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Bioactivity analysis of *Sarcolobus globosus* Wall., a mangrove plant of the Sundarbans

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ABSTRACT

The aspiring prospect for bioactive metabolites has guided this very study to screen for antioxidant, antibacterial and cytotoxic activities of leaf, and bark extracts of Sundarbans mangrove plant, *Sarcolobus globosus* that might lead to novel drug, agrochemicals and nutraceuticals. Methanolic bark extract of *S. globosus* revealed the highest antioxidant properties among all the extracts in DPPH free radical scavenging activity (IC_{50}:26.04 µg/ml), reducing efficiency (EC_{50}:77.72 µg/ml), total phenolic content (47.25 ± 0.002 mg GAE/g of extract), total flavonoid content (101 ± 0.003 mg GAE/g of extract) and total tannin content (93.5 ± 0.007 mg GAE/g of extract). Free radical scavenging activity and reducing power are significantly correlated with total phenol, flavonoid and tannin content. For evaluation of antibacterial activity, Gram positive bacteria (*Bacillus megaterium*, *Staphylococcus aureus*, and *Micrococcus* sp.) and Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Vibrio cholerae*, *Salmonella paratyphi*, *Pseudomonas* sp., and *Proteus* sp.) were used in disc diffusion bioassay. Both extracts possessed significant ($P < 0.05$) inhibition competency against most of the bacteria. Test for cytotoxic activity was carried out by means of brine shrimp lethality bioassay and the chloroformic bark extract demonstrated greater cytotoxicity (LC_{50}19.487 µg/ml) than standard vincristine sulphate (LC_{50} 26.68 µg/ml). Further exploration is required focusing especially the bark extract that could be utilized as source and template for the synthesis of new potential pharma and agrochemicals.

Introduction

As part of an attempt to discover novel lead compounds for pharma and agrochemicals, plant extracts have become the target to spot secondary metabolites with respective biological activities. Therefore, a number of simple bioassays have been established for screening purposes of such bioactive compounds from extracts (Hostettmann, 1991). Oxidative injury plays the vital role in the initiation of numerous neurodegenerative diseases such as stroke, Alzheimer's disease, etc. (Senol *et al.*, 2010). Antioxidants provide protection by inhibiting lipid peroxidation and scavenging radicals and thus terminate the progress of many chronic diseases. Natural antioxidants including phenolic acids, flavonoids, vitamins and carotenoids found in higher plants are being used for pharmaceutical as well as food and feed formulations as active compounds (Qi *et al.*, 2005; Athukorala *et al.*, 2006; López *et al.*, 2007). In parallel, the extensive emergence of multidrug resistant bacteria (MDR) is making the researchers interested to find unique entities to eliminate these bacteria. In this case, plant can exhibit the path by being a crucial source of diversified chemical compounds against MDR (Tchinda *et al.*, 2017).

However, due to excessive use and lack of adequate knowledge of other detrimental by-products possessing by some plants, harmful impacts have been observed through the use of local medicinal plants and that's why, it is necessary to determine the toxicity of medicinal plants (Olowa and Nuñeza, 2013). Moreover, general bioassay regarding toxicity is considered a useful tool for preliminary assessment as well as detection of cytotoxic (Siqueira *et al.*, 1998), antimalarial (Pérez *et al.*, 1997), insecticidal (Oberlies *et al.*, 1998), antitumor (Meyer *et al.*, 1982), anti-parasitic (Ziegler *et al.*, 2002) and anti-rodent compounds (Lyoussi *et al.*, 2018).

Mangroves inhabit the intertidal forest wetlands at the interface between land and sea with numerous physical stress conditions including high salinity, extreme tides, variation in moisture, or biological stress factors. Therefore, to cope with these extreme environments, it is

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assumed that they would produce exceptional natural products on their own (Salini, 2015). Many mangrove plant species have their uses in folk or traditional medicine as cures for various ailments. As a consequence, traditional uses of mangroves draw the attention of the scientific communities to find out the pharmaceutical products to combat several serious diseases (Lin *et al.*, 1999; Bandaranayake, 2002). One important medicinal plant, *S. globosus* Wall. (Asclepiadaceae), known as Baoalilata or Caw phal in Bengali, is a prostrate or climbing shrub growing in the mangrove forest of the Sundarbans estuary, situated in the southwest of Bangladesh (Naskar, 2004; Hossain, 2014). In traditional medicine, the plant has been used to treat rheumatism, dengue and fever (Kuddus *et al.*, 2011).

