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Original Article

Antibacterial, Antioxidant and Cytotoxic Properties of *Crinum asiaticum* Bulb Extract

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Antibacterial effect of *Crinum asiaticum* bulb extract (1mg/disc) was tested on four Gram- positive and six Gram-negative bacteria by disc diffusion method using kanamycin (30 ìg/disc) as standard antibiotic disc. The bulb extract (250-1000mg/disc) showed significant zone of inhibition against all Gram-positive and Gram-negative bacteria ranging from 12-14 mm in diameter. Antioxidant potential of the same extract was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method. The extract showed remarkable free radical scavenging effect (95.96%) providing the IC_{50} value of 5.62 for the bulb extract and 5.46 for ascorbic acid (standard antioxidant) at the concentration of 1000 ìg/ml. The bulb extract was found to be (LC_{50} value 94.06 µg/ml) in Brine-Shrimp lethality test.

Key words: *Crinum asiaticum*; DPPH; Kanamycin; Scavenging; Gallic acid.

Introduction

Crinum asiaticum locally known as Bara kanur, is an evergreen herb that is widely distributed in China, Hongkong, India, Srilanka, Myanmar, Thailand, Malaysia and Mainland Japan[1-3]. It is also found in hilly areas of Bangladesh especially in Chittagong. It has been used by the tribes of Chittagong Hill tracts for their treatment of pain, swelling carbuncle, piles, earache, arthritis, skin disease, cold and cough disorders, vomiting, worms infestation, disuria, polyuria, bowel complains, throat disorder, colic, dyscrasia, flatulence, fever[1-3]. Traditional uses of the leaves and roots of this plant include emetic, diaphoretic and purgative. Leaves of smeared *C. asiaticum* with warmed castor oil are useful remedies for repelling inflammations and swellings at the end of toes and fingers. The plant is also used to treat inflammed joints and sprains. Slightly warmed juice of the leaves, mixed with a little salt, has been used for earache and other ear complaints. Roasted bulb is used as rubefacient in rheumatism. Bruised leaves of this plant act as an efficient insect repellent[1-3].

Although, the traditional uses of the *C. asiaticum* are now popularized, however, very few scientific evaluations of this plant have been documented so far. This study was, therefore, taken to test the antimicrobial, antioxidant and cytotoxic activity of *C. asiaticum* bulb extract.

Materials and Methods

Collection of Plant

The bulbs of *C. asiaticum* were collected from Chittagong Hill tracts, Bangladesh, in the month of January 2009. The plant was taxonomically identified and authenticated by Dr. Shaikh Boktear Uddin, Assistant Professor, Department of Botany, University of Chittagong. The voucher specimen is preserved in Bangladesh National Herbarium under the plant accession no. 34545.

Preparation of Plant Extract

The fresh bulbs of *C. asiaticum* (Syn: *Crinum amabile*) were minced into small pieces, air dried at room temperature for about 10 days, ground into powder (536.46g) and extracted with methanol (99% Anal-R), being macerated at room temperature (23±5)ºC for 15 days with occasional stirring. Methanol was filtered off through a cotton plug and concentrated under reduced pressure below 50ºC through rotatory vaccum evaporator (RE200 Sterling, UK). The concentrated bulb extract (35g, yield 6.5 % w/w) was stored at 4ºC.

Bacterial strains and antibiotic disc

Four Gram-positive (*Bacillus cereus*, *Bacillus subtilis, Bacillus megaterium* and *Staphylococcus aureus)* and six Gram-negative (*Escherichia coli, Salmonela typhi, Salmnela paratyphi, Pseudomonous sp (I), Pseudomonous sp (II)* and *Shigella sonnei)* bacterial strains were used in the present study (Table 1). All the strains were collected from the Department of Microbiology, University of Chittagong. All the strains were grown in Mueller-Hinton agar medium. Kanamycin (30ìg/disc, Oxoid, England) was used as a standard antibiotic disc.

Preparation of discs

The discs of about 4 mm in diameter were cut by punching machine from Whatman No.1 filter paper. The discs were taken in a petri dish and sterilized by autoclaving, dried in oven at 180ºC.

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Preparation of extract solution

A measured amount of *C. asiaticum* bulb extract was dissolved in definite volumes of methanol to give solution of known concentration (100µg/µl). Methanol was chosen as solvent because the crude extract is completely dissolved in methanol and it has no inhibitory effect on the cultures.

