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Biological activity studies of the aerial parts of *Phyllanthus niruri* L.

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ABSTRACT

Phyllanthus niruri L. is a small annual plant grows up to 30-40 cm tall. It is an herbaceous plant that is widely distributed in the tropical and sub-tropical regions. The aerial parts of *P. niruri* growing in Bangladesh were investigated for moisture content and ash content, total phenolic content, total flavonoid content, total antioxidant capacity (TAC), cytotoxicity, relative fatty acid compositions and antibacterial activity. The moisture and ash content of the dried leaves were 5 and 7%, respectively. The extracts of *P. niruri* contained a good amount of phenolic and flavonoid content and revealed significant antioxidant capacity. The highest TAC was shown by dichloromethane fraction (131.3±9.20) and it was followed by 80% ethanol extract (126.1±6.25) and then ethyl acetate fraction (122.5±10.65). The highest amount of phenolic content was present in ethyl acetate fraction, whereas the lowest amount was in hexane fraction. Cytotoxicity was found in 80% ethanol extract, dichloromethane and aqueous fractions. Fatty acids like lauric acid (0.5%), tetradecanoic acid (1%) palmitoleic acid (37%),octadecanoic acid (57%) cis-9-oleic acid (5%) were identified and quantified in hexane fraction of *P. niruri* by GC-FID and octadecanoic acid was predominant among all acids. The antibacterial activity of 80% ethanol extract and aqueous fraction of *P. niruri* was tested against two Gram negative (*Escherichia coli*, *Salmonella typhi*) and two Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacteria following agar diffusion method. The results revealed weak antibacterial activity.

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1. Introduction

Plants provide us medical, environmental and economic supports to sustain on earth. From ancient times, herbal medicines made by potential ingredients of plants like leaves, roots or bark have been used to relieve from various ailments. In recent years, plants are still contributing an important role to health care (Saeed et al., 2012). Plants generate various and high diversity of compounds or products known as secondary metabolites and these metabolites are not required for plant's primary metabolism or energy production. Most of the plants produce phenolics, flavonoids, terpenoids, tannins and steroids compounds known as secondary metabolites (Schäfer and Wink, 2009). Reactive oxygen species (ROS) such as hydroxyl ion, singlet oxygen, hydrogen peroxide and superoxide ion are produced normally in cells during metabolism that can acutely damage to DNA, lipid, proteins and enzymes and finally can create various diseases including cancer, stroke. Natural antioxidants like phenolics, flavonoids, polyphenols, tannins, proanthocyanidins in plants may protect these damages and diseases (Babaa and Malik, 2015; Adebiyi et al., 2017). *Phyllanthus niruri* L. locally known as Bhui Amla belongs to Phyllanthaceae family. It has medicinal properties like antioxidant, hepatoprotective, inhibitor of stone formation, antidiabetic, antiviral, antibacterial and immune activation properties (Ranilla et al., 2012; Colpo et al., 2014; Colombo et al., 2014). The plant is very much familiar in Brazilian folk medicine as quebra pedra (stone breaker) and it has strong action on kidney stones and gallstones. The medicinal properties of *P. niruri* are associated with its active compounds like lignans, phyllanthin, hypophyllanthin alkaloids, terpenes, tannins, coumarins, saponins glycosides, ellagitannins flavonoids, polyphenols and phenylpropanoids (Colombo et al., 2014). The genus *Phyllanthus* includes shrubs, trees and rare herbs and comprises more than 600 species (Kaur et al., 2017). The leaves are 7–12 cm long, alternate, sessile oblong. It has small off-white-greenish flowers, which are solitary, auxiliary, pedicellate, apetalous and monoecious. *P. niruri* is closely related to *P. amarus* in appearance and phytochemical contents but a recent cladistic analysis indicated that the *Phyllanthus* genus is paraphyletic thus these two problematic and confusing species, *P. niruri* and *P. amarus*, are two individual species (Jantan et al., 2018). This paper reports investigation of moisture content and ash content, total phenolic content, total flavonoid content, total antioxidant capacity (TAC), cytotoxicity, relative fatty acid composition and antibacterial activity in the aerial parts of *P. niruri* growing in Bangladesh.