The present study was designed to enrich the scientific data on *S. globosus* as a potential source providing potent bioactivity. In the light of above context, the goal of this study was to evaluate antioxidant, antibacterial and cytotoxic activities possessing by bark and leaf of this plant. For better understanding of such biological activities, polar solvent methanol and non-polar solvent chloroform were used to extract the bioactive metabolites from *S. globosus*.

Materials and Methods

Collection of plant material

The plant sample of *S. globosus* was collected from Dhangmaree, Chadpai range, East zone of the Sundarbans East Division, Khulna, Bangladesh. Collected plant samples were identified and representative specimens (AA-KU-2018014) were deposited at the herbarium of the Forestry and Wood Technology Discipline, Khulna University, Khulna, Bangladesh.

Extraction

Leaf and bark of the plant were separated and cut into small pieces followed bygentle washing with distilled water. After completion of sun drying, the plant materials were ground into powdered form with a grinder and kept in a dry, cool and dark place in a suitable airtight container until further analysis. The powder of plant parts was transferred into different clean, flat-bottomed glass jars and soaked into chloroform (Merck, Germany). They were then sealed and kept for a period of 5 days in a dark room. Individual mixtures were filtered using white cotton material. After filtration, sample was re-extracted using methanol (Merck, Germany) and filtered in the similar manner. Filtrates were evaporated yielding the chloroformic and methanolic extracts, respectively and stored in a refrigerator for experimental uses.

Determination of antioxidant activity

Measurement of DPPH free radical scavenging activity

The free radical scavenging property of extracts was evaluated by DPPH assay established by Brand-Williams *et al.* (1995). Different concentrations (1.75, 3.13, 6.25, 12.5, 25, 50, 100, 200 and 400µg/ml) of the extracts and the positive control of Quercetin (Sigma Aldrich, Germany) in ethanol (Merck, Germany) were prepared by serial dilution. Then, 2 mL of 0.004% DPPH (Sigma Aldrich, Germany) solution was added in each test tube of different concentrations. DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank. The test tubes were kept for 30 minutes at dark to complete the reaction and after this the absorbance of each sample was measured at 517nm and recorded (Gupta *et al.*, 2003). The experiment was carried out in triplicate.

Percent scavenging activity was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{absorbance of blank - absorbance of sample}}{\text{absorbance of blank}} \times 100$$

Determination of reducing power

The reducing power assay of extracts was conducted following Afrin *et al.* (2016). Various concentrations of extracts (25, 50, 100, 200, 400 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] (purchased from UNICHEM, China). After 20 minutes incubation at 50°C, 2.5 ml of trichloroacetic acid (10%) was added to the mixture and centrifuged at 3000 rpm (605 × g) for 10 minutes. Before the absorbance was measured at 700 nm, the 2.5 ml upper layer of the solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ (Merck, Germany). Blank was prepared in the same way as the sample without addition of extract or standard. Increased absorbance of the reaction mixture indicated increased reducing power. Reducing power with absorbance 1.0 is equal to100% of reductivity. The effective concentration obtaining 0.5 of absorbance or 50% reductivity, is assumed to be EC_{50} (µg/mL) (Sugahara *et al.*, 2015).

Determination of total phenol, flavonoid, tannin content

Total phenol content of the extracts was measured by applying the Folin-Ciocalteu (FC) assay (Petros and UY, 2010). FC reagent was purchased from Merck, India. In this assay, 1 ml of extract was added to 9 ml of distilled water and then 1 mL of FC reagent (10-fold diluted with distilled water) was mixed with it. After 5 min, 10 ml of 7% Na_2CO_3 (Merck, India) was added to the mixture, kept for 30 minutes and then the absorbance was measured at 750 nm using UV spectrophotometer. The total phenolics was calculated from the calibration curve

of gallic acid (Merck, India) and expressed as mg gallic acid equivalent (GAE)/g of plant extract.

