Antimicrobial, Antioxidant and Cytotoxic Activities of *Callistemon citrinus* (Curtis) Skeels

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ABSTRACT: The petroleum ether, ethyl acetate and methanol extracts of the stem bark of *Callistemon citrinus* were subjected to screenings for antimicrobial and antioxidant activities and brine shrimp lethality. The ethyl acetate extract and its column fractions XVIII and XIV exhibited moderate antimicrobial activity, while the methanol extract revealed significant antioxidant activity having IC_{50} of 3.84 µg/ml. The methanol extract and fraction-II showed potent cytotoxic its cytotoxicity with the LC_{50} of 11.27 and 11.35 µg/ml, respectively.

Keywords: *Callistemon*, Myrtaceae, Antimicrobial, Antioxidant, Cytotoxic.

INTRODUCTION

Callistemon citrinus also known as "Crimson Bottlebrush" belongs to the family Myrtaceae and comprises over 30 species. They are woody aromatic trees or shrubs widely distributed in the wet tropics, notably Australia, South America and tropical Asia, but are now spread all over the world. In China *Callistemon* species, especially *C. viminalis*, are used in Traditional Chinese Medicine (TCM) for treating hemorrhoids.[1] *Callistemon* species are also used as weed control and as bioindicators for environmental management.[2,3] Previous phytochemical investigations of members of this genus resulted in the identification of *C*-methyl flavonoids, triterpenoids and phloroglucinol derivatives.[4-8] Furthermore, piceatannol and scirpusin B isolated from the stem bark of *C. rigidus* growing in Japan, showed inhibitory effects on mouse α-amylase activity.[9] In addition to antimicrobial, antistaphylococcal, antithrombin, insects repellent and nematicidal activities as well as larvicidal and pupicidal values have also been documented for the genus.[10-15] So far no systematic chemical and biological investigations have been carried out on the stem bark of this plant and as this plant have medicinal properties, we investigated on *C. citrinus* growing in Bangladesh and we, here in, report the results of our preliminary studies.

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MATERIALS AND METHODS

Collection of plant materials. The stem bark of *Callistemon citrinus* was collected from Gajipur district, Bangladesh in March 2010. A voucher specimen (DACB 32919) for the plant sample has been deposited in Bangladesh National Herbarium for future reference, where the plant was identified. About 1.5 kg of stem bark was dried for 15 days and ground to a coarse powder.

Extraction The ground material (1 kg) was sequentially extracted with petroleum ether, ethyl acetate and methanol at room temperature for 10 days with regular shaking and stirring to facilitate extraction of compounds. After successful extraction all the extractives were filtered and then concentrated through evaporation by using a Buchi rotavapor. Three extractives were found which were analyzed by TLC over silica gel. Lots of spots were found for ethyl acetate extract. So, the ethyl acetate extract was fractionated using solvent systems of increasing polarity by flash column chromatography. The solvent systems for elution were petroleum ether, petroleum ether-ethyl acetate, ethyl acetate-methanol

and finally methanol. The fractions (I-XXVII) were used for the biological investigations as well as isolation of compounds using preparative Thin Layer Chromatography (TLC).

BIOASSAYS

Determination of antibacterial and antifungal activities. *In vitro* antibacterial and antifungal activities of different extractives and fractions of *C. citrinus* were evaluated in the present study by disc diffusion method[16] using 13 human pathogenic bacteria and 3 fungi. All of the bacterial and fungal strains were collected as pure culture from the Biochemistry and Molecular Biology Lab, Department of Biochemistry and Molecular Biology, University of Dhaka. The activity was measured by determining the zone of inhibition. Kanamycin disc (Oxoid, England) with concentration of 30 µg/disc was used as positive control. The extractives and fractions dissolved in methanol were applied separately to discs (6 mm in diameter) so that each disc contains 300 µg of test sample and dried, well.