2. Materials and methods

2.1. Sample collection

The leaves with aerial parts of *P. niruri* (Fig. 1) were collected from Munshiganj, Dhaka during Nov-Dec 2019 and was identified by a Professor from Department of Botany, University of Dhaka, and voucher specimen was kept in the organic research lab, Department of Chemistry, University of Dhaka. Since the plant is an annual herb

and grows during high rainfall, limited amount of sample was collected. The leaves then were washed with running water and dried at room temperature followed by oven dry and ground into powder.

Fig. 1. *P. niruri* leaves with aerial parts

2.2. Chemicals and reagents

Solvents, reagents were procured from E. Merck (Germany) to use during the research work. Rotary vacuum evaporator (Heidolph, Germany) was used to dry extracts at reduced pressure removing organic solvents. For freeze drying a freeze-drier (LABCONCO, USA) was used. Absorbances were assessed by a double beam UV-Visible spectrophotometer (SHIMADZU UV – 1800).

2.3. Extraction

Dried powdered of samples (28 g) were extracted in thrice and three days interval with 80% ethanol following maceration process and 6.7 g dry crude extract was collected. It was suspended in water and partitioned by n-hexane, dichloromethane (DCM), ethyl acetate and water. Finally, dried n-hexane (790 mg), DCM (169 mg), ethyl acetate (517 mg) and aqueous (3.5 g) fractions were collected.

2.4. Moisture and ash content

Moisture and ash content were determined according to the standard Association of Official Analytical Chemists (AOAC 2000) method. Each sample (5 g) was taken into different crucibles and placed for moisture content (3 hours, 105°C) in the oven and for ash content placed in the carbolite furnace (6 hours, 700°C) (Biswas et al., 2021).

2.5. Total phenolic content (TPC)

The total phenolic content (TPC) was determined by the modified Folin-Ciocalteu method (Wolfe et al., 2003; Saroar et al., 2020). 0.5 ml of different extract and fractions (80% ethanol, n-hexane, dichloromethane (DCM), ethyl acetate and water) of *P. niruri* was taken separately in different test-tube, then 5 ml of Folin-Ciocalteu's reagent (1: 10 v/v distilled water) and 4 ml sodium carbonate solution were added. The mixer solution was then vortexed for 15 seconds and allowed to stand for 30 min at 40°C. Absorbance was measured at maximum 765 nm against the blank in a double beam UV-Visible spectrophotometer (UV-1800). The total phenolic content was determined and expressed as mg gallic acid equivalent per gram of dry extract/fraction using the equation obtained from a standard gallic acid calibration curve, $y = 0.0054x + 0.0142$, $R^2 = 0.9985$.

2.6. Total flavonoid content (TFC)

Aluminum chloride colorimetric method was used for the determination of total flavonoid content of the *P. niruri* extract and fractions (Chang et al., 2002; Nesa et al., 2021). 5ml of each extract (80% ethanol, n-hexane, dichloromethane (DCM), ethyl acetate and water) was individually mixed with 2.5 ml of Aluminum Trichloride ($AlCl_3$) solution. They were allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 430 nm with a double beam spectrophotometer. The total flavonoid content was determined as mg Quercetin equivalent per gram of dry extract/fraction using the equation obtained from a standard quercetin calibration curve $y = 0.0409x - 0.0348$; $R^2 = 0.984$.

2.7. Total antioxidant capacity (TAC)

The total antioxidant capacity of the extract and fractions (80% ethanol, n-hexane, dichloromethane (DCM), ethyl acetate and water) were evaluated by the phosphomolybdenum assay method (Prieto et al., 1999; Saroar et al., 2020). The subsequent formation of a green phosphate-Mo (V) complex in acidic condition was visible. The 0.3 ml of each extract was allowed to mix with 3.0 ml of the reagent solution. This reaction mixture was incubated at 95°C for 90 min. The absorbance was measured at 695 nm using a spectrophotometer against a blank solution. The total antioxidant capacity was measured and expressed as mg ascorbic acid equivalent per gram of dry extract/fraction using the equation obtained from a standard ascorbic acid calibration curve, $y = 0.0044x - 0.0177$, $R^2 = 0.997$.

