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In vitro evaluation of antimicrobial, cytotoxic and antioxidant activities of Crude methanolic extract and other fractions of *Sterculia villosa* barks

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ABSTRACT

Crude methanolic extract and other fractions of *Sterculia villosa* barks were investigated for their antimicrobial, cytotoxic and antioxidant activity in this study. Antimicrobial activity of different extract was evaluated by measuring the diameter of the zone of inhibition against gram-positive & gram-negative bacteria and fungi using Ciprofloxacin as a standard antimicrobial agent. Free radical scavenging activity for the same extracts was determined by DPPH assay where BHT was used as positive control and Cytotoxicity was determined by Brine Shrimp nauplii where the minimum inhibitory concentration was assessed by serial dilution technique. Mild antimicrobial activity was found; crude methanolic extract showed effect against all the organisms, while other extracts showed effect for some of the organisms. The LC_{50} value for cytotoxicity assay was found 0.3, 2.95, 3.76, 35.33 & 55.98 µg/ml for CSV, PESV, CTSV, DCMSV & EASV extracts respectively where LC_{50} value of Vincristine Sulfate was 0.544 µg/ml. Ethyl acetate fraction showed good antioxidant properties and except Pet Ether fraction all other extracts showed considerable antioxidant activity. The bark of *Sterculia villosa* can be considered for further research for finding potent compounds of antioxidant, antimicrobial and cytotoxic activity.

INTRODUCTION

Infectious disease is one of main causes of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States (Pinner *et al.*, 1996). Besides, an increase in antibiotic resistance in nosocomial and community acquired infections has been found common now-a-days with the most dramatic increases are occurring at the age of 25–44 year (Pinner *et al.*, 1996). Several research programs also showed results of drug resistance to human pathogenic bacteria from all over the world (N'guessan *et al.*, 2007; Lu *et al.*, 2007; Mbwambo *et al.*, 2007; Andremont, 2001). Due to these facts, in recent times extensive attention has been made on finding alternative antimicrobials from natural source. It is scientifically proved that free radicals induce oxidative damage to biomolecules. This damage causes cancer, aging, neurodegenerative diseases, atherosclerosis, malaria and several other pathological events in living organisms (VanWagenen *et al., 1993).* Antioxidants which scavenge free radicals are known to possess an important role in preventing these free radical induced-diseases. There is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and diseases. A number of reports on the isolation and testing of plant derived antioxidants have been described during the past decade. Natural antioxidants constitute a broad range of substances including phenolic or nitrogen containing compounds and carotenoids (Auroma, 1998).

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Synthetic antioxidant such as *tert*-butyl-1-hydroxytoluene (BHT), butylatedhydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) are widely used as food additives to increase shelf life, especially lipid and lipid containing products by retarding the process of lipid peroxidation (Shahidi *et al.,* 1992). However, BHT and BHA are known to have not only toxic and carcinogenic effects on humans, but abnormal effects on enzyme systems (Wichi, 1988).

Therefore, the interest in finding antioxidant from natural source with minimized side effects has greatly increased in recent years (Inatani *et al.,* 1983). Therefore, there is a need for more effective, less toxic and cost effective compounds from natural sources. Several medicinal plants with ethno-botanical uses have been used traditionally in the treatment of diseases and have been exploited for different desired traits (Patel *et al.,* 2010; Okoro *et al.,* 2010; Lagnika *et al., 2011)*.

The present study was conducted on *Sterculia villosa* (Family: Sterculiaceae), which is Sub-tropical to tropical medium-sized, deciduous tree with gray-white bark. Locally it is known as Udal, Chala & Chandul. It has diuretic, cooling and aphrodisiac properties (Kumar *et al., 2004*). Also used by Indians for traditional remedy of Inflammation (Nasma *et al., 2009)*. Flavonoids, terpenoids, phenolics, and histamines were reported to exist in other species of *Sterculia* (Anjaneyulu and Raju, 1987). This study was performed on different extracts of *Sterculia villosa* bark (crude methanolic, Carbon tetrachloride, Pet ether, Dichloromethane & Ethyl acetate extracts) to evaluate its cytotoxic, antimicrobial & antioxidant activity.

MATERIALS AND METHODS

Collection of plant materials

Plant sample of *Sterculia villosa* was collected from Rangamati on 10 September, 2011. Bark of *Sterculia villosa* was washed properly, cut into small pieces and then air dried for several days. The pieces were then oven dried for 24 hours at considerably low temperature and then grounded into coarse powder in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka using high capacity grinding machine.