Determination of antioxidant activities. Free radical scavenging activity of the plant extractives against stable DPPH [2,2-diphenyl-2-picrylhydrazyl] were determined spectrophotometrically.[17] The antioxidant activity of petroleum ether, ethyl acetate, methanol extractives and fraction-II of ethyl acetate extractive were evaluated by comparing with standard (ascorbic acid) on the basis of scavenging activity of the stable DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical. 175 ml solution of DPPH (20 µg/ml) was prepared by dissolving 3.5 mg of DPPH in spectral grade methanol in a 250 ml volumetric flask. Ascorbic acid with same concentration (20 µg/ml) was used as standard. Different concentrated solutions of extractives were made by dissolving dried extractives ranging from 1µg/ml to 500 µg/ml in spectral grade methanol solvent. DPPH solution (freshly prepared) was added to each solutions and kept in dark for 30 minutes to complete the reactions. After 30 minutes, absorbance of each test tube was taken by a spectrophotometer at 517 nm and inhibition of free radical DPPH oxidant was calculated in percentage (I %), as follows: (I %) = (1 – A_{sample} /A_{blank}) x 100. The IC_{50} was determined from % inhibition Vs concentration graph (Table 2).

Determination of cytotoxic activities. Cytotoxic activity of plant extractives were determined by brine shrimp lethality assay.[18] For determining cytotoxic activity 4.00 mg of each extractive of sample (petroleum-ether, ethyl acetate, methanol extractives and fraction-II) were dissolved in 10 ml pure dimethyl sulfoxide (DMSO) to get the initial concentration 400 µg/ml and from which solution with different concentrations 400**,** 200**,** 100**,** 50**,** 25**,** 12.5**,** 6.25, 3.125, 1.563, 0.781 µg/ml were obtained by serial dilution. Measured amount of the vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml from which solutions with decreasing concentration were made by serial dilutions using DMSO to get 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125 and 0.0390 µg/ml. The test samples were then applied against *Artemia salina* in a 24 hrs *in vitro* assay. Vincristine sulphate was used as the positive control and DMSO as the negative control for the brine shrimp nauplii. The LC_{50} was determined from % of mortality Vs log concentration graph (Table 3).

The bioassays were performed in triplicate. The zone of inhibition, IC_{50} and LC_{50} were calculated as mean ± SD (n=3) for all the assays.

RESULTS AND DISCUSSION

From the above experiment it was found that all the gram positive bacterial strains exhibited promising sensitivity (Table 1) towards *C. citrinus* extractives. The ethyl acetate and petroleum ether showed moderate activity (9-10 mm) to *B. subtilis, B. cereus, B. megaterium* and *S. aureus*.

In the case of gram negative bacteria, high antimicrobial activity was found for fraction-XIV and fraction-XVIII. *E. coli, V. parahaemolyticus, V. mimicus, Sh. dysenteriae* were found to be highly sensitive (11 mm) to fraction-XIV, whereas, *V. mimicus* and *P. species* showed high sensitivity also to fraction-XVIII. In the case of fungi, *C. albicans, A. niger, S. cerevisiae* showed higher sensitivity to

fraction-II, fraction-XVIII, fraction-XXVI and petroleum ether extract.

In DPPH the IC_{50} of methanol extract, petroleum ether, ethyl acetate extractives and fraction-II, of *C. citrinus* were evaluated 3.84 µg/ml, 10.50 µg/ml, 9.53 µg/ml, 21.91 µg/ml, respectively (Table 2).

Table 1. Antibacterial and antifungal activities of *C. citrinus* extractives at 300 µg/disc and kanamycin at 30 µg/disc.

