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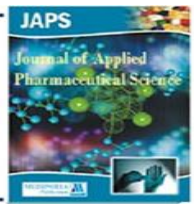

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Evaluation of antioxidant, antibacterial, antifungal and cytotoxic effects of *Clausena suffruticosa* ethanolic root extract

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ABSTRACT

Clausena suffruticosa (Family: Rutaceae) root ethanolic extract was subjected to analyze for its antioxidant effect by DPPH free radical scavenging method, antibacterial & antifungal effect by disc diffusion technique and cytotoxic effect by brine shrimp lethality test. The extract showed very significant antioxidant activity with the LC_{50} value of 3.28µg/ml. In antibacterial screening, moderate zone of inhibition (7.5-9.0mm in diameter) was observed against gram positive *Bacillus subtilis, Bacillus cereus, Streptococcus aureus, Bacillus polymyxa & Bacillus megaterium* and promising zone of inhibition (10.0-13.0mm in diameter) against gram negative *Salmonella typhi, Shigella flexneri,, Proteus sp. & Escherichia coli. Klebsiella sp.* and *Shigella sonnei* did not show sensitivity. The MIC values against these bacteria were ranged from 3,000 to 4,500µg/ml. Root extract showed significant zone of inhibition against *Aspergillus ustus, Aspergillus niger and Aspergillus ochraceus* in antifungal assay. In the cytotoxicity assay, LD_{50} and Chi-square value of the ethanolic extract against brine shrimp nauplii were 546.94µg/ml and 1.9932 demonstrating potent cytotoxic effect of the extract.

Key words: *Clausena suffruticosa*; Antioxidant; Antibacterial; Antifungal; Cytotoxic.

INTRODUCTION

Clausena suffruticosa Rob (family: Rutaceae) is popularly known as Sadi Urisha, has been commonly used in Chakma herbal medicine. It is rarely distributed in Chittagong Hill tracts of Bangladesh, Eastern Himalayan regions and Kashi Hill of India and Burma. Traditionally, the roots, leafs and seeds have been used for some chronic diseases like paralysis, tumors and disease related to kidney and liver, pain, bleeding and fever. It is also used for mumps, viral pneumonia, cerebrospinal meningitis etc. But no scientific documentation has been on the uses of this plant except on its local uses advised by Chakma Herbalists. We have attempted to evaluate the antioxidant, antibacterial, antifungal and cytotoxic effects of *Clausena suffruticosa* (C. *suffruticosa*) root ethanol extract in this study.

MATERIAL AND METHODS

Plant material collection

Plant materials were collected from various regions of Khagrachari, Chittagong Hill tracts of Bangladesh. The plant was taxonomically identified by Dr. Md. Mostofa Kamal Pasha (Professor and Taxonomist, Department of Botany, University of Chittagong, Chittagong, Bangladesh) and identification was confirmed by Sarder Nasir Uddin (Taxonomist, Bangladesh National Herbarium, Ministry of Environment and Forest, Dhaka, Bangladesh). A voucher specimen is preserved in Bangladesh National Herbarium with the accession No. DACD- 32909.

The fresh roots of *C. suffruticosa* were washed with water immediately after collection. Then chopped into small pieces, air dried at room temperature for about 10 days and pulverized into powder (1kg) which was macerated in 6L pure ethanol for 7 days at room temperature (23±5)^{0}C. After 7 days, extract was filtered off through cotton plug and finally with a Whatman No. 1 filter paper. Then extract was concentrated under reduced pressure below 50^{0}C through rotatory vacuum evaporator (RE200 Sterling, UK). The concentrated extract (45 gm blackish-green, 4.5% w/w yield) was stored at 4^{0}C.

Preparation of Media

Mueller-Hinton agar media (Hi media, India), final P^H 7.3±0.2 (at 25^{0}C), was used for antibacterial and MIC determination. On the other hand, potato dextrose agar media (Hi media, India), final P^H (at 25^{0}C) 5.6±0.2 and artificial seawater (3.8 % NaCl solution) were used for the determination of antifungal and cytotoxic effect.