2.8. Cytotoxicity assay on cancer cell line

The cytotoxicity assay of *P. niruri* extract and fractions (80% ethanol, n-hexane, dichloromethane (DCM), ethyl acetate and water) (5mg/2ml in 2.5% DMSO) was assessed against a human cervical carcinoma cell line, HeLa which was maintained in DMEM (Dulbecco's modified Eagles medium) containing 1% penicillin-streptomycin (1:1) and 0.2% gentamycin and 10% fetal bovine serum (FBS). Cells (4×104/200 µL) were seeded onto 48-well plate and incubated at 37°C + 5% CO_2. Next day, 50 µL sample (filtered) was added in each well. Cytotoxicity was examined under an inverted light microscope after 48 hours of incubation. Samples were dissolved in 2.5% DMSO and duplicate wells were used for each sample (Sik et al., 2018; Nesa et al., 2021).

2.9. Relative fatty acid compositions

The fatty acids in the oil from n-hexane fraction were made into their methyl ester by saponification with methanolic NaOH followed by esterification with BF_3-MeOH complex, and analysed by GC-FID. The relative percentage of methyl ester of fatty acids in oil was identified by comparing their retention time with that of methyl ester of fatty acid standard (Apurba et al., 2019).

2.10. Antibacterial activity

The antibacterial activity of 80% ethanol extract and aqueous fraction (1mg/ml in 2.5% DMSO) of *P.niruri* was tested against two Gram negative (*E. coli*, *Salmonella typhi*) and two Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacteria following agar diffusion method by Kirby-Bauer (Bauer et al., 1966).

3. Results and discussion

3.1. Moisture and ash content

Moisture content is much important as it affects their physical properties, chemical stability, technological processing, storage or distribution. Comparatively high amount of moisture content estimates its perishability during processing or storage. The moisture content of dried powder leaves with aerial parts of *P. niruri* is (5±0.5) %. However, the ash content of that materials is (7±0.3) %. This study meets the research reported by Samali that dried materials of *P. niruri* plant showed moisture content (12.40±0.5) % and ash content (7±0.45) % (Samali et al., 2012).

3.2. Total phenolic content (TPC)

The concentration of gallic acid solution (0-400 ppm) was used for making calibration curve (Fig. 2). TPC of 80% ethanol extract,

hexane, dichloromethane (DCM), ethyl acetate and aqueous fractions were around 81.6±1.9, 56.65±4.8, 156.65±2.9, 497.4±14.04 and 173.3±12.2 mg GAE/g of dry extract/fraction, respectively (Fig. 3). The results showed that the ethyl acetate fraction exhibited the highest TPC as compared to 80% ethanol extract, hexane, dichloromethane (DCM) and aqueous fractions. Higher phenolic content is responsible for bioactivity and expected to exhibit good result of antioxidant. The crude extract of this plant, 80% ethanol, contains around 80 mg where methanol extract (crude) contains 72 mg reported by Nimmi (Nimmi et al., 2012).

3.3. Total flavonoid content (TFC)

Total flavonoid content (TFC) is the process to measure the amount of flavonoid compounds present in the extracts. The concentration of quercetin (0-20 ppm) was used for making calibration curve (Fig. 4). TFC of 80% ethanol extract, hexane, dichloromethane (DCM), ethyl acetate and aqueous fractions were around 60.1±1.95, 40.9±1.5, 51.9±1.0, 42.0±1.40 and 49.9±1.0 mg QE/g of dry extract/fraction, respectively (Fig. 5). The results showed that the 80% ethanol extract exhibited the highest TFC as compared to the hexane, dichloromethane (DCM), ethyl acetate and aqueous fractions. Ramandeep reported that flavonoid content of 80% methanol crude extract was found 61.41 µg quercetin equivalent/mg of dry extract (Ramandeep et al., 2017). However, Amin reported that TFC of aqueous and ethanol crude extracts were determined as 34.6±0.001 and 123.9±0.002 mg quercetin equivalent/ g of dry extract, respectively (Amin et al., 2012).

