Antimicrobial, Antioxidant and Cytotoxic Activities of *Citrus Hystrix* DC. Fruits

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Citrus hystrix DC. (Bengali name- Satkara; Family-Rutaceae) is a small and bushy tree, about 3-5 m tall, which grows well in Jayantapur, Gowainghat and Moulobibazar of Bangladesh,[1] Khasia hills of Assam, India[2] and South East regions of Asia. It has folkloric reputation to be used in flu, fever, hypertension, abdominal pains and diarrhoea in infants.[3] The fruits are used as pickle as well as in cooking. The fruit juice is rubbed onto the skin to soften or mixed with bath water to control body odor.[4] It is known as medicinal lime for killing land leeches as an anti-leech rub.[5] The fruits are also used in shampoo as an insecticide for washing the head as a hair shampoo.[6] In addition to be used in cosmetic products, the fruits have been reported to have anti-inflammatory and anti-fertility effects.[7,8] The stem bark of *C. hystrix* showed mild to moderate antimicrobial activity[9] while the methanolic extract of leaves is known to inhibit the herpes virus[3] and also used as mosquito repellent.[10] Previous phytochemical studies on the leaves of *C. hystrix* led to the isolation of antitumor glyceroglycolipids, 1,2-di-*O*-α-linolenoyl-3-*O*-ß-galactopyranosyl-*sn*-glycerol (DLGG) and 1-*O*-α-linolenoyl-2-palmitoyl-3-galactopyranosyl-*sn* glycerol (LPGG)[11] as well as α-tocopherol.[12] The fruit oil contained citronellal, geranial and d-limonene.[7] In this investigation, we are reporting the antimicrobial, antioxidant and cytotoxic activites of the fruits of *C. hystrix* for the first time.

The fruits of *C. hystrix* were collected from the Bandar bazar of Sylhet in the month of December, 2008. A voucher specimen (accession no.-34181) for this collection has been deposited in Bangladesh National Herbarium, Dhaka. The dried and powdered fruits of *C. hystrix* (700 g) were soaked in 2.0 L methanol for 7 days with occassional shaking and stirring and filtered through a cotton plug followed by Whatman filter paper number-1. The extract was then concentrated by using a rotary evaporator at reduced temperature and pressure. A portion (5.0 g) of the concentrated methanol extract (ME) was fractioned with *n*-hexane, carbon tetrachloride and dichloromethane by the modified Kupchan partitioning method.[13] Evaporation of solvents yielded *n*-hexane (HX, 1.75 g), carbon tetrachloride (CT, 0.425 g), dichloromethane (DCM, 0.475 g) and aqueous soluble (AQ, 2.35 g) materials.

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The antimicrobial activity of the extractives was determined against the test organisms (Table 1) by the disc diffusion method.[14] Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipettes and the residual solvents were completely evaporated. Discs containing the test materials were placed onto nutrient agar medium uniformly seeded with the test microorganisms. Standard discs of kanamycin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. These plates were kept at low temperature (4ºC) for 24 hours to allow maximum diffusion of the test materials and kanamycin. The plates were then incubated at 37ºC for 24 hours to allow maximum growth of the organisms. The test material having antimicrobial activity will show a clear, distinct zone of inhibition was visualized surrounding the discs. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The experiment was carried out in triplicate and the mean values were taken.

Table 1. Antimicrobial activity of extractives of *C. hystrix* fruits at 400 µg/disc.

