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Chemical and Biological Investigation of *Phyllanthus niruri*. Isolation of a Pure Compound from Crude Dichloromethane Extract

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Abstract

The crude *n*-hexane (HX), dichloromethane (DCM), methanol (ME) and ethyl acetate (EA) extracts of *Phyllanthus niruri* were subjected to antioxidant, antibacterial and antifungal activities and cytotoxicity against brine shrimp nauplii. The DCM, ME and EA extracts exhibited significant antimicrobial activities, whereas, HX extract did not show any sort of sensitivity. On the other hand, the brine shrimp lethality with LC_{50} values was 3.801, 7.244, 13.183 and 23.980 µg/mL for HX, DCM, ME and EA extracts, respectively. Among the four crude extracts, only EA extract showed potential antioxidant activity with IC_{50} value of 29.5µg/mL. A pure compound was isolated from DCM extract and the structure of the compound was elucidated as stigmasterol by means of ^{1}H NMR spectroscopy.

Key words: *Phyllanthus niruri*, Antimicrobial activity, Cytotoxicity, Antioxidant property, Stigmasterol.

I. Introduction

Phyllanthus niruri is a herb, popularly known as Bhui-amla in Bengali, belonging to the genus *Phyllanthus*, family *Euphorbiaceae*. It is known for a wide variety of phytochemicals and pharmacological properties. It is an ingredient of almost 175 Ayurvedic formulations. This herb has been used as traditional medicine for household remedy against various human ailments. *Phyllanthus niruri* is also used for the preparation of various health care and personal products like hair oil, dye, face cream, tooth powder etc.[1]

Phyllanthus niruri plant usually grows up to a height of 40-60 cm, quite glabrous and stems are often branched at the base. The leaves are numerous, elliptic oblong obtuse, with acute tip. The flowers are yellowish, very numerous and axillary. *Phyllanthus niruri* is a winter weed distributed throughout the hotter part of Indian subcontinent including Bangladesh, and in most West African countries such as Fiji, Peru, Haiti, Sudan, Nigeria, Brazil, Mexico, West Indies etc.[2] *Phyllanthus niruri* has been reported for various ethno medical uses such as antiviral[3], antidiabetic[4], antioxidant[5] antimalarial[6], antispasmodic[7] and antibacterial activities.[8] Previous phytochemical studies with *Phyllanthus niruri* revealed that it contains tannin[9], lignans[10] and flavanones.[11]

The current study deals with the antibacterial, antifungal and antioxidant activities of *Phyllanthus niruri*, and has included the test for cytotoxicity, using brine shrimps. Furthermore, an attempt has been taken to isolate the components from different extracts and we also report here the isolation of a pure compound from the dichloromethane extract of the whole plant.

II. Materials and Methods

Plant materials: *Phyllanthus niruri* plants were collected from the Curzon Hall campus of Dhaka University, Bangladesh in 2008 and the plants were thoroughly washed with clean water to remove earthy matters. The plants were sun-dried for several days and then oven-dried for 24 hrs at 40 °C for better grinding. The dried plants were then ground into coarse powder. A voucher specimen has been deposited in the Department of Botany, University of Dhaka.

Extraction: About 50 gm of the coarse powder of the whole plant was successively extracted with *n*-hexane, dichloromethane, methanol and ethyl acetate (based on increasing polarity) for one week at room temperature with occasional shaking. The individual extracts were then filtered off through a cotton plug followed by filter paper. The volume of each filtrate was reduced using Buchii rotary evaporator at low temperature, which afforded *n*-hexane (4.7 gm), dichloromethane (3.2 gm), methanol (4.7 gm) and ethyl acetate (3.8 gm) soluble materials.