Extraction of the plant material

About 600 gm of the powdered material was taken in a clean, round bottomed flask (5 liters) and soaked in 2.5 liter of methanol. The container with its content was sealed by foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixtures were then filtered through a fresh cotton plug and finally with a Whatman No.1 filter paper. The volume of the filtrate was then reduced using a Büchi Rotavapor at low temperature and pressure.

Solvent-solvent partitioning

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by (Kupchan and Tsou, 1973). The crude extract (5 gm) was dissolved in 10% aqueous methanol. It was then extracted with Pet Ether (PE), Carbon Tetrachloride (CTC), Dichloromethane (DCM), finally with Ethyl Acetate (EA). All four fractions were evaporated to dryness and were used for the study.

Antimicrobial Activity

Test Organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both gram positive and gram-negative organisms were taken for the test and they are listed in the following (Table 1).

Preparation of the Medium

Nutrient agar medium (DIFCO) was used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures. Nutrient agar medium was prepared by using the composition of 0.5gm Bacto peptone, 0.5gm Sodium chloride, 1.0 gm Bacto yeast extract, 2.0gm Bacto agar & sufficient quantity to 100 ml of distilled water. To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25 °C) was adjusted at 7.2-7.6 using NaOH or HCl. 10 ml and 5 ml of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively.

The test tubes were then capped and sterilized by autoclaving at 15 lbs pressure at 121°C for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glassware were sterilized by autoclaving at a temperature of 121°C and a pressure of 15 lbs/square inches for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized by UV light.

Preparation of Subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37 °C for their optimum growth. These fresh cultures were used for the sensitivity test.

Preparation of the Test Plate

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized

agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

Preparation of Discs

Calculated amount of each test sample was dissolved in specific volume of solvent (Methanol) to obtain the desired concentrations of 400 µg/disc in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried. Standard Ciprofloxacin (5µg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative controls.

Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24 hours.

Brine Shrimp Lethality Bioassay

Brine shrimp nauplii (*Artemia salina*) were used as the test organism. Sea water was prepared with minor alteration of chemical composition as described by Culkin (Culkin *et al., 1965*). 38 gm sea salt (pure NaCl) was weighed, dissolved in one liter of distilled water and filtered off to get a clear solution. Ten test tubes were used for ten different concentrations (one test tube for each concentration) of test samples and ten test tubes were taken for ten concentrations of standard drug Vincristine and another one test tubes for control test.

All the test samples (methanol extract, pet ether, carbon tetrachloride, dichloromethane and ethyl acetate fraction) of 4mg were dissolved in DMSO and solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.78125 g/ml were obtained by serial dilution technique. Vincristine sulfate and DMSO were used as the positive control and negative control respectively. Measured amount of the Vincristine sulphate was dissolved in DMSO to get an initial concentration of 40µg/ml from which serial dilutions were made using DMSO to get 20µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 0.625µg/ml, 0.3125µg/ml, 0.15625µg/ml and 0.078125 µg/ml which were used as positive control. 100 µl of DMSO was added to each of three pre-marked glass vials containing 5 ml of simulated sea water and used as negative control. If the brine shrimps in these vials show mortality, then the test is considered as invalid as the nauplii died due to other reason than the cytotoxicity of the compounds. 10 shrimp nauplii were used in each test tube by using Pasteur pipette (Meyer *et al.,* 1982). The median lethal concentration (LC_{50}) of the test samples after 24 hrs of exposure were determined from a plot of % of the dead shrimps against the logarithm of the sample concentration.

DPPH Assay

DPPH was used to evaluate the free radical scavenging activity (antioxidant potential) of various compounds and medicinal plants (Jayaprakash and Rao, 2000; Choi *et al.,* 2000). T*ert*-butyl-1-hydroxytoluene (BHT) was used as positive control. Calculated amount of BHT was dissolved in methanol to get a mother solution having a concentration 1000µg/ml. Serial dilution was made from mother solution to get different concentration from 500.0 to 0.977 µg/ml. 4 mg of DPPH was weighed and dissolved in methanol to get a DPPH solution having a concentration 20 µg/ml.