Assay for Antibacterial Activity

Antibacterial activity of plant extract was determined by disc diffusion method[4]. Discs (4 mm in diameter) impregnated in known amount of test substances (500 ìg/discs) were placed on Mueller-Hinton agar medium uniformly seeded with the test organisms and kept at low temperature (4ºC) for two to four hours to allow maximum diffusion of compound. The diffusion occurred according to the physical law that controls the diffusion of molecules through agar gel[5]. The plates were then incubated at 37ºC for 24 hours to allow maximum growth of the microorganisms. Antibacterial effects were determined by measuring the diameter with a transparent scale in millimeter based on appearance of the clear zones of inhibition on the discs. The results were compared with the control antibiotic, Kanamycin.

Assay for Antioxidant Activity

The antioxidant activity of *C. asiaticum* extract was tested on the basis of its stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging effect according to established procedure[6,7] using ascorbic acid as a positive control (BDH, England). Ascorbic acid solution (1ml) and different concentrations (10, 50, 100, 200, 400, 600 and 800 ìg/ml in methanol) of 1ml of *C. asiaticum* solution were mixed with 3 ml of 0.4 mM DPPH solution. The mixtures were kept in dark for 30 minutes to measure the absorbance at 517 nm using a UV-Visible Spectrophotometer (Cintra, Australia). Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The procedure also involves the degree of decolorization of DPPH from purple to yellow indicating the scavenging efficiency of the extract. The scavenging activity against DPPH was calculated by the following equation:

$$\text{Scavenging activity (\%)} = [(A - B) / A] \times 100$$

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). Percent (%) of scavenging activity was plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis.

Assay for Cytotoxicity

Cytotoxic activity of plant extract was determined by Brine-Shrimp lethality test[8]. Shrimp eggs were added to the artificial "sea water" (25g salt/ liter water) in the larger compartment of an unequally divided tank which was darkened by covering it with Aluminum foil[9]. The chamber was kept under illumination using a table lamp for 48 h for the eggs to hatch into shrimp larvae. The illuminated compartment attracts shrimp larvae (nauplii) through perforations in the dam. Twenty shrimps larvae were added to 5 ml of sea water in 5 test tubes and 200,100, 50, 25, 10 µg/ml solutions of extracts, prepared from 500mg of crude through serial dilution, were added to these test tubes. Each concentration was tested in triplicate. A control containing 5 ml of DMSO solvent was used for each solvent. The test tubes were maintained under illumination. After 24 hours have elapsed, survivors were counted with the aid of a 3X magnifying glass. From the % lethality of brine shrimp, the probits (probability unit) were calculated for each concentration by using "BioStat-2007" software. Probits were then plotted against corresponding log concentration of bulb extract to get LC_{50} (lethal concentration 50) value through regression analysis.

Qualitative Phytochemical Tests

Alkaloid test: Crude ethanol extract of *C. asiaticum* bulb was subjected to analyze for the occurrence and existence of alkaloid in the extract. Extract (0.5 g) was neutralized by adding 1 or 2 drop of dilute H_2SO_4. The resulting solution was treated with a very small amount of Mayer's reagent (potassiomercuric iodide solution), Wagner's reagent (Iodopotassium iodide) and Hager's reagent (1% picric acid solution)[10]. The color of precipitates formed in each case was noted (Table 4).

Flavonoid test: A small amount of the extract and few drops of concentrated hydrochloric acid were mixed and immediate color development was observed minutely[11].

Results and Discussion

The methanol extract of *C. asiaticum* were found to be active against all tested organisms except *B. cereus*, *Pseudomonas sp* and *Shigella sonnei* with lower concentration (250mg/disc) of plant extract. The zone of inhibition for the extract of different concentrations against four Gram-positive and six Gram-negative bacterial strains are summarized in Table 1. The Kanamycin, the antibiotic control showed pronounced antibacterial activity against all the test organisms. It is clear that extract showed antimicrobial activity in a dose-dependent manner where the extract concentration (1000mg/disc) showed the largest zone of inhibition to Gram-positive bacteria. However, the zone inhibitory effect of the same concentration was lower to Gram-negative organisms.