Chemical investigation of crude extracts: The concentrated crude dichloromethane extract (3.2 gm) was subjected to column chromatography for fractionation on silica gel (Kieselgel 60, mesh 70-230) and eluted with 5:1 hexane/ethyl acetate solvent system to afford 48 fractions (each 20 ml). Fractions 4-8, 9-12 and 16-17 were combined separately to give 0.732 gm (A), 0.436 gm (B) and 0.546 gm (C) materials, respectively. These three fractions thus obtained were analyzed by TLC and those with satisfactory resolution of components were subjected to PTLC to obtain pure compounds. On the other hand, thin layer chromatography (TLC) screening of crude *n*-hexane, crude methanol and crude ethyl acetate extracts showed tailing with different solvent systems and seemed difficult to isolate the components from each extract. Thus, phytochemical investigations of these three crude extracts were avoided but investigations for antimicrobial, cytotoxicity and antioxidant properties were conducted for each extract.

Microorganisms: Thirteen bacteria (5 Gram positive and 8 Gram negative) and three fungi, collected from the stock cultures of the Institute of Nutrition and Food Science, University of Dhaka, were used for the antimicrobial assays.

Antimicrobial tests: Antibacterial and antifungal activities were tested by the disc-diffusion method.[12] The crude extracts (HX, DCM, ME and EA) were dissolved separately in methanol and applied to sterile filter paper discs at

400µg/disc and carefully dried to evaporate the residual solvent. Discs containing the test materials were then placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard disc of kanamycin (30µg/disc) and blank discs (impregnated with methanol followed by evaporation) were used as positive and negative controls, respectively. These plates were then kept at low temperature (4 °C) for 24 hours to allow maximum diffusion of test samples. The plates were then incubated at 37 °C for 24 hours to allow maximum growth of the organisms. The test materials having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the disc. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition in millimeter. The experiment was carried out in triplicate and the average zone of inhibition was calculated.

Brine shrimp lethality test: Brine shrimp lethality bioassay technique of Meyer[13] was applied for the determination of cytotoxic property of the plant extracts of *Phyllanthus niruri*. The crude *n*-hexane (HX), dichloromethane (DCM), methanol (ME) and ethyl acetate (EA) extracts were separately dissolved in DMSO. Four mg of each of the crude extracts (HX, DCM, ME and EA) was dissolved in DMSO and solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.78125 µg/mL were obtained by serial dilution technique. Vincristine sulphate and DMSO were used as the positive and negative control, respectively. Then the solutions were added to the premarked vials containing ten live brine shrimp nauplii in 5 mL simulated sea water. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From the data, the percent (%) of lethality of the brine shrimp was calculated for each concentration. The median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

Antioxidant activity test: The free radical scavenging activity (antioxidant potential) of different crude extracts (HX, DCM, ME and EA) of *Phyllanthus niruri* on the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was estimated by the method of Brand-Williams[14]. Two mL of methanol solution of the crude extracts (2 mg) at different concentrations such as 500, 250, 125, 62.5, 31.25, 15.625, 7,831, 3.906, 1.953 and 0.977 µg/mL were mixed with 3 ml of DPPH methanol solution (20µg/mL). After 30 min reaction period at room temperature in dark place, absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by plant extracts as compared to that produced by the standard antioxidant agents of *tert*-butyl-1-hydroxytoluene (TBHT) and ascorbic acid (ASA). Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph obtained by plotting inhibition percentage against extract concentration.

III. Results and Discussion

Antimicrobial activity: The result of the antimicrobial activities of crude *n*-hexane (HX), dichloromethane (DCM), methanol (ME) and ethyl acetate (EA) extracts of *Phyllanthus niruri* has been summarized in Table 1. Present investigation showed that the ME, DCM and EA extracts have the potent to moderate sensitivity against almost all the bacteria and fungi (except *Staphylococcus aureus* and *Escherichia coli*), whereas crude HX extract did not show any antimicrobial activity.

Table. 1. Antimicrobial activity of the plant extracts of *Phyllanthus niruri*

Test Microorganisms	Diameter of zone of inhibition (mm)				
	HX	DCM	ME	EA	Kanamycin
Gram positive bacteria					
Bacillus cereus	-	9	9	8	26
Bacillus megaterium	-	9	10	8	24
Bacillus subtilis	-	9	9	8	25
Staphylococcus aureus	-	-	-	-	23
Sarcina lutea	-	8	8	7	22
Gram negative bacteria					
Escherichia coli	-	-	-	-	25
Pseudomonas aeruginosa	-	7	7	7	23
Salmonella paratyphi	-	8	9	8	25
Salmonella typhi	-	8	8	9	25
Shigella boydii	-	7	7	7	23
Shigella dysenteriae	-	7	7	7	25
Vibrio mimicus	-	8	8	8	24
Vibrio parahemolyticus	-	8	8	7	25
Fungi					
Candida albicans	-	7	8	7	25
Aspergillus Niger	-	8	8	8	25
Sacharomyces cerevacae	-	8	7	7	23