By using an aluminium chloride colorometric assay (Petros and UY, 2010), total flavonoid content of the extracts was determined, where 1 ml of extract was added to distilled water (5 ml); 0.3 ml 5% $NaNO_2$ (Merck, India) was then added to the mixture followed by the addition of 0.6 ml 10% $AlCl_3$ (Merck, India) and 2 ml 1M NaOH (Merck, India). Standard (quercetin) solution (25, 50, 100, 200, 400 µg/ml) was prepared for creating standard calibration curve. The absorbance was measured at 510 nm; the total flavonoid was calculated from the calibration curve of quercetin and expressed as mg quercetin equivalent (QE)/g of plant extract.

In accordance with the Folin-Denis method as described by Polshettiwar and Ganjiwale (2007), total tannin content was determined. One ml of extract solution (100 µg/ml) was mixed with 7.5 ml of distilled water and 0.5 ml of FC reagent (Merck, India). After 5 minutes, 1ml of 35% Na_2CO_3 was added and the final volume was adjusted to 10 ml with distilled water. The mixture was kept at room temperature for 30 minutes and absorbance was recorded at 725 nm. For calibration curve, gallic acid was used as standard.

Determination of antibacterial activity

Antibacterial activity of *S. globosus* extracts was evaluated by disc diffusion method (Bauer *et al.*, 1966). Three Gram-positive (*Micrococcus* sp., *Staphylococcus aureus* (ATCC 25923), *Bacillus megaterium* (ATCC 14581)) as well as seven Gram-negative bacteria (*Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27833), *Salmonella typhi* (ATCC 13311), *Vibrio cholerae* (ATCC 9458), *Salmonella paratyphi* (ATCC 9150), *Pseudomonas* sp. and *Proteus* sp.) used in this study were collected as pure cultures from the Biochemistry Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh. The bacterial isolates were cultured in nutrient broth at 37 °C for 24 hours. The sterile filter paper discs were prepared by adding desired concentration (250 and 500 µg/disc) of extracts on the disc with the help of a micropipette. Bacterial broth culture was spread over the nutrient agar medium. Sample impregnated discs, standard Erythromycin disc (10 µg/disc) and negative control discs were placed gently on the solidified agar plates, freshly seeded with the test organisms with the help of sterile forceps. Finally, the plates were incubated overnight at 37˚C and then checked for the zone of inhibitions.

Screening of cytotoxic activity

Brine shrimp lethality bioassay was carried out for the cytotoxicity test and vincristine sulphate was used as positive control (Meyer *et al.*, 1982; Afrin *et al.*, 2016). The eggs of the brine shrimp, *Artemia salina* and sea water were collected from BRAC Prawn Hatchery, Bagerhat, Bangladesh. *S. globosus* extracts were dissolved in DMSO and each test tube contained 4 mL of sea water with different concentrations of extracts (5, 10, 20, 40, 80, 160, 320 µg/ml). The final volume for each test tube was adjusted to 10 mL with artificial sea water and 10 living nauplii were transferred into each tube. After 24 hours, the number of survived nauplii was recorded. The lethal concentration (LC_{50}) values of the plant extracts were obtained by a plot of percentage of the dead nauplii against the concentrations of the extracts.

Statistical analysis

The results were expressed as means ± standard deviation (SD). P values < 0.05 were considered as the threshold level of significance. Experimental results of antioxidant activity evaluation were analyzed for Pearson's correlation coefficient. Statistical analysis for disc diffusion method was evaluated by two-way ANOVA followed by Tukey's multiple comparisons test. Regression analysis was conducted for analyzing the data obtained from brine shrimp lethality bioassay to observe the relationship between different samples and vincristine sulphate as standard. The statistical analysis was carried out using Graph Pad Prism 6.

Results and Discussion

Antioxidant related activity

As shown in Figure 1, with the increase of concentration, both extracts and standard provided enhanced free radical scavenging activities. Figure 2 shows the reducing activities of various extracts in comparison to quercetin as standard. The higher the absorbance of the reaction mixture, the higher would be the reducing power. Hence, *S. globosus* extracts exhibited concentration-dependent reducing power.