Test microorganisms	Diameter of zone of inhibition (mm)				
	ME	CT	DCM	AQ	KAN
Gram positive bacteria					
Bacillus cereus	21.33 ± 0.57	11.66 ± 1.52	15.66 ± 0.62	19.66 ± 0.64	35.66 ± 0.58
B. megaterium	23.66 ± 0.57	11.66 ± 0.57	17.00 ± 1.00	21.66 ± 1.52	38.66 ± 0.64
B. subtilis	16.33 ± 0.23	11.66 ± 0.57	17.33 ± 0.50	15.33 ± 0.23	35.66 ± 0.57
Staphylococcus aureus	16.33 ± 0.23	15.66 ± 1.15	21.66 ± 0.57	13.66 ± 0.57	35.33 ± 1.52
Sarcina lutea	17.00 ± 1.00	13.66 ± 1.15	20.00 ± 1.00	12.66 ± 2.08	37.33 ± 1.61
Gram negative bacteria					
Escherichia coli	9.66 ± 0.57	13.66 ± 1.52	18.66 ± 3.90	16.00 ± 1.00	33.33 ± 0.23
Pseudomonas aeruginosa	15.66 ± 1.52	13.00 ± 1.00	17.66 ± 1.52	11.00 ± 1.00	34.00 ± 1.00
Salmonella paratyphi	17.33 ± 0.23	10.00 ± 1.00	18.00 ± 1.00	14.00 ± 1.00	35.00 ± 1.00
S. typhi	9.33 ± 0.23	10.33 ± 0.23	17.00 ± 1.00	10.00 ± 1.00	33.00 ± 1.00
Shigella dysenteriae	15.33 ± 1.52	13.00 ± 1.00	9.33 ± 1.41	10.33 ± 0.23	35.66 ± 1.52
Sh. Boydii	20.00 ± 1.00	12.31 ± 1.35	10.66 ± 1.41	18.33 ± 0.23	34.66 ± 2.08
Vibrio mimicus	15.33 ± 1.52	11.00 ± 1.00	15.00 ± 1.00	15.33 ± 1.15	34.66 ± 1.57
V. parahemolyticus	15.33 ± 1.15	10.00 ± 1.00	17.33 ± 1.15	9.33 ± 0.23	34.66 ± 1.08
Fungi					
Candida albicans	14.33 ± 1.52	16.66 ± 2.08	20.00 ± 1.00	12.33 ± 0.23	38.33 ± 1.52
Aspergillus niger	20.66 ± 0.57	13.33 ± 1.15	17.66 ± 0.57	11.00 ± 1.00	36.00 ± 1.00
Sacharomyces cerevaceae	20.33 ± 1.52	9.66 ± 0.57	16.66 ± 0.70	12.33 ± 1.35	35.33 ± 3.65

The diameters of zones of inhibition are expressed as mean ± S.D. (n = 3); a diameter less than 7.00 mm was considered inactive; ME: crude methanolic extract; CT: carbon tetrachloride soluble fraction of the methanolic extract; CF: chloroform soluble fraction of the methanolic extract; AQ: aqueous soluble of methanlic extract fraction; KAN: kanamycin (30 µg/disc)

The antioxidant activity (free radical scavenging activity) of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method developed by Brand-Williams et al., 1995.[15] In this experiment, 2.0 mg of each of the extract was dissolved in methanol. Solution of varying concentrations such as 500, 250, 125, 62.50, 31.25, 15.62, 7.81, 3.91, 1.95, and 0.98 µg/ml were obtained by serial dilution technique. An aliquot of two ml of the extract in methanol was mixed with 3 ml of a DPPH-methanol solution (20 µg/ml) and was allowed to stand for 20 minutes for the reaction to occur. The absorbances were determined at 517 nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

$$\% \text{ inhibition} = [\, 1 - (ABS_{sample} / ABS_{control})] \times 100$$

Then % inhibitions were plotted against concentrations used and from the graph the IC_{50} was calculated by using tert-butyl-1-hydroxytoluene (BHT) as a positive control. The experiment was carried out in triplicate and the mean values were taken. Brine shrimp lethality bioassay[16] technique was applied for the determination of cytotoxicity of the fruit extractives. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day *in vivo* assay. For this experiment, 4 mg of each of the methanol crude extract, *n*-hexane, carbon tetrachloride, dichloromethane and aqueous soluble fractions were dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution. Vincristine sulphate was used as positive control. The experiment was carried out in triplicate and the mean values were taken.