The diameter of zone of inhibition is expressed as mean ± SD (n=3); a diameter less than 7 mm was considered as inactive; HX: crude *n*-hexane extract; DCM: crude dichloromethane extract; ME: crude methanol extract; EA: crude ethyl acetate extract; KAN: Kanamycin; '-' indicates no activity.

Among the Gm-positive bacteria, *Bacillus cereus*, *Bacillus megaterium* and *Bacillus subtilis* showed significant sensitivity[15] (9-10 mm) toward dichloromethane and methanol extracts. On the other hand, rest of the microorganisms showed the moderate sensitivity toward DCM, ME and EA extracts with average zone of inhibition of 7-8 mm.

Brine shrimp lethality: It was found from the result of the brine shrimp lethality test (Table 2) that the crude extracts (HX, DCM, ME and EA) of *Phyllanthus niruri* exhibited toxicity towards brine shrimp. Test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase of the concentration for each sample. The percent mortality of the brine shrimp nauplii was calculated for every concentration for each sample. A plot of log concentration of the sample versus percent of mortality showed an approximate linear correlation between them. The positive control groups showed non linear mortality rates at lower concentrations and linear rates at higher concentrations. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the test samples. LC_{50} obtained from the best-fit line slope were 0.3229, 3.801, 7.244, 13.183 and 23.980 µg/mL for vincristine sulphate (Std.), HX, DCM, ME and EA extracts respectively. In comparison to positive control (vincristine sulphate), *n*-hexane, dichloromethane, methanol and ethyl acetate extracts were found to be highly cytotoxic although the cytotoxicity exhibited by *n*-hexane (HX) extract was promising.

Table. 2. Brine shrimp lethality of the crude extracts of *Phyllanthus niruri*

Sample	LC_{50} (µg/mL)	Regression equation	R^2
VS	0.323	y = 29.797x + 64.624	0.927
HX	3.801	y = 21.316x + 37.419	0.935
DCM	7.244	y = 31.377x + 22.872	0.975
ME	13.183	y = 38.013x + 7.598	0.898
EA	23.98	y = 27.734x + 11.415	0.842

The values of LC_{50} are expressed as mean ± SD (n=3). VS: vincristine sulphate (Std.); HX: crude *n*-hexane extract; DCM: crude dichloromethane extract; ME: crude methanol extract; EA: crude ethyl acetate extract.

Antioxidant potential: Different crude extracts (HX, DCM, ME and EA) of *Phyllanthus niruri* were subjected to free radical scavenging activity to evaluate the antioxidant potential of *Phyllanthus niruri*, where *tert*-butyl-1-hydroxytoluene (TBHT) and ascorbic acid (ASA) was used as reference standard. The results are summarized in Table 3. It has been observed that EA extract showed the highest antioxidant activity with IC_{50} value of 29.5µg/mL, which is comparable to that of the reference standard TBHT (27.5µg/mL). On the other hand, HX and ME extracts showed mild antioxidant activity, whose IC_{50} values were 292.0µg/mL and 235µg/mL, respectively. Antioxidant property of DCM extract was insignificant (429µg/mL).