Assay of antioxidant effect

The antioxidant activity of *C. suffruticosa* root ethanol extract and the standard antioxidant ascorbic acid was assessed on the basis of the radical scavenging effect of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical activity according to the method described by Brand-William *et al.* (1995) with slight modifications. *C. suffruticosa* root ethanol extract with different concentrations (10, 50, 100, 200, 400, 600, 800 and 1000 µg/ml) were prepared in ethanol. Ascorbic acid was used as standard in 1-100 µg/ml solution. 0.004 % of DPPH solution was prepared in ethanol and 5 ml of this solution was mixed with 5 ml of extract solution and standard solution separately. These solution mixtures were kept in dark for 30 min. The degree of DPPH purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract. The absorbance of the mixture was determined at 517 nm using UV-Visible Spectrophotometer (Cintra, Australia) and ascorbic acid was served as a positive control. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The scavenging activity against DPPH was calculated using the following equation: Scavenging activity (%) = [(A-B)/A] x100, Where *A* was the absorbance of control (DPPH solution without the sample), *B* was the absorbance of DPPH solution in the presence of the sample (extract/ascorbic acid). Then, % scavenging activity was plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis.

Assay of antibacterial effect

In vitro antibacterial screening was carried out by disc diffusion method (Bauer *et al*, 1966), which is a qualitative to semi-qualitative test. The bacterial suspension turbidity adjusted to McFarland standard number 0.5, in Mueller Hinton Broth (Hi-media, India). With a sterile cotton swab bacterial culture was streaked on previously prepared Mueller Hinton agar plate (Hi-media, India). Dried and sterilized paper discs were treated separately with desired concentration of previously prepared ethanolic solution of the root extract using a micropipette dried in air under aseptic condition and placed at equidistance in a circle on the seeded plate. The concentrations of root extract used were 2mg/disc and 3mg/disc. These plates were kept for 4-6 hours at low temperature and the test materials diffuse from disc to the surrounding medium by this time. The plates were then incubated at 37^{0}C for 18 hours. The diameter of zone of inhibition produced by root extract was then compared with standard antibiotic Kanamycin (30µg/disc). Each sample was used in triplicate for the determination of antibacterial activity. Blank disc impregnated with solvent ethanol followed by drying off was used as negative control.

Determination of MIC

Minimum inhibitory concentrations (MIC) of crude extract of the *C. suffruticosa* were performed by macrodilution method (Saha and Rahman, 2008). The crude extract was dissolved in 30% dimethyl sulfoxide (DMSO) to obtain 10% (w/v) solution. For MIC test of the selected bacteria, the extract was first diluted in sterilized Mueller-Hington broth to the highest concentration of 10,000µg/ml and then dilution were performed at concentration of 5000µg, 4000µg/ml, 3500µg/ml, 3000µ/ml, 2500µ/ml, 2000µg/ml, 1500µg/ml, 1000µg/ml, 750µg/ml, 500µg/ml and 250µg/ml in screw caped tube containing broth medium. Bacterial suspensions of the test organism were prepared in sterilized Mueller-Hington broth. Then 1ml of the dilution was added to each sterilized screw capped tube containing 1ml of compound suitably diluted in the sterilized broth medium to give final volume of 2ml. Culture medium without samples and others without microorganisms were used in the tests as control. Tubes were incubated at 37^{0}C for 20-24 hours and growth was indicated by turbidity.