	Zone of inhibition (mean ± SD) after 24 hours of inhibition									
Micro-organisms	Kana mycin	Ethyl acetate extract	Petroleum ether extract	Methanol extract	Fraction -II	Fraction -V	Fraction – VI	Fraction – XIV	Fraction - XVIII	Fraction - XXVI
Gram positive bacteria										
Bacillus subtilis	25.5	9 ± 0.5	9.5 ± 0.4	-	9 ± 0.6	9 ± 0.4	9 ± 0.3	10.5 ± 0.5	10 ± 0.3	-
B. cereus	26	10 ± 0.5	9.5 ± 0.6	-	8.5 ± 0.5	8.5 ± 0.4	8.5 ± 0.3	10 ± 0.4	10.5 ± 0.3	-
B. megaterium	25	9.5 ± 0.4	9 ± 0.3	-	-	-	9.5 ± 0.5	11 ± 0.6	10 ± 0.3	-
Sarcina Lutia	35	-	-	-	10 ± 0.4	-	9 ± 0.5	10.5 ± 0.6	9.5 ± 0.5	-
Staphylococcus aureus	35	10.5 ± 0.6	9 ± 0.5	-	-	10.5 ± 0.3	9 ± 0.3	11 ± 0.4	10 ± 0.4	9 ± 0.3
Gram negative bacteria										
Vibrio parahaemolyticus	27	9.5 ± 0.5	10 ± 0.6	-	9.5 ± 0.4	9 ± 0.5	9 ± 0.4	11 ± 0.6	10 ± 0.3	-
V. minicus	26	9.5 ± 0.4	8.5 ± 0.5	-	-	8.5 ± 0.6	9.5 ± 0.4	11 ± 0.3	11 ± 0.5	10 ± 0.4
Echerichea coli	26	9 ± 0.3	9.5 ± 0.3	-	8.5 ± 0.5	-	9 ± 0.4	11 ± 0.5	9 ± 0.4	-
Salmonella typhi	24	9 ± 0.4	9 ± 0.3	-	9 ± 0.5	-	9 ± 0.3	10.5 ± 0.6	10 ± 0.5	9.5 ± 0.4
S. para typhi	25	9 ± 0.3	10 ± 0.3	-	9.5 ± 0.4	8 ± 0.3	9 ± 0.4	10 ± 0.5	10 ± 0.4	-
Shigella boydii	25	10 ± 0.6	11 ± 0.5	-	9 ± 0.3	-	9 ± 0.4	10 ± 0.6	9.5 ± 0.5	-
Sh. dysenteriae	26	10 ± 0.5	8.5 ± 0.3	-	-	-	8.5 ± 0.6	11 ± 0.3	9.5 ± 0.4	-
Pseudomonus sp.	26	8 ± 0.3	9 ± 0.4	-	-	-	9.5 ± 0.3	10.5 ± 0.6	10 ± 0.4	-
Fungi	**Zone of inhibition (mean ± SD) after 48 hours of incubation**									
Candida albicans	13	9.5 ± 0.5	11 ± 0.3	-	9.5 ± 0.4	-	9 ± 0.3	10 ± 0.5	9 ± 0.6	9 ± 0.4
Aspergilus niger	13	9 ± 0.6	10 ± 0.5	-	10 ± 0.3	-	-	9 ± 0.6	9 ± 0.5	-
Saccharromyces cerevisiae	16	-	10 ± 0.4	-	10 ± 0.5	9 ± 0.6	-	10 ± 0.3	10 ± 0.4	9 ± 0.3

"-"= Indicates no zone of inhibition.

Table 2. Antioxidant screening of *C. citrinus* extractives.

Test samples	IC50 (µg/ml)	Regression line	R2	Activity
Ascorbic acid (standard)	10.92	Y = 28.74x + 31.07	R2 = 0.950	Standard
Petroleum ether extract	10.50	Y = 28.64x + 20.75	R2 = 0.988	Moderate
Ethyl acetate extract	9.53	Y = 27.99x + 22.59	R2 = 0.996	Higher
Methanol extract	3.84	Y = 21.63x + 37.36	R2 = 0.995	Highest
Fraction-II	21.91	Y = 24.42x + 17.26	R2 = 0.993	Lower

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

Test sample	LC50 (µg/ml)	Regression line	R^2	Activity
Vincristine sulphate	0.539	Y = 36.55x + 59.80	R^2 = 0.981	Standard
Petroleum-ether extract	21.05	Y = 31.96x + 7.710	R^2 = 0.969	Lower
Ethyl acetate extract	12.11	Y = 35.93x + 11.08	R^2 = 0.974	Moderate
Methanol extract	11.35	Y = 35.75x + 12.28	R^2 = 0.960	Moderate
Fraction - II	11.27	Y = 43.66x + 4.068	R^2 = 0.973	Moderate

The ascorbic acid used as standard showed IC_{50} of 10.92 µg/ml. By comparing the results with ascorbic acid, it was found that the methanol and ethyl acetate extractives showed highest free radical scavenging activity with IC_{50} 3.84 µg/ml and 9.53 µg/ml, respectively. From this investigation it can be concluded that, the plant has potential role in scavenging free radicals due to antioxidant property.