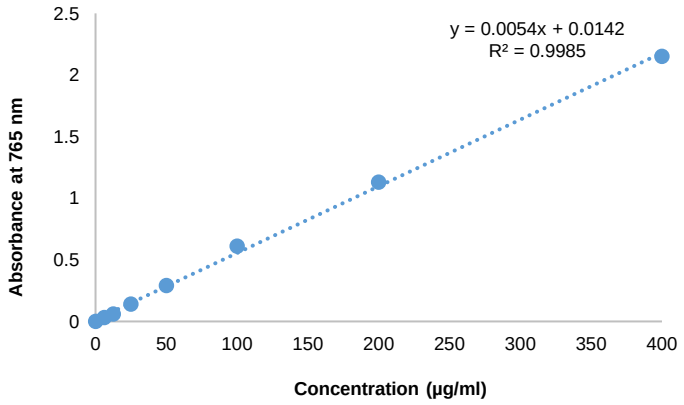

Fig. 2. Calibration curve of standard gallic acid

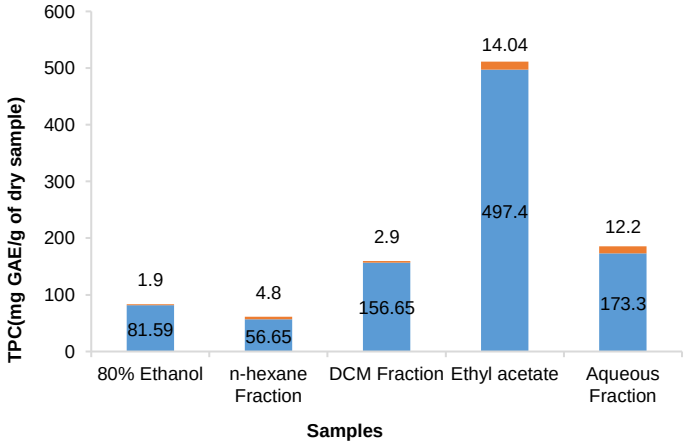

Fig. 3. TPC of the extract and fractions of *P. niruri* (values are mean±SD)

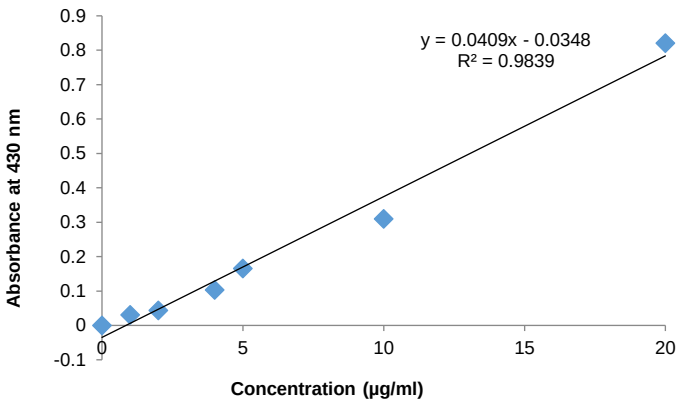

Fig. 4. Calibration curve of standard quercetin

3.4. Total antioxidant capacity (TAC)

To determine antioxidant capacity ascorbic acid solution as standard ranging (0-100) ppm was used to draw calibration curve (Fig. 6). Total antioxidant capacity of 80% ethanol extract, hexane, dichloromethane (DCM), ethyl acetate and aqueous fractions were found to be 126.1±6.25, 74.5±6.90, 131.3±9.20, 122.5±10.65 and 58.6±8.50 mg AAE/g of dry extract/fraction. Dichloromethane fraction showed the highest antioxidant capacity comparing with other extract and fractions. On the other hand, the lowest amount containing 74.5±6.90 mg was present in the n-hexane fraction (Fig. 7). It was reported that TAC of methanol crude extracts of *P. amarus* and *P. urinaria* grown in India revealed 245±25.9 and 364±29.4 mg AAE/g of dry extract, respectively (Kumaran and Joel, 2007).