Assay of antifungal effect

The poisoned food technique (Grover & Moore, 1962) was used to screen for antifungal activity. Potato dextrose agar was used as a culture medium. From this required concentration of extract was taken by sterilized pipette in a sterilized petriplate and then 15 ml medium was poured into the petriplate and mixed well and allowed to solidify. Inoculation was done at the center of each plate with 5 mm mycelium block for each fungus. The mycelium block was prepared with the help of cork borer from the growing area of a 5 days old culture of the test fungi on PDA. The blocks were placed at the center of each petriplate in an inverted position to get greater contact of the mycelium with the culture medium. The inoculated plates were incubated at (25±2)^{0}C. The experiment was repeated for three times. Proper control (PDA without extract) was also maintained. Diameter of fungal colonies was measured after 5 days of incubation. The average of three measurements was taken as colony diameter of the fungus in millimeters. The percentage inhibition of mycelial growth of the test fungus was

calculated by the following formula:

$$I= (C-T)/C \times 100$$

where, I=Percentage of Inhibition, C=Diameter of the fungal colony in control, T=Diameter of the fungal colony in treatment.

Assay of cytotoxicity effect

Cytotoxic activity of ethanolic roots extract was determined by Brine-Shrimp Lethality assay as described by Meyer *et al*., (1982). Simple zoological organism (Artemia saline) was used as a convenient monitor for the screening. The eggs of the brine shrimp were collected from Institute of Marine Science and Fisheries, University of Chittagong, Bangladesh and hatched in artificial seawater (3.8% NaCl solution) for 48 hours to mature shrimp called nauplii. Then 30 mg of *C. suffruticosa* root extract were separately dissolved in 3 ml of DMSO, and from these 1000, 500, 250, 125 and 62.5 µg/ml were prepared by serial dilution.

Each concentration was tested in triplicate, giving a total of 15 test tubes for each sample. A control containing 5 ml of DMSO solvent was used for each solvent. The final volume of the solution in each test-tube was made up to 5 ml with seawater immediately after adding shrimp larvae. The test tubes were maintained under illumination. After 24 hours have elapsed, Survivors were counted with the aid of a 3x magnifying glass. The LD_{50} values were calculated from Probit Chart using computer software "BioStat-2007".

RESULT AND DISCUSSION

The antioxidant activity of the extracts was assessed by the DPPH free radical scavenging assay as shown in Table 1. The root extract exhibited significant DPPH free radical scavenging effects compared to standard ascorbic acid. Where, IC50 value of ascorbic acid was 5.46µg/ml and root extract was 3.28µg/ml.

Table 1. DPPH free radical scavenging effect of *C. suffruticosa* root extract (CSRE) and ascorbic acid (AA).

Sample Con. (µg/ml) CSRE/AA	Log Con.	Absorbance		Percent (%) of scavenging activity		IC_{50} (µg/ml)	
		CSRE	AA	CSRE	AA	CSRE	AA
Control	-	0.8146	0.8146	-	-		
10	1.00	0.3588	0.4012	55.95	50.78		
50	1.70	0.2699	0.2149	66.86	73.62		
100	2.00	0.0378	0.1281	95.35	84.27		
200	2.30	0.0458	0.0804	94.37	90.13	3.28	5.46
400	2.60	0.0479	0.0609	94.11	92.52		
600	2.78	0.0566	0.0446	93.05	94.52		
800	2.90	0.0256	0.0239	96.85	97.06		
1000	3.00	0.0110	0.0109	98.64	98.66		

The results of antibacterial activity of root extract against the test bacteria are presented in Table 2. Root extract showed highest activity against *Proteus sp*. and lowest activity against *Bacillus subtilis* but resistance to *Klebsiella sp*. and *Shigella sonnei*. On the other hand, *Proteus sp.* showed resistance against Kanamycin but sensitivity to root extract.

Table 2: In-vitro antibacterial activity of CSRE and Kanamycin.