The solution was prepared in the amber reagent bottle and kept in the light-proof box. Methanolic extract of the barks of *Sterculia villosa* was partitioned with Petroleum ether, Carbon tetrachloride, Dichloromethane & Ethyl acetate. Calculated amount of different fractions (2mg) were measured and were dissolved in methanol to get a mother solution having a concentration 1000 µg/ml. Serial dilution was made using the mother solution to get different concentration from 500.0 to 0.977 µg/ml.

2.0 ml of a methanol solution of the sample (Control / extractives) at different concentration from 500.0 to 0.977µg/ml were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ ml). After 30 minutes reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. Inhibition of free radical DPPH in percent (I %) was calculated as follows-

$$(I\ \%) = (1- A_{sample}/A_{blank}) \text{ X } 100$$

Where, A_{blank} is the absorbance of control reaction (containing all reagents except the test material). Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.

RESULTS

Evaluation of Antimicrobial Activity

The antimicrobial potency of the test agents were measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale which are presented in (Table 2).

Table. 1: List of micro-organisms used for Antimicrobial Activity.

Gram positive bacteria	Gram negative Bacteria	Fungi
Bacillus cereus	*Escherichia coli*	*Aspergillusniger*
Bacillus megaterium	*Salmonella paratyphi*	*Candida albicans*
Bacillus subtilis	*Salmonella typhi*	*Sacharomycescerevacae*
Sarcinalutea	*Shigellaboydii*	
Staphylococcus aureus	*Shigelladysenteriae*	
	Pseudomonas aeruginosa	
	Vibrio mimicus	
	Vibrio parahemolyticus	

Table. 2: Antimicrobial activity of test samples of *Sterculia villosa*.

	Diameter of the zone of inhibition (mm)					
Test organisms	**CSV**	**CTSV**	**PESV**	**DCMSV**	**EASV**	**Ciprofloxacin**
Gram positive Bacteria						
Bacillus cerus	8	7	-	-	-	42
Bacillus megaterium	7	-	8	-	-	45
Bacillus subtilis	8	-	8	8	-	40
Staphylococcus aureus	7	8	8	8	8	40
Sarcina lutea	8	-	-	9	-	42
Gram negative Bacteria						
Escherichia coli	8	8	9	9	8	43
Pseudomonas aeruginosa	8	8	9	9	-	45
Salmonella paratyphi	8	9	8	9	8	44
Salmonella typhi	8	-	-	9	-	41
Shigella boydi	7	8	8	9	8	44
Shigella dysenteriae	8	8	-	9	8	42
Vibrio mimicus	8	8	9	9	8	41
Vibrio parahemolyticus	8	7	-	7	7	42
Fungi						
Candida albicans	8	9	9	9	8	40
Aspergillus niger	8	8	7	9	8	42
Saccharomyces cerevaceae	8	8	8	*8*	7	42

Table. 3: LC_{50} values of the test samples of *Sterculia villosa* bark.

Test Samples	Regression lines	R^2 values	LC_{50} (µg/ml)
1.Vincristine Sulfate	y=33.223x+58.787	0.9581	0.544
2.Crude Methanol Extract	y=15.07x + 72.78	0.89	0.3
3.Pet ether extract	y= 12.74x+82.24	0.907	2.95
4.Carbon tetrachloride extract	y=30.51x + 21.44	0.935	3.76
5.DCM Extract	y=41.92x -14.9	0.983	35.33
6.Ethyl acetate extract	y=40.33x -20.50	0.938	55.98

Table. 4: Percentage inhibition of different fractions of *Sterculia villosa* bark.

SL	Absorbance of blank	Conc. (µg/ml)	Inhibition (%)					
			BHT	**CSV**	**PESV**	**CTSV**	**DCMSV**	**EASV**
1.	0.425	500	83.52	79.05	91.85	93.88	79.53	94.12
2.		250	75.06	66.82	51.06	93.41	79.06	93.18
3.		125	69.41	64.24	34.82	93.17	78.35	92.47
4.		62.5	57.88	63.05	24.24	92.47	78.12	92.00
5.		31.25	50.35	61.88	22.35	70.12	63.05	89.64
6.		15.625	43.76	43.05	21.88	52.24	42.35	84.70
7.		7.813	32.71	33.88	21.17	37.88	24.70	57.64
8.		3.906	20.71	28.23	20.7	28.47	17.65	42.35
9.		1.953	12.94	26.11	20.47	25.88	16.94	35.53
10.		0.977	8.47	12.23	20.00	20.94	11.06	28.23

Table. 5: IC_{50} values of standard and different partitions of *Sterculia villosa* bark.