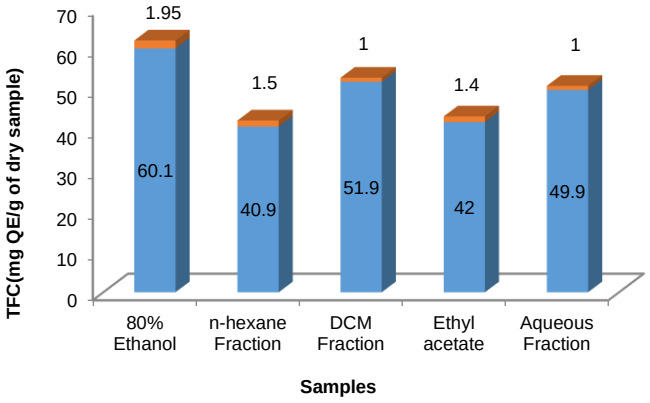

Fig. 5. TFC of the extract and fractions of *P. niruri* (Values are mean±SD)

3.5. Cytotoxicity assay

To inaugurate new pharmaceutical drug, it is necessary to study toxicity. Studies of toxicity in appropriate way are used to assess health risk to human. Cytotoxicity assay of 80% ethanol extract, n-hexane, DCM, EtOAc and aqueous fractions of *P. niruri* were assessed against HeLa, a human cervical carcinoma cell line. Hela, the oldest and most common human cell line, is an immortal cell line. Out of five different extracts 80% ethanol extract, DCM and aqueous fractions were found to be cytotoxic against Hela cell lines (Fig. 8). At a concentration of 1 mg/ ml in 2.5% DMSO solution, ethanol extract, DCM and aqueous fractions showed toxicity on Hela cell line but Eze reported that oral administration of methanol extract of *P. niruri* on a group of mice did not cause death or signs of acute intoxication even at doses up to 5000 mg/kg after 24 h of observation (Eze et al., 2014).

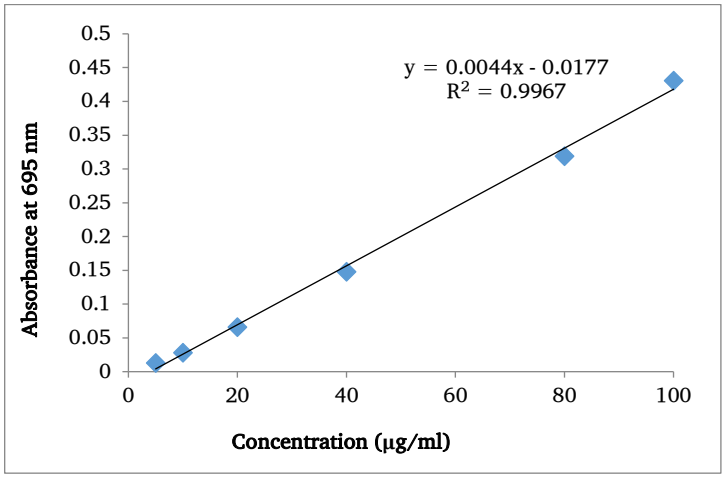

Fig. 6. Calibration curve of standard ascorbic acid (AA)

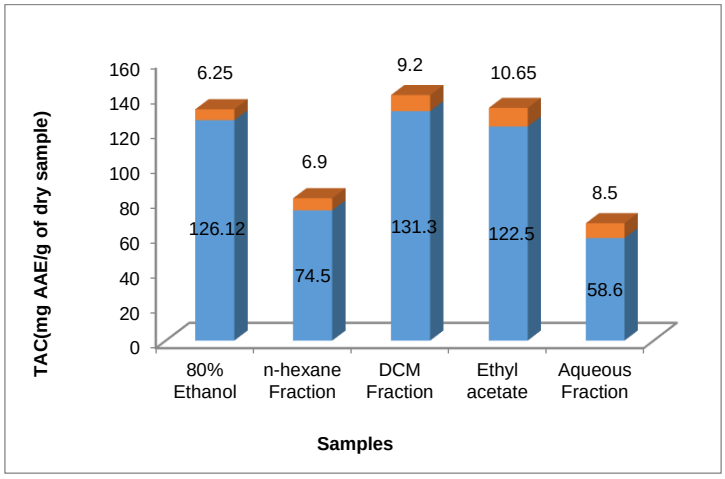

Fig. 7. TAC of the extract and fractions of *P. niruri* (values are mean±SD)