Test organism		Source ID	Zone of inhibition(diameter in mm)		
			CSRE (2mg/disc)	CSRE (3mg /disc)	Kanamycin (30µg/disc)
Gram positive	*Bacillus subtilis*	BTCC 17	6	7.5	21
	Staphyl. aureus	BTCC 43	7.5	8	24
	Bacillus cereus	BTCC 19	7	8	18
	B. polymyxa.	BTCC 16	7	9	21
	B.megaterium	BTCC 18	7.5	8.5	28
	Klebsiella sp.	ICDDR'B	-	-	12
Gram negative	*Salmonella typhi*	ICDDR'B	9	10	27
	Shigella flexneri	ICDDR'B	8	9	9
	Shigella sonnei	ICDDR'B	-	-	8
	Proteus sp.	ICDDR'B	11	13	-
	E. coli	ICDDR'B	8	10	22

The MIC value against Gram positive and Gram-negative bacteria ranged from 3,000 to 5,000µg/ml, respectively (Table 3). *C. suffruticosa* root extract showed strong antifungal activity against a number of tested fungi (Table 4). In cytotoxic bioassay, the LC_{50} value of root extract was 104µg/ml whereas that of vincristine sulfates was 0.76µg/ml.

Table-3: Minimum inhibitory concentration of CSRE and Kanamycin.

Organism	MIC Value of CSRE (µg/ml)	MIC Value of Kanamycin (µg/ml)
Bacillus subtilis	5,000	4
Staphylococcus aureus	4,000	16
Bacillus cereus	3,500	8
B. polymyxa.	4,000	8
B. megaterium	4,000	8
Salmonella typhi	4,000	16
Shigella flexneri	4,000	16
Proteus sp.	3,000	8
E. coli	3,500	4

Table-4: In-vitro antifungal activity of CSRE and fluconazole.

Organism	Source	ID	% of inhibition		
			CSRE (4mg)	CSRE (5mg)	Fluconazole (100µgm)
Aspergillus ustus	DSM	63535	32.92	37.50	40
A. niger	DSM	737	40.69	41.86	64
A. ochraceus	DSM	824	42.68	44.66	38

Table-5: Brine shrimp lethality test for CSRE.

Dose (µg/ml)	Log dose	Total	Alive	Death	Lethality (%)	Actual (%)	Probit	Chi-square
62.5	1.796	20	20	0	0	0.0125	0.006	0.09
125	2.097	20	18	2	5	0.1	0.046	1.21
250	2.398	20	16	4	10	0.2	0.18	0.01
500	2.699	20	13	7	35	0.35	0.45	0.52
1000	3.000	20	6	14	65	0.7	0.75	0.07
2000	3.301	20	0	20	100	0.9875	0.92	0.07

Cytotoxic effect of the extract is summarized in the Table 5. Regression analysis for the brine shrimp lethality is shown in Figure 2. Antioxidant activity of *C. suffruticosa* extract was measured by DPPH free radical scavenging method and their

scavenging activity was compared with the standard antioxidant ascorbic acid. Both ascorbic acid and extract showed a dose dependent activity. However, extract showed very strong DPPH free radical scavenging effect in regard to ascorbic acid. Among seven different concentrations used in the study (10 to 1000µg/ml) 1000µg/ml showed the highest scavenging activity 98.64% whereas ascorbic acid of the same concentration showed 98.66% which are very close to each other (Table 1 and Figure 1). Percent (%) scavenging activity was plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis. IC_{50} value of ascorbic acid and extract was found 5.46µg/ml and 3.28µg/ml, respectively (Figure 1).

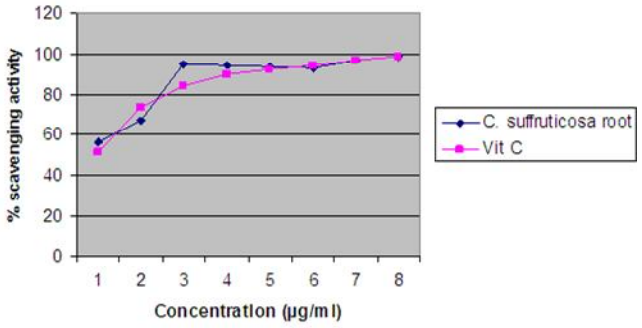

Fig.1 Relative % scavenging activity of standard antioxidant ascorbic acid and CSRE extract.