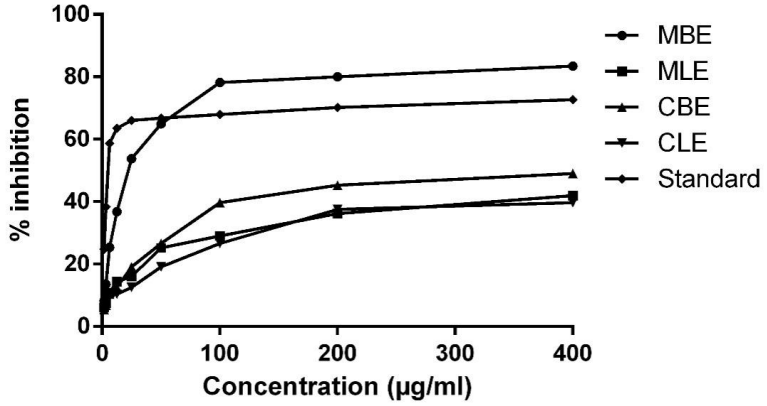

Fig. 1 DPPH free radical scavenging activity of different extracts of *S. globosus* and quercetin (MBE-methanolic bark extract, MLE-methanolic leaf extract, CBE-chloroformic bark extract, CLE-chloroformic leaf extract).

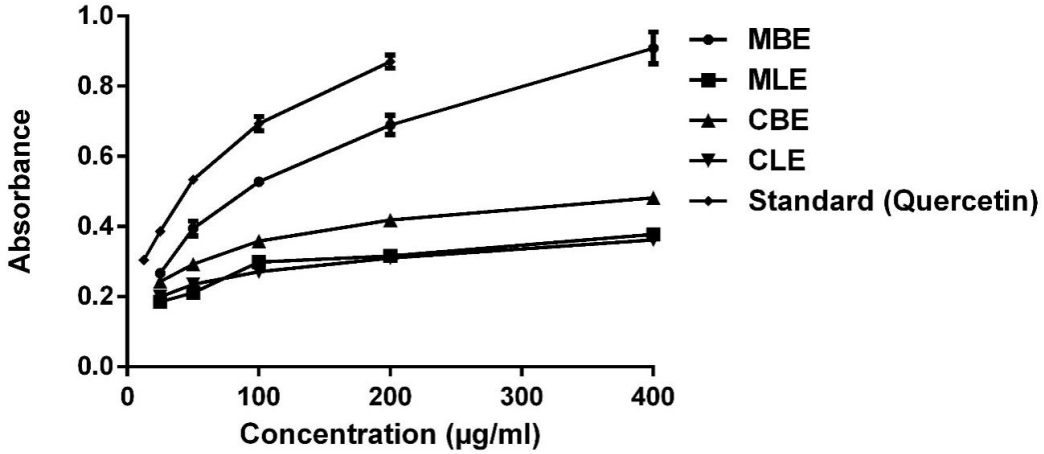

Fig. 2 Reducing power of different types extracts of *S. globosus*. (MBE-methanolic bark extract, MLE- methanolic leaf extract, CBE-chloroformic bark extract, CLE-chloroformic leaf extract)

Table 1 depicts the summarized results of antioxidant activities as IC_{50} value of DPPH free radical scavenging activity, EC_{50} value of reducing power as well as total phenolic content (mg GAE/g), total flavonoid content (mg QE/g) and total tannin content (mg GAE/g) of all extracts. Methanolic bark extract has maximum inhibitory activity (IC_{50}~26.04 µg/ml) against the DPPH free radical among all extracts. As can be seen in Table 1, 50% effective concentration of standard was 37.87µg/ml. Although the reducing power of methanolic bark (EC_{50}~77.72µg/ml) was lower than the standard, it gave higher activity than other extracts.

The amount of total content of phenolics varies in different extracts ranging from 10.75 to 47.25 mg GAE/g (Table 1). The total phenolic content of extracts is in descending order of MBE > CBE > MLE > CLE while total flavonoid content of different extract varies from 59 to 121.25 mg GAE/g with a decreasing order of MBE > MLE > CBE > CLE. Phenolic compounds of plants fall into several categories; flavonoids are the most dominant class of phenolic compounds among these categories which have potent antioxidant activities (Nunes *et al.*, 2012). Moreover, antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups (Sharififar *et al.*, 2009). Consequently, variation in reaction pattern and structure of flavonoids might be the reason of no DPPH scavenging activities in MLE and CLE, in spite of possessing moderate amount of flavonoid.