Table. 3. IC_{50} values of standard and test samples of *Phyllanthus niruri*

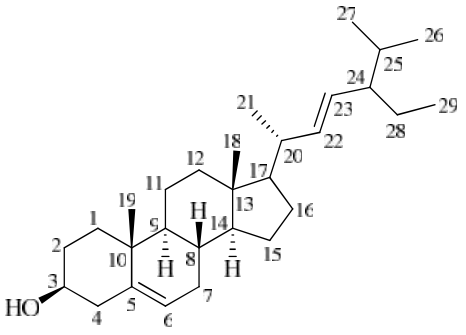
Sample	IC_{50} (µg/mL)
TBHT (Standard)	27.5 ± 0.54
ASA	5.8 ± 0.21
HX	292.0 ± 2.03
DCM	429.0 ± 1.24
ME	235.0 ± 0.57
EA	29.5 ± 2.56

The values of IC_{50} are expressed as mean ± SD (n=3). TBHT: *tert*-butyl-1-hydroxytoluene; ASA: ascorbic acid; HX: crude *n*-hexane extract; DCM: crude dichloromethane extract; ME: crude methanol extract; EA: crude ethyl acetate extract.

Isolation of a pure compound from crude dichloromethane extract: We have mentioned earlier about the separation of three fractions A, B and C from the dichloromethane extract by fractionation on silica gel column chromatography. These three fractions still contained some mixture of compounds as observed from TLC and thus subjected to PTLC technique for purification. After developing the PTLC plate for each fraction with hexane : ethyl acetate (5:1) as eluent, spraying with vanillin-sulfuric acid followed by heating at 110 °C for several minutes to give blue or purple colored spots were then scrapped out and filtered off, using dichloromethane. Volume of each filtrate was reduced to give DCM-1 (27 mg, brownish paste, R_f = 0.72), DCM-2 (10 mg, colorless paste, R_f = 0.61) and DCM-3 (22 mg, greenish paste, R_f = 0.50) from fractions A, B and C, respectively. The ^{1}H NMR spectra revealed that only DCM-2 was in the pure form, whereas, DCM-1 and DCM-3 were found to have impurities. Due to trivial amount of DCM-2, antimicrobial, cytotoxicity and antioxidant investigations were not done for this sample.

The ^{1}H NMR spectrum (400MHz, $CDCl_3$) of DCM-2 displayed a multiplet for one proton at δ 3.58, indicative of H-3 of the steroidal nucleus and a broad singlet at δ 3.72 indicated the presence of –OH group at C-3. The typical signal for H-6 of the steroidal skeleton was evident from a multiplet at δ 5.35 that integrated for one proton. The olefinic protons H-22 and H-23 appeared as characteristic downfield signals at δ 5.14 and δ 5.05, respectively with a coupling constant of 15.0, which is indicative for *trans*-coupling with the olefinic proton and vicinal coupling with neighboring methene proton. Two tertiary methyl groups at δ 0.66 and δ 1.03 are assignable for C-13 and C-10, respectively. In addition, two doublets centered at δ 0.80 and δ 0.83 could be ascribed to the methyl groups at C-25 and another doublet at δ 0.87 integrating for three protons was demonstrative of a methyl group at C-20. The triplet at δ 0.8 could be demonstrated to the methyl group at C-28. All these spectral features were found to be compatible with the structure of stigmasterol (**1**)[16] and the structure of compound **1** has been represented in Chart-1.

Chart-1. Chemical structure of compound **1**



Stigmasterol

IV. Conclusion

It has been found from the above discussion that the different crude extracts of *Phyllanthus niruri* have significant antimicrobial and cytotoxic activities, which supports the traditional uses of this herb for the treatment of bacterial and fungal infections. The high lethality to brine shrimp nauplii indicates that this plant contains potential bioactive compounds. Potent antioxidant property of crude ethyl acetate (EA) extract of this plant will explore its significant utility in reducing the diseases or disorders caused by oxidative stresses. In the present study, we have isolated stigmasterol, which will be applied for antitumor and antiproliferative studies after large scale isolation. A thorough chemical study is also required to isolate the molecules that are responsible for antimicrobial and antioxidant activities.

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1. Rastogi, R. P. and B. N. Mehrotra. 1991. Compendium of Indian Medicinal Plants, Vol. II, Central Drug Research Institute, Lucknow and publications and Information Directorate, New Delhi, India.
2. Prajapati, N. D., S. S. Purohit, A. K. Sharma and T. Kumar, 2007. A Handbook of Medicinal Plants-A Complete Source Book. First Edition, Agrobios: Jodhpur, India, *392*.
3. (a) Meixa, W., C. Haowei and L. Yanjin, 1995. Herbs of the genus *Phyllanthus* in the treatment of chronic hepatitis B observation with three preparation from different geographic sites, *J. Lab. Clin. Med. 126(**2**), 350-360*.