Free radicals from oxidative stress are involved in many disorders like atherosclerosis, angina pectoris, neurodegenerative diseases and cancer. Antioxidants due to their scavenging activity are useful for the management of those diseases. DPPH stable free radical method is a sensitive way to determine the antioxidant activity of plant extracts (Koleva *et al*, 2002; Suresh *et al*, 2008). The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured. The therapeutic potential of natural medicinal plants as an antioxidant in reducing such free radical induced tissue injury, suggests that many plants have antioxidant activities that can be therapeutically useful (Kanatt *et al*, 2007).

Ascorbic acid, the standard antioxidant in this study, acts as a chain breaking scavenging agent that impairs the formation of free radicals in the process of intracellular substances formation throughout the body, including collagen, bone matrix and tooth dentine (Beyer, 1994; Aqil et al, 2006). The quantitative determination of antioxidants explored that high quantity of scavenging substances are found to be (Prakash et al, 2009) in *C. suffruticosa* which plays the key role in showing free radical scavenging activity of this plant.

Antibacterial activity of *C. suffruticosa* extract was evaluated on four Gram positive and six Gram negative bacteria by disc diffusion method using Kanamycin (30µg/disc) as standard antibiotic disc. Plant extract showed zone of inhibition to almost all the strains (at dose 2mg and 3mg/disc) except *Klebsiella sp. and Shigella soneii.* Crude extract at the concentration of 2mg/disc showed 6, 7.5, 7.0, 7.0 & 7.5mm diameter zone of inhibition against Gram positive *Bacillus subtilis, Staphylococcus aureus, Bacillus cereus, Bacillus polymyxa & Bacillus megaterium*, respectively & 9.0, 8.0, 11.0, 8.0 diameter against Gram negative *Salmonella typhi, Shigella flexneri, Proteus sp. & E. coli* (Table 2). On the other hand, standard antibiotic Kanamycin (30µg/disc) showed significant antibacterial activity against all tested gram positive and Gram negative bacteria except to *Proteus sp.* (Table 2). Results implicated that the Gram negative bacteria were more sensitive to the extract than the gram positive bacteria. This is possibly because of the presence of outer membrane that serves as an effective barrier in gram-negative species (Nikaido, 1999; Adesokan *et al*, 2007)

Proteus sp., Salmonella typhi, Shigella flexneri, S. aureus and Bacillus megaterium were the most susceptible bacteria, an observation that may be attributed to the presence of single membrane of the organism which makes it more accessible to permeation by active principles of the extract of *C. suffruticosa*. In contrast, *Klebsiella sp.* and *Shigella sonnei* showed the least susceptibility to the extract. This may be due to the fact that these organisms have intrinsic resistance from a restrictive outer membrane barrier and transenvelope multidrug resistance pumps (MDRs) (Girish and Satish, 2008). The results of present research highlights, the fact that the organic solvent extracts exhibited greater antimicrobial activity because the antimicrobial principles were either polar or non-polar and they were extracted only through the organic solvent medium (Mohanasundari *et al*, 2007; Britto, 2001). The present observation suggests that the organic solvent extraction was suitable to verify the antimicrobial properties of medicinal plants and they are supported by many investigators (Krishna *et al*, 1997; Singh and Singh, 2000; Narayan *et al*, 2008). The present study justifies the claimed uses of *C. suffruticosa* in the traditional system of medicine to treat various infectious diseases caused by the microbes. The obtained results may provide a support to use of the plant in traditional medicine. Based on this, further chemical and pharmacological investigations to isolate and identify minor chemical constituents in *C. suffruticosa* and to screen other potential bioactivities may be recommended.