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Traditional medicinal plants are used primarily water as the solvent but in our studies we found that plant extracts in organic solvent (methanol) provided more consistent antimicrobial activity compared to those extracted in water. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and in addition to their intrinsic bioactivity.[12] Methanolic extracts of plants generally possess terpines and phenolics, which are reported by different workers as antimicrobial compounds[13-14]. Phytochemicals exert their antimicrobial activity through different mechanisms, tannins for example act by iron deprivation, hydrogen bounding or non specific interactions with vital proteins such as enzymes[15]. Some

Table 1. *In vitro antibacterial activity of C. asiaticum bulb extract*

Test organism	Antibacterial activity (Diameter of zone of inhibition in mm) Extract (µg/disc)			Kanamycin (30 µg/disc)
	250	500	1000	
Gram-positive organism				
Bacillus cereus	-	11	13	28
Bacillus subtilis	9	12	14	32
Bacillus megaterium	9	11	12	26
Streptococcus aureus	11	12	13	30
Gram-negative organism				
Escherichia Coli	10	10	12	30
Salmonella typhi	11	13	14	30
Salmonella paratyphi	9	11	12	30
Pseudomonous sp (I)	-	9	11	29
Pseudomonous sp (II)	9	11	12	30
Shigella sonnei	-	10	11	28

alkaloids like indoloquinoline alkaloid and cryptolepine[16]cause cell lysis and morphological changes of *S. aureus*, but the antimicrobial effects of the alkaloid may be through another mechanism, since these compounds are known to be a DNA intercalator and an inhibitor of DNA synthesis through topoisomerase inhibition[17]. Therefore, the phytochemical differences between different plants cause the difference in the antibacterial activities of their extract's composition[12,18]. A phytochemical investigation of the bulbs of *Crinum asiaticum L*. var. sinicum Baker resulted in the isolation of two new alkaloids, asiaticumines A and B (1 and 2, resp.), together with 21 known compounds, including nine alkaloids, four amides, five phenolic compounds, and three flavonoids. All 23 compounds were isolated for the first time from *Crinum asiaticum* L. var. sinicum Baker[19]. However, presence of such alkaloids and flavonoids in our phytochemical screening (Table 4) supports our observations that the antibacterial activity exerted by the extract is due to either of these secondary metabolites.

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity of the *C. asiaticum* bulb extract and ascorbic acid is depicted in Table 2. Both ascorbic acid and bulb extract showed a dose dependent activity. However, bulb extract showed very strong DPPH free radical scavenging effect compared to ascorbic acid. Bulb extract and ascorbic acid promoted the highest scavenging activity 95.96% and 98.66%, respectively, at the concentration of 1000ìg/ml (Table 2). Regression analysis from the plot of (%) scavenging activity versus log concentration showed the IC_{50} value of ascorbic acid (5.46 ìg/ml) and bulb extract (5.62 ìg/ml), respectively.

This method has developed by utilizing the stable DPPH radical to determine the antioxidant activity of natural products[20]. The odd electron in the DPPH free radical gives a purple color with maximum absorption at 517 nm. The color turns to yellow as the molar absorptivity of the DPPH radical reduces from 9660 to 1640 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. IC_{50} value denotes the concentration of sample required to scavenge 50% of the DPPH free radical[21].

Table 2. *DPPH free radical scavenging activity of C. asiaticum bulb extract and ascorbic acid*

Sample con^n(µg/ml)	$Logcon^n$	Absorbance		% Scavenging activity		IC_{50} (µg/ml)	
		Bulb Extract	Ascorbic acid	Bulb Extract	Ascorbic acid	Bulb Extract	Ascorbic acid
Control	-	0.8146	0.8146	-	-		
10	1	0.3201	0.4012	60.70	50.79		
50	1.7	0.2893	0.2149	64.49	73.62		
100	2	0.2645	0.12815	67.53	84.27	5.62	5.46
200	2.3	0.215	0.0804	73.61	90.13		
400	2.6	0.1426	0.0609	82.49	92.52		
600	2.78	0.1135	0.0446	86.07	94.52		
800	2.9	0.0619	0.0239	92.40	97.07		
1000	3	0.0329	0.0109	95.96	98.66		

Table 3. *Brine shrimp lethality for the methanol extract of C. asiaticum bulb*

Dose(ìg/ml)	Log dose	Total(n)	Alive	Death	% Lethality	Actual %*	Probit[a]
10	1.000	20	20	0	0.0	1.25	0.994
25	1.398	20	19	1	5.0	5	3.866
50	1.699	20	17	3	15.0	15	3.964
100	2.000	20	13	7	35.0	35	4.622
200	2.301	20	0	20	100.0	98.75	6.838

[a] probit were calculated using statistical software "Biostat 2007"
*Actual % = Actual formulas (n is the number of animals in a group): For the 0% dead, 100 (0.25/n), for the 100% dead, 100 (n-0.25) /n

The closeness of IC_{50} value of *C. asiaticum* bulb extract (5.62 ìg/ml) with that of ascorbic acid (5.46 ìg/ml) indicates the similar efficiency of extract, to neutralize free radicals, is as like as that of ascorbic acid (Fig. 1). Different research suggests that most of the plant extracts showing antioxidant activity are due to the presence of phenolic compounds[22-23]. Flavonoids are a group of phytochemicals found in varying amounts in foods and medicinal plants which have been shown to exert potent antioxidant activity against the superoxide radical. Antioxidant activity of flavonoids (Table 4) is due to their redox properties which allow them to act as reducing agents, hydrogen donors and singlet oxygen quencher. In addition, they have metal chelating potentials[24]. The phenolic compounds, identified in the extract, might contribute to the antioxidant activity of *C. asiaticum* bulb extract.

