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Fatty acid analysis, cytotoxicity, antimicrobial and antioxidant activities of different extracts of the flowers of *Nyctanthes arbor-tristis* L.

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Abstract

The fatty acid analysis and biological activity of n-hexane, dichloromethane, ethyl acetate and methanol extracts of *Nyctanthes arbor-tristis* L. flowers are reported. Five fatty acids namely palmitic (44.15%), stearic (19.34%), arachidic (15.06%), behenic (9.77%) and lignoceric (11.69%) acids were identified. From cytotoxicity test, the LC_{50} values (the median lethal concentration) for n-hexane, dichloromethane, ethyl acetate and methanol extracts as well as for standard vincristine sulphate were found 7.05, 4.67, 3.14, 5.53 and 0.50 µg/ml, respectively. Antibacterial activity results of different extracts were compared with standard antibiotic ciprofloxacin and tetracycline by measuring the zone of inhibition diameter in millimeter. The dichloromethane and ethyl acetate extracts showed significant antibacterial activity. From antioxidant activity test, IC_{50} values (50% inhibitory concentration) of n-hexane, dichloromethane, ethyl acetate, methanol extracts and ascorbic acid were found to be 291.92 mg/ml, 45.74 µg/ml, 21.86 µg/ml, 64.30 µg/ml and 3.98 µg/ml, respectively.

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Introduction

Medicinal plants have attracted much attention recently for being potent sources of biologically active compounds or substances (Peyvast and Khorsandi, 2007; Miladi and Darnak, 2008; Malik *et al.,* 2012; Ahmad *et al.,* 2014). Day by day the frequency of life-threatening infections has increased worldwide. Many infectious microorganisms are being resistant to synthetic drugs. Resistance to antimicrobial agents is growing in a wide variety of pathogens and multiple drug resistance is becoming common in diverse organisms. This situation leads scientists to discover new antimicrobial substances from various medicinal plants and also isolate active ingredients through extraction, isolation and characterization of their constituents (Chew *et al.,* 2012; Ullah *et al.,* 2013).

Nyctanthes arbor-tristis L. is a well known medicinally important plant of Bangladesh and its neighboring countries. As for the medicinal use, the whole plant is used for treatment of cancer (Kirtikar and Basu, 2002). Flowers of *Nyctanthes arbor-tristis* L. are carminative, astringent and used in ophthalmic purposes (Rani *et al.,* 2012). Juice of flowers is used as a tonic in preventing graying of hair and hair fall (Girach *et al.,* 1994). Several reports are available in the literature describing the use of the flower extracts of *Nyctanthes arbor-tristis* L. Antimicrobial activity (Syam *et al.,* 2015), antioxidant and polyphenolic agent identification (Nagavani *et al.,* 2010) and DPPH free radical scavenging capacity of flower extract (Thakur *et al.,* 2017; Jyothi *et al.,* 2018; Bhardwaj and Sharma, 2018) are among many other reports in recent years. But to our knowledge the fatty acid analysis of flower extract has not yet been reported.

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The present research is to study fatty acid compositions in the flowers of *Nyctanthes arbor-tristis* L. as well as to study cytotoxicity, antibacterial and antioxidant activity of different extracts of the flowers of this plant. Medicinal plants or plant parts are commonly used in herbal industries as medicine and herbal preparations/ formulations in our country. This study will help to develop chemical and biological profiling of the herbal extracts which will ensure herbal identity and improve the quality of plant-based products.

Materials and methods

General experimental procedures

All the solvents and chemicals used for this research were analytical reagent grade, procured from E. Merck (Germany), BDH (England), AppliChem (Germany) and Sigma Aldrich (Germany). Gas chromatography analyses were performed with a SHIMADZU 2010 Plus gas chromatograph equipped with a flame ionization detector (FID) and a fused silica (5% phenyl/95% polydimethylsiloxane) capillary column (length 30m, inner diameter 0.25 mm, film thickness 0.25 µm) using hydrogen as carrier gas (1.0 ml/min). The injector temperature was 250 °C and the column oven was programmed between 50-220 °C at 4 °C/min. The detector (FID) was operated at 260 °C. The absorbance of prepared solutions (extractive or control) of different concentrations for antioxidant activity was performed by using a Parkin Elmer Lambda-25 UV-VIS spectrophotometer (USA). Quartz cells (1 cm × 1 cm) were used as sample holder to record the spectrum.

Flowers of Nyctanthes arbor-tristis were collected from BCSIR campus, Dhaka, Bangladesh. The flowers were dried, powdered and extracted successively with n-hexane, dichloromethane, ethyl acetate and methanol at room temperature according to the published procedure (Haque *et al.,* 2019). The resulting extracts were filtered, concentrated, dried and stored in a desiccator for use in subsequent experiments.

Identification and quantification of fatty acids

The esterification of fat was carried out by a modified procedure using trifluoride methanol (BF_3–MeOH) complex (Griftin, 1960; Metecalfe and Schmitz, 1961; AOAC, 1984). The n-hexane extract (200 mg) was methylated by heating with BF_3-CH_3OH reagent (5 ml) for 10 min. Methyl esters of fatty acids were isolated by partitioning between water and n-hexane. The esterified fatty acids were taken for GC analysis.

Fatty acid methyl esters (Sigma-Aldrich) of capric acid, caprilic acid, lauric acid, myristic acid, palmotelic acid, palmitic acid, linolic acid, oleic acid, stearic acid, arachidic acid, behenic acids and lignoceric acid were used as standard for the identification of sample peaks. The fatty acids were identified by comparison of retention times with the standard fatty acids chromatogram. The peak areas were calculated by software of the instrument. The relative percentages of fatty acids were calculated by using the following formula:

Relative % of individual fatty acid = (Individual area/ Total areas for all fatty acids) × 100

Brine shrimp lethality bioassay

Brine shrimp lethality bioassay of crude extracts of n-hexane, dichloromethane, ethyl acetate and methanol were screened for cytotoxicity by the Mayer's method (Mayer *et al.,* 1982; McLaughlin *et al.,* 1998). Test samples of 4 mg were dissolved in 200 µL of pure dimethylsulfoxide (DMSO) to prepare stock solutions. Then 100 µL of stock sample solution was taken in a test tube each containing 5 ml of simulated seawater and 10 shrimp nauplii. The concentration of prepared