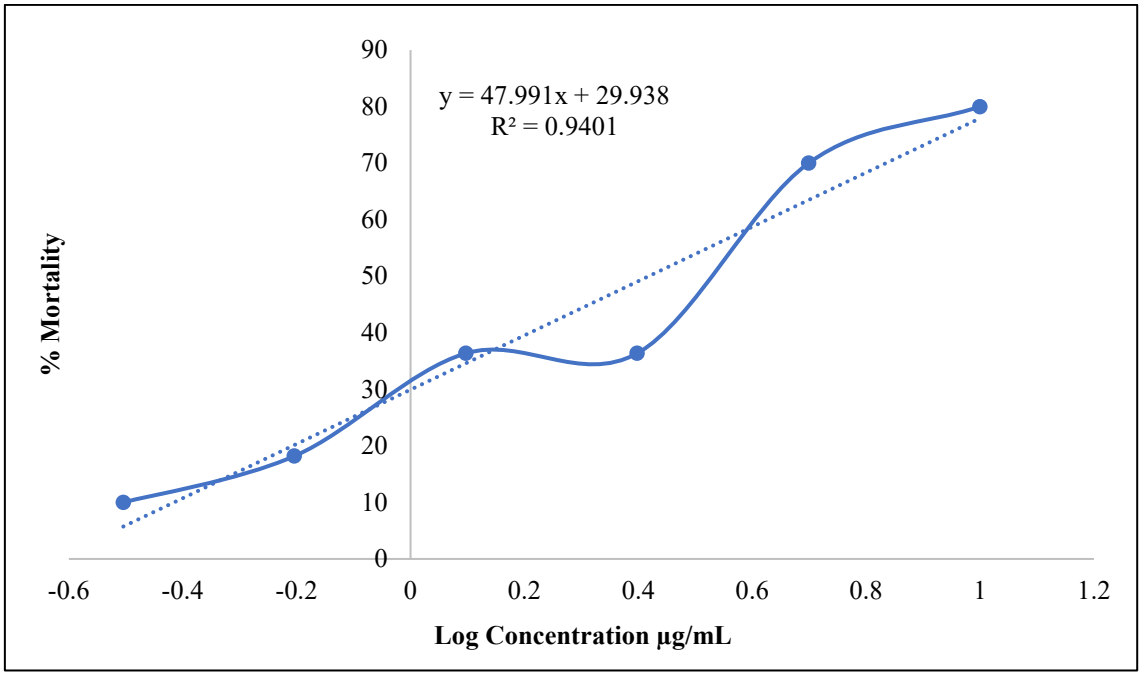

Figure S6 Calibration curve for Vincristine sulfate.

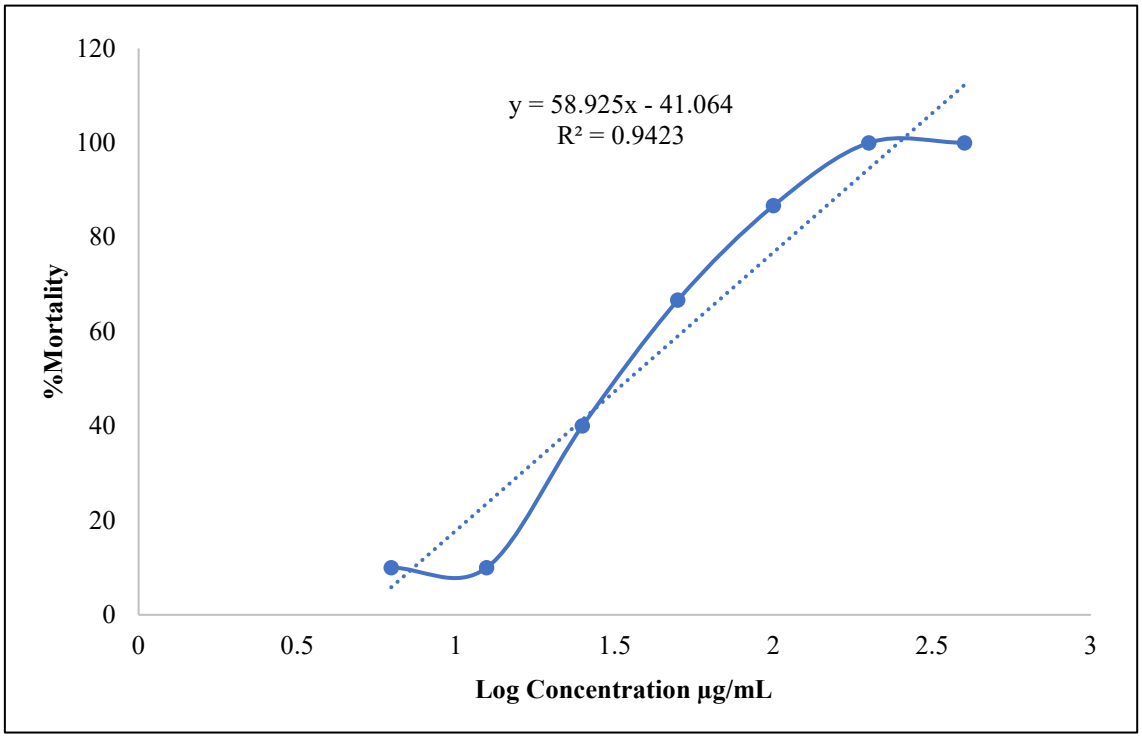

Figure S7 Calibration curve for *S. placentarium* methanol extract (SPM).