In the case of total tannin content, highest total tannin content (93.5 mg GAE/g) belonged to MBE (Table 1). Additionally, the ranking order for total tannin content of the extracts is MBE > MLE > CBE > CLE. Li *et al.* (2009) and Kumar *et al.* (2014) found significant correlation between DPPH activity and total phenolic content (correlation coefficient r = 0.760 and r = 0.994, respectively). As can be observed in Table 2, DPPH scavenging activity and reducing power of *S. globosus* extracts are significantly correlated with total phenolic, flavonoid and tannin contents.

Bioactivity of S. globosus

Table 1. Antioxidant properties of different types of extracts from *S. globosus*

Extracts	DPPH IC_{50} (µg/ml)	Reducing power EC_{50} (µg/ml)	TPC (mg GAE/g)	TFC (mg QE/g)	TTC (mg GAE/g)
CLE	3162.28	4859.54	10.75 ± 0.005	59 ± 0.001	3.5 ± 0.003
CBE	498	503.58	17 ± 0.001	63.25 ± 0.004	7.25 ± 0.001
MLE	2,344.22	2320.34	13 ± 0.002	79.5 ± 0.009	10.125 ± 0.005
MBE	26.04	77.72	47.25 ± 0.002	121.25 ± 0.013	93.5 ± 0.007
Standard (Quercetin)	7.65	37.87	-	-	-

CLE=Chloroformic leaf extract, MLE=Methanolic leaf extract, CBE=Chloroformic bark extract, MBE=Methanolic bark extract, TPC=Total phenolic content, TFC=Total flavonoids content, TTC=Total tannin content, GAE=Gallic acid equivalent, QE=Quercetin equivalent, IC_{50}=50% inhibition concentration. EC_{50}=50% effective concentration

Therefore, these phytochemicals might be some of the major contributors responsible for the antioxidant efficacy of *S. globosus*. DPPH scavenging activity is significantly correlated with phenol ($p \leq 0.001$, r = 0.9822) (Table 2). Reports have suggested that there is a correlation between the total phenolic content and antioxidant activity of plant extracts (Zhang and Wang, 2009). However, low level of correlation was found between the flavonoid content and the antioxidant activities of the extracts in this study. Different extraction methods and antioxidant assays may be responsible for this lower correlation with flavonoid content.

Antibacterial activity

The data pertaining to the antibacterial potential of the plant extracts are presented in Table 3. For the interpretation of antibacterial assay results, we have implemented the scale of measurement according to Carović-Stanko *et al.* (2010) which considers zone of inhibition value of >15 mm as strongly inhibitory, 10-15 mm as moderately inhibitory and <10 mm as not inhibitory (Carović-Stanko *et al.*, 2010). The antibacterial activity was detected at 25 µg/µl and 50 µg/µl concentrations, where highest activities were found at 50 µg/ml concentrations.

Table 2. Correlation among antioxidant activities and total phenolic, flavonoid and tannin contents of *S. globosus* extracts

	Correlation Coefficient r		
Parameter	Phenol	Flavonoid	Tannin
DPPH assay	0.987**	0.895*	0.995**
Reducing power	0.998***	0.872*	0.982**

* indicates significance at $p \leq 0.05$, ** indicates significance at $p \leq 0.01$, *** indicates significance at $p \leq 0.001$.

Table 3. Mean values of inhibition zone diameter (mm) of *S. globosus* extracts and standard.