Solvent- Solvent+ 80% EtOH extract hexane fraction

DCM fraction Ethyl acetate fraction Aqueous fraction

Fig. 8. Images of cells of extract and fractions

3.6. Relative fatty acid compositions

It was found that lauric acid (0.5%), tetradecanoic acid (1%) palmitoleic acid (37%), octadecanoic acid (57%) cis-9-oleic acid (5%) were identified and quantified in the extract and fractions of *P. niruri* and octadecanoic acid was predominant among all acids. GC-MS analysis of the ethanol extract of *P. emblica* revealed 22 fatty acids where octadecanoic acid was the highest amount with 22.93% and tetradecanoic acid was found as 1.40% (Asmilia et al., 2020). Ali reported that the ether extract of *P. fraternus* was found to contain 42 chemical compounds by GC-MS, where the main constituents were saturated fatty acids (31.14%), unsaturated fatty acids (29.28%), long chain aldehyde (11.36%) and fatty alcohols (5.37%) (Ali et al., 2018). The data presented here show the correlation of the fatty acids among the Phyllanthus species.

3.7. Antibacterial activity

Bacteria are microorganisms which have different physiological functions. Some bacteria have beneficial and some have harmful effects to human beings (Rahman et al., 2015). The results showed weak activity of the extracts (Fig. 9). Amin stated that following disk diffusion method the water extract of *P. niruri* exhibited significant antibacterial activity against Gram-positive bacteria like *Staphylococcus aureus* (20 mm inhibition zone) and *Streptococcus agalactiae* (12 mm inhibition zone) and Minimal inhibitory concentration (MIC) values were found 0.5 mg/ml and 1 mg/ml, respectively However, no activities were shown by the extract against Gram-negative bacteria like *Escherichia coli* and *Klebsiella pneumonia* (Amin et al., 2012). Literature says that the plant used in the present study was found to contain potential antibacterial activity but current study showed weak activity.

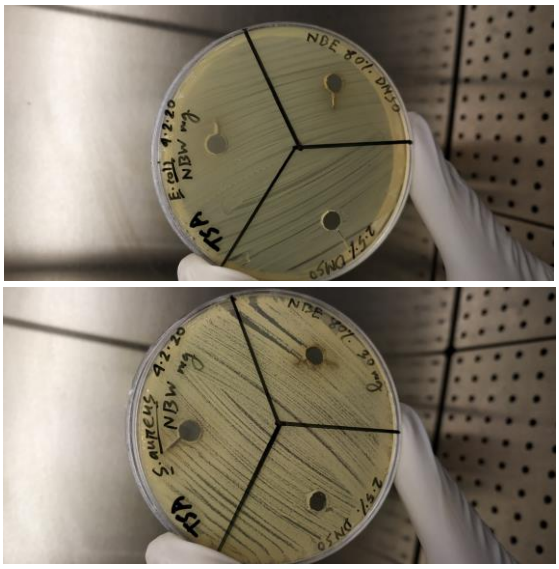

Fig. 9. Antibacterial activity of 80% ethanol extract and aqueous fraction of *P. niruri*

4. Conclusion

The data obtained from the present study suggest that the presence of bioactive phytochemicals in the 80% ethanol extract, DCM and ethyl acetate fractions of *P. niruri*. Total phenolics and flavonoids content were also found to be very rich in these extracts. Octadecanoic acid was predominant fatty acid followed by palmitoleic acid present in hexane extract.

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Conflict of interest

Authors declare that there is no conflict of interest.

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