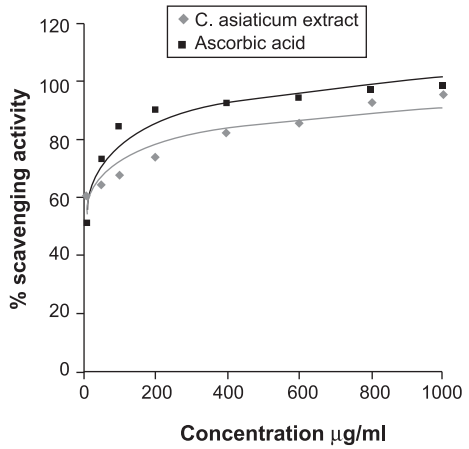

Figure 1. *Comparative % scavenging activity of C. asiaticum bulb extract and ascorbic acid.*

Table 4. *Observation on qualitative phytochemical screening*

Name of the test	Reagent	Change of colors	Indication
	Mayer's reagent	White or creamy white precipitate	++
Alkaloid test	Wagner's reagent	Brown or deep brown precipitate	++
	Hager's reagent	Yellow crystalline precipitate	++
Flavonoid	Conc. HCl	Development of color	++

"++" indicates the presence.

Bulb extract showed lethality in a dose dependent manner in Brine-shrimp test. Percent mortality of brine shrimp observed at 10, 25, 50, 100, 200 µg/ml of extract were 1.25, 5, 15, 35 and 98.75% (Table 3). LC_{50} value of *C. asiaticum* bulb extract was found 94.06µg/ml at 95% confidence limit where the lower and upper limits were 75.29 and 119.17 µg/ml (Fig. 2).

Brine shrimp lethality assay, a general bioassay[8], is an indication of cytotoxicity, antibacterial activities, pesticidal effects and various pharmacologic actions[25]. In this study, the LC_{50} value of the extract was found very significant (94.06 ìg/ml) which indicates that the methanol extract of *C. asiaticum* bulb has high pharmacological actions[26]. It also indicates that the plant might have the potentiality to kill cancer cells[27].

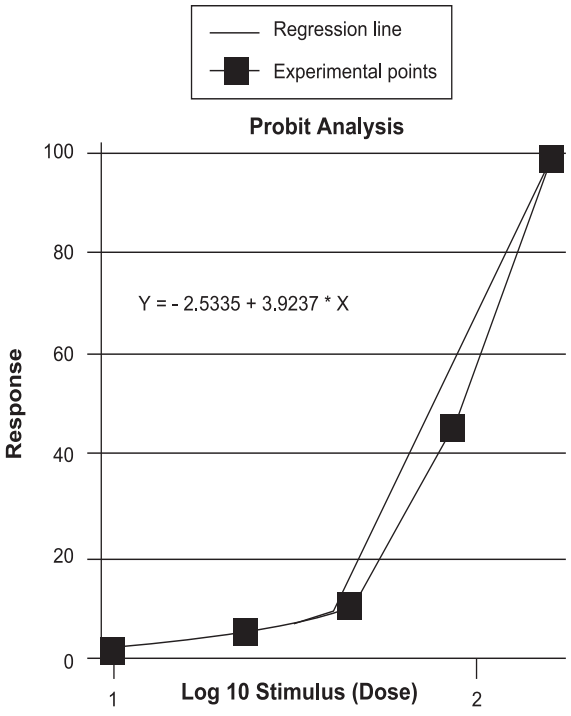

Figure 2. *Regression line for determining the LC_{50} value of methanol extract of Crinum asiaticum bulb*

The results of the study demonstrate that the methanol extract of *C. asiaticum* bulb exhibits very potential antibacterial effect in experimental models which support the claims by traditional medicine practitioners. On the basis of the results, it can be used as a good source of microbiological references although principle compounds responsible for such action are unknown. However, further studies are still necessary to verify the above results in other experimental models to conclude whether the effect observed is truly authentic for aforementioned effect. Phytochemical investigation is also proposed in order to isolate the active fraction and to explore their individual action in regard of the above functions.

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