The methanol extract of the fruits of *C. hystrix* as well as its Kupchan fractions demonstrated various degrees of bioactivities when subjected to antimicrobial, antioxidant and cytotoxicity screenings. In case of antimicrobial screening, the fruit extractives of *C. hystrix* exhibited mild to strong antimicrobial activity. The zone of inhibition produced by the crude methanol extract and its carbon tetrachloride, dichloromethane and aqueous soluble fractions were ranged from 9.33-23.66, 9.66-16.66, 9.33-21.66 and 9.33-21.66 mm, respectively (Table 1). However, the *n*-hexane soluble fraction was found to be insensitive to microbial growth (data not shown in Table 1).

In the antioxidant assay, free radical scavenging activity of various fractions of the crude methanol extract was also evaluated. Table 2 shows the antioxidant activity of the test samples. The IC_{50} values for the methanolic crude extract and its *n*-hexane, carbon tetrachloride, dichloromethane and aqueous sluble partitionates were found to be 32.00 ± 2.00, 30.00 ± 1.00, 73.00 ± 4.00, 238.00 ± 7.00 and 51.00 ± 3.00 µg/ml, respectively. By the way, the IC_{50} value exhibited by the standard (BHT) was 9.50±0.50 µg/ml. This demonstrates the *C. hystrix* fruits as a potential source of antioxidant.

Table 2. Antioxidant activity of extractives of *C. hystrix* fruits.

Samples	IC_{50} (µg/ml)
BHT	9.50 ± 0.50
ME	32.00 ± 2.00
HX	30.00 ± 1.00
CT	73.00 ± 4.00
DCM	238.00 ± 7.00
AQ	51.00 ± 3.00

The values of IC_{50} are expressed as mean ± S.D. (n = 3); BHT: *tert* butyl-1-hydroxy toluene (Std); ME: crude methanolic extract; HX: *n*-hexane soluble fraction of methanolic extract; CT: carbon tetrachloride soluble fraction of methanolic extract; DCM: dichloromethane soluble fraction of methanolic extract; AQ: aqueous soluble fraction of methanolic extract.

Following the procedure of Meyer, the lethality of methanolic crude extract and its *n*-hexane, carbon tetrachloride, dichloromethane and aqueous soluble partitionates to brine shrimp was determined after 24 hours of exposure. The LC_{50} were found to be 0.80 ± 0.09, 1.12±0.03, 1.92 ± 0.07, 3.84 ± 0.13 and 3.20 ± 0.04 µg/ml for methanol crude extract, *n*-hexane, carbon tetrachloride, dichloromethane and aqueous soluble materials, respectively (Table 3). In comparison with the LC_{50} 0.32 ± 0.05 µg/ml of the positive control (vincristine sulphate). The cytotoxicity exhibited by the methanol crude extract and its *n*-hexane and carbon tetrachloride soluble fractions was significant. The bioactivities exhibited by the extractives of *C. hystrix* fruits support the traditional uses of this plant in different diseases as well as its popular uses in foods and cosmetics.

Table 3. Brine shrimp lethality bioassay of *C. hystrix* extractives.

Samples	LC_{50} (µg/ml)
VS	0.32 ± 0.05
ME	0.80 ± 0.09
HX	1.12 ± 0.03
CT	1.92 ± 0.07
DCM	3.84 ± 0.13
AQ	3.20 ± 0.04

The values of LC_{50} are expressed as mean ± S.D. (n = 3); VS: vincristine sulphate (std); ME: crude methanolic extract; HX: *n*-hexane soluble fraction of methanolic extract; CT: carbon tetrachloride soluble fraction of methanolic extract; DCM: dichloromethane soluble fraction of methanolic extract; AQ: aqueous soluble fraction of methanolic extract.

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