In the brine shrimp lethality bioassay the mortality rate of brine shrimp was found to be

increased with the increase of concentration of each sample (Table 3). Vincristine sulphate was used as standard. The LC_{50} obtained from the best-fit line slop for vincristine sulphate, methanol, ethyl acetate, petroleum-ether extractives and fraction-II were 0.539, 11.35, 12.11, 21.05 and 11.27 µg/ml, respectively. Fraction-II and methanol extract showed potent cytotoxic activity.

From the above findings, it can be concluded that *C. citrinus* should be subjected to systematic chemical and biological investigations to isolate and characterize the bioactive compounds.

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REFERENCES

1. Ji, T. 2009. Traditional chinese medicine pills for treating hemorrhoid, *CN 101352524 A 20090128*.
2. Wheeler, G.S. 2005. Maintenance of a narrow host range by *Oxypos vitiosa:* A biological control agent of melaleuca, *Biochem. Syst. Ecol.* ***33,*** 365-383.
3. Burchett, M., Mousine, R. and Tarran, J. 2002**.** Phytomonitoring for urban environmental management. *Air Pollut. Plant Biotechnol.* 61-91.
4. Wrigley, J.W. and Fagg, M. 1993. *Bottlebrushes, Paperbarks and Tea Trees and all other plants in the Leptospermum alliance*; Angus & Rovertson: Sydney, Australia, p. 352.
5. Wollenweber, E., Wehde, R., Dorr, M., Lang, G. and Stevens, J.F. 2000. C-methyl flavonoids from the leaf waxes of some Myrtaceae. *Phytochemistry* ***55,*** 965-970.
6. Huq, F. and Misra, L.N. 1997. An alkenol and C-methylated flavones from *Callistemon lanceolatus* leaves. *Planta Med*. **63,** 369-370.
7. Varma, R.S. and Parthasarathy, M.R. 1975.Triterpniods of *Callistemon lanceolatus*. *Phytochemistry* ***14,*** 1676-1676.
8. Younes, M.E. 1975. Triterpenoids from the leaves of *Callistemon lanceolatus*. *Phytochemistry* ***14,*** 592.
9. Lounasmaa, M., Puri, H.S. and Widen, C. 1977. Phloroglucinol derivatives of *Callistemon lanceolatus* leaves*. Phytochemistry* **16,** 1851-1852.
10. Kobayashi, K., Ishihara, T., Khono, E., Miyase, T. and Yoshizaki, F. 2006. Constituents of stem back of *Callistemon rigidus* showing inhibitory effects on mouse a-amylase activity. *Biol. Pharm. Bull.* **29,** 1275-1277.
11. Saxena, S. and Gomber, C. 2006. Antimicrobial potential of *Callistemon rigidus*. *Pharm. Biol*. **3,** 194-201.
12. Gomber, C. and Saxena, S. 2007. Anti-staphylococcal potential of *Callistemon rigidus. CE Med.* **2,** 79-88.
13. Chistokhodova, N., Nguygen, C., Calvino, T., Kachriskaia, I., Cuuingham, G. and Miles, D.H. 2002. Antithrombin activity of medicinal plants from Floida. *J. Ethnopharmacol.* ***82,*** 277-280.
14. Aisien, M.S.O., Imasuen, A.A., Wagbatsoma, V.A. and Ayinde, B.A. 2004. Preliminary evaluation of repellant activity of some plant essential oils against *Simulium damnosum* s.l. the vector of human onchocerciasis. *Intern J. Trop. Insect Science* **24,** 196-199.
15. Sangwan, N.K., Verma, B.S., Verma, K.K. and Dhindsa, K.S. 1990. Nematicidal activity of some essential plant oils. *Pestic. Science* **3,** 331-335.
16. Bauer, A.W., Kirby, W.M.M., Serris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Path.* **45,** 493-496.
17. Brand-Williams, W., M.E. Cuvelier and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *LWT Food Science Technol.* **28,** 25-30.
18. Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E. and McLaughlin, J.L.1982. A convenient general bioassay for active plant constituents. *Planta Med.* **45,** 31-34.