Bacteria	Conc. (µg/ml)	CLE	MLE	CBE	MBE	Erythromycin
B. megaterium	25	7±0	9.25±0.5	7.5±0.58	8.75±0.5	28.75±0.5
	50	10.5±0.5	13.25±0.95	11.5±0.58	12.25±0.95	
S. aureus	25	-	-	7.75±0.5	-	28±1.41
	50	-	-	10.75±0.95	-	
Micrococcus	25	7.25±0.5	10.5±0.58	8.75±0.5	-	21.75±0.98
	50	9.25±0.58	13.75±0.5	13.75±0.5	-	
E. coli	25	8.75±0.5	9.75±0.95	7.5±0.58	7.25±0.5	15±0.82
	50	11.75±0.5	12.5±0.58	10±0.81	10.5±0.58	
P. aeruginosa	25	7.75±0.5	11.25±0.5	8.5±0.58	11.75±0.5	27.75±0.96
	50	13.75	14.75±0.5	14.25±0.95	15.5±0.58	
S. typhi	25	-	11.5±0.58	8.75±0.5	8.75±0.5	29.5±1.29
	50	7.5±0.58	16.25±0.5	14.75±0.5	12.75±0.5	
V. cholerae	25	8.75±0.5	9.25±0.5	13.75±0.5	7.5±0.75	32.25±0.96
	50	10.75	13.25±0.5	21.5±0.58	10.75±0.5	
S. paratyphi	25	-	8.75±0.5	11±0.82	8.5±0.57	18±0.82
	50	7.5±0.58	12.75±0.5	15.5±0.58	11.75±0.5	
Pseudomonas	25	7.25±0.5	8.25±0.5	8.5±0.58	9.75±0.5	28.5±1.29
	50	12.75±0.5	10.5	10.75±0.5	11.5±0.58	
Proteus	25	-	7.25±0.5	10.5±0.58	-	32.75±0.98
	50	8.75±0.5	9.75±0.5	14.5±0.58	7±0	

MBE-methanolic bark extract, MLE-methanolic leaf extract, CBE-chloroformic bark extract, CLE-chloroformic leaf extract; Significant at P< 0.05.

Antibacterial activity revealed that chloroformic extract of bark strongly inhibited the growth of *V. cholerae* (ZOI=21.5 mm) and *S. paratyphi* (ZOI=15.5 mm). Moreover, *P. aeruginosa* (ZOI=15.5 mm) and *S. typhi*

(ZOI=16.25 mm) were strongly sensitive to methanolic bark and leaf, respectively. In the case of leaf, mehanolic extract exhibited larger zone of inhibition than chloroformic extract at respective concentrations. From analyzing the results, it can be stated that there were moderate to strong activities against all the test bacteria in the chloroformic extract from bark. Based on statistical analyses, the antimicrobial action of the extracts for concentrations 250 ìg/disc and 500 ìg/disc (Table 3) differed significantly for any of the strains tested except the cases where there is no zone of inhibition at both concentrations. Moreover, the diameter of the inhibition at 250 ìg/disc and 500 ìg/disc was significantly different from the diameter of zone for standard erythromycin.

Cytotoxic activity

Brine shrimp lethality activity of *S. globosus* extracts are listed in Table 4. The bark chloroform extract is the most active extract among all extracts tested, presenting an LC_{50} of 19.487 µg/ml which is less than the standard (LC_{50} 26.68 µg/ml). In addition, chloroformic leaf extract exhibits LC_{50} of 28.872 µg/ml, which is very close to the standard value. These extracts can be regarded as a promising candidate for plant-derived antitumor, anti-parasitic and anti-rodent compounds.

Table 4. Brine-shrimp bioassay of different extracts of *S. globosus*

Extracts	LC_{50} (µg/ml)	Regression equation	R^2
CBE	19.487	y = 40.57x – 2.325	0.988
MBE	495.709	y = 16.34x + 5.960	0.987
CLE	28.872	y = 39.16x – 7.192	0.933
MLE	288.4	y = 24.62x – 9.439	0.984
VS	26.68	y = 52.79x – 25.29	0.972

CLE=Chloroformic leaf extract, MLE=Methanolic leaf extract, CBE=Chlorofornic bark extract, MBE=Methanolic bark extract, VS=Vincristine sulphate.

Conclusion

Since a moderately polar and a non-polar solvent system were employed for extraction, bioactivity namely antioxidant, antibacterial and cytotoxicity, were found at different levels. All the extracts showed varying degrees of antimicrobial activity on test microorganisms. The promising and novel finding of this study was that bark extracts either methanolic or chloroformic showed noticeable activity in all the tests, in some cases highest activities were observed. In antioxidant evaluation, methanolic bark demonstrated the highest results, whereas chloroformic bark extracts gave strong activity than the standard in cytotoxic assay. Furthermore, the distinctive result of this study provides striking baseline information for the potential and constructive use of this plant and generates our anticipation that detailed investigation for pharmacological activity may lead to the isolation of interesting pharma and agrochemicals of plant origin through elucidation of the identity of compounds responsible for respective activity.

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