The MIC values of the crude extract obtained from *C. suffruticosa* are summarized in Table 3. The range of MIC values of ethanol extracts bacteria was 3000µg to 5000µg. The lowest MIC (2500µg) was recorded against *Bacillus subtilis* and the highest MIC (5000 µg) recorded against *Proteus sp*.

C. suffruticosa showed a significant degree of anti-fungal activity (Table 4). The maximum anti-mycotic activity 44.66% was

shown against *A. ochraceus*. Plant natural compounds are important source of fungi toxic compounds and they may provide a renewable source of useful fungicides that can be utilized in anti-mycotics drugs against infection of *A. ochraceus*. The effect of extract against *A. niger* was also higher implying that this plant can be utilized as anti-mycotics drugs against infection of *A. niger* in patients with pulmonary tuberculosis **(**Sunita and Mahendra, 2008). Moderate anti-mycotic effect was found against *Aspergillus ustus* at the concentration of 4 and 5mg/ml. Fluconazole was used as standard antifungal agent to compare the potentials of extract (Table 4). There are, however, alarming reports of opportunistic fungal infections (Bauer *et al*, 1996) which describe that the resistance of the organisms increased due to indiscriminate use of commercial anti-microbial drugs commonly used for the treatment of infectious disease. This situation forced the researchers to search for new anti-microbial substance from various sources including medicinal plant (Bauer *et al*, 1996). Our research findings revealed that medicinal plant *C. suffruticosa* can play a vital role in combating fungal resistance.

Brine shrimp lethality is a general bioassay which is indicative of cytotoxicity, antibacterial activities, pesticidal effects and various pharmacologic actions (MacLaughin *et al*, 1991)**.** In this research, **s**ix different concentrations (62.5, 125, 250, 500 and 1000µg/ml) of *C. suffruticosa* extract were used to determine its cytotoxicity by brine shrimp lethality bioassay (Table 3). The extract showed lethality in a dose dependent manner. The mortality 0%, 10.00%, 35.00%, 65.00% and 100% were observed at the extract concentration of 62.5, 125, 250, 500, and 1000 µg/ml, respectively. "BioStat-2007" computer software was used to calculate the probits for each concentration. Actually, the notion of probit was introduced by Chester Ittner Bliss (1934) to transfigure the data, such as the percentage of a pest killed by a pesticide, into a 'probability unit' (or probit) through plotting the killed percentages against the logarithm of the doses to obtain more or less a straight line. However, probits were then plotted against corresponding log concentration of extract and from the plot LD_{50} (Lethal Dose 50) value was calculated by regression analysis (Table 3 and Figure 2). LD_{50} value of *C. suffruticosa* ethanol extract was found 546.94µg/ml at confidence limit 95% (Table 3). The LD_{50} value found in this study to be very significant suggesting the ethanol extract of *C. suffruticosa* has high potentiality to kill cancer cells as well as pests (MacLaughin *et al*, 1991).

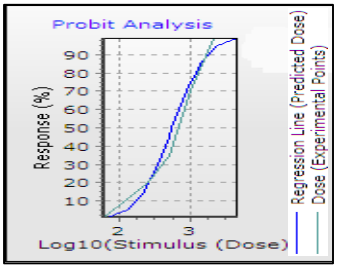

Fig. 2 Probit analysis for brine shrimp treated with CSRE.

CONCLUSION

This study delineates that *C. suffruticosa* extract possesses some potentials in free-radical scavenging activity as ell as antibacterial & antifungal effect. Since, crude ethanol extract of *C. suffruticosa* showed antibacterial and cytotoxic effect, it can be assumed that different active secondary metabolites were present in this extract and perhaps some of these compounds may function in a synergistic manner. However, further studies are necessary to elucidate the mechanism lying with these effects. However, this is the first report on this sample and it may serve as a footstep regarding the biological and pharmacological activities of this sample.

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