Test sample	IC_{50} (µg/ml)
BHT	31.72
Crude Methanolic extract	27.28
Pet ether extract	177.62
Carbon tetrachloride extract	10.34
Dichloromethane extract	23.70
Ethyl acetate extract	3.76

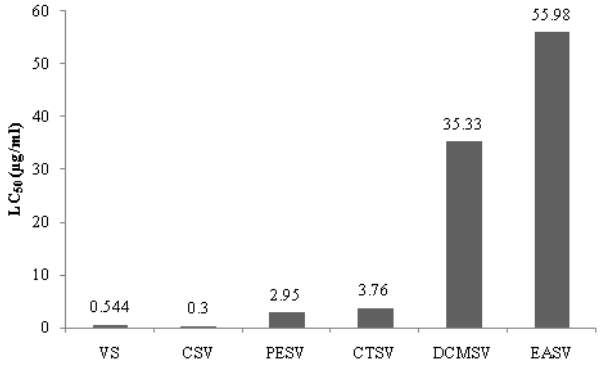

Fig. 1: Comparative cytotoxic study of different fractions of *Sterculia villosa*.

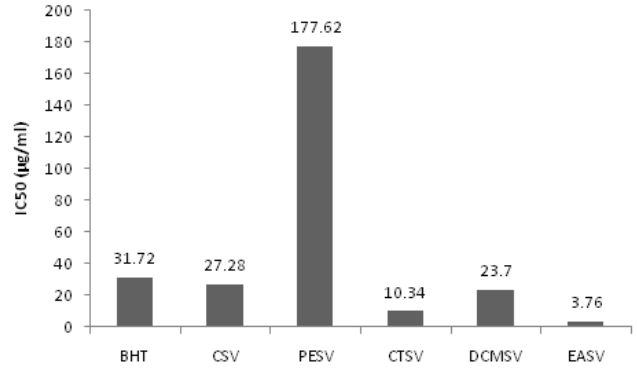

Fig. 2: IC_{50} values of the standard and different partitions of *Sterculia villosa*.

Brine Shrimp Lethality Bioassay

The methanolic crude extract and the different fractions of the methanolic extract of the bark of *Sterculia villosa* were subjected to brine shrimp lethality bioassay following the procedure of Meyer (Meyer *et al*., 1982). The lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best -fit line was obtained from the curve data by means of regression analysis. Vincristine Sulphate (VS) was used as positive control and the LC_{50} for VS was found as 0.544 µg/ml. Compared with the negative control, VS (positive control) gave significant mortality and the LC_{50} values of the different extractives were compared with negative control which has been shown in (Table 3; Figure 1).

Evaluation of Antioxidant activity

The methanolic crude extract and the different fractions of the bark of *Sterculia villosa* were subjected to determine free radical scavenging activity using activity of BHT as standard (Table 4 & 5; Figure 2).

DISCUSSION

In the last few decades, multiple antibiotic drug resistance in pathogenic bacteria has been increased worldwide (Perez *et al.,* 1990). Due to repeated use of antibiotics and drugs against new strains of pathogenic bacteria. These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. It is this last solution that would encompass the development of new antimicrobials (Fauci, 2005). Due to this fact, finding new generation of drugs from natural source of origin gaining greater interest (Gericke, 2002; Burt, 2004). The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the in-vitro fungal and bacterial growth. In this investigation, antimicrobial activity of different plant extracts of the bark of *Sterculia villosa* was studied and found mild antimicrobial responses. Only crude methanolic extracts showed activity against all micro-organisms used in the study whereas PESV, CTSV, DCMSV & EASV extract showed response against some of the organisms.

The test results of brine shrimp lethality bioassay revealed strong mortality in vitro toxicity compared to Vincristine Sulfate. Pet ether and Carbon tetrachloride fraction also showed some mortality and found LC_{50} value 2.95 & 3.76 µg/ml respectively.

From the DPPH assay, it was observed that the ethyl acetate fraction (IC_{50} 3.76 µg/ml) of *Sterculia villosa* bark has shown good free radical scavenging activity compared to standard BHT (31.72 µg/ml). Whereas methanolic crude extract (27.28 µg/ml), carbon tetrachloride (10.34 µg/ml) and dichloromethane (23.7 µg/ml) fractions also have shown free radical scavenging activity, but less than ethyl acetate fraction. Pet ether fraction has no free radical scavenging activity.

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