   (b) Naik, A. D. and A. R. Juvekar, 2003. Effects of alkaloidal extract of *Phyllanthus niruri* on HIV replication, *Indian J. Med. Sci., **57**, 387-393*.
4. Raphael, K. R., M. C. Sabu and R. Kuttan. 2000. Antidiabetic activity of *Phyllanthus niruri*. *Amla Res. Bull. **20**, 19-25*.
5. Harish, R. and T. Shivanandappa. 2006. Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*. *J. Food Chem. 95(**2**), 180-185*.
6. Neraliya, S. and R. Gaur. 2004. Juvenoid activity in plant extracts against filarial mosquito *Culex quinquefasciatus*. *J. Med. Arom. Plant Sci. 26(**1**), 34-38*.
7. Calixto, J. B., A. R. S. Santos, V. C. Filho and R. A. Yunes. 1998. A Review of the Plants of the genus *Phyllanthus*: Their Chemistry, Pharmacology, and Therapeutic Potential. *Med. Res. Rev. 18(**4**), 225-258*.
8. (a) Ekwenye, U. N. and N. U. Njoku. 2006. Antibacterial Effect of *Phyllanthus niruri* (Chanca Piedra) on Three Enteropathogens in Man. *Int. J. Mol. Med. & Adv. Sci. 2(**2**), 184-189*.

   (b) Venugopalan, V., M. S. Dinesh and K. S. Geetha. 2010. Enhancement of Antimicrobial Potential of *Phyllanthus niruri* by Fermentation. *J. Herb. Med. & Technol. 4(**2**), 167-175*.
9. Masturah, M., M. Hasan, W. R. W. Daud, H. Singh and M. J. Jahim. 2007. Extraction of hydrolizable tannis *Phyllanthus niruri Linn*: Effect of Solvent and Extraction Methods. *Separation & Purification Technology, 52(**3**), 487-496*.
10. Murugaiyah, V. and K. L. Chan. 2006. Antihyperuricemic lignans from the leaves of *Phyllanthus niruri*. *Planta Medica, 72(**14**), 1262-1267*.
11. Shakil, N. A., J. P. Kumar, R. K. Pandey and D. B. Saxena. 2008. Nematicidal pernyllated flavanones from *Phyllanthus niruri*. *Phytochemistry, 69(**3**), 759-764*.
12. Bauer, A. W., W. M. M. Kibry, J. C. Sheris and M. Truck, 1966. Antibiotic susceptibility testing by a standard single disc method. *Am. J. Clin. Pathol. **45**, 493-496*.
13. Meyer, B. N., N. R. Ferringni, J. E. Puam, L. B. Lacobsen, D. E. Nichols and J. L. McLaughlin, 1982. Brine shrimp: a convenient general bioassay for active constituents. *Planta Med. **45**, 31-32*.
14. Brand-Williams, W., M. E. Cuvelier, C. Berset, 1995. Use of free radical method to evaluate antioxidant activity. *Lebensm Wiss Technol., **28**, 25-30*.
15. Schinor, E. C., M. J. Salvador, J. C. Tomaz, E. M. F. Pral, S. C. Alfieri, S. de Albuquerque, I. Y. Ito and D. A. Dias, 2006.

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16. Colorless paste; ^{1}H NMR ($CDCl_3$) δ 0.66 (3H, s, position-18), 0.80 (3H, t, position-29), 0.80 (3H, d, *J* 6.0 Hz, position-26), 0.83 (3H, d, *J* 6.0 Hz, position-27), 0.87 (3H, d, *J* 6.5 Hz, position-21), 1.03 (3H, s, position-19), 3.58 (1H, m, position-3), 3.72 (1H, s, –OH, position-3), 5.05 (1H, dd, *J* 15.0 Hz, *J* 8.3 Hz, position-23), 5.14 (1H, dd, *J* 15.0 Hz, *J* 8.3 Hz, position-22), 5.35 (1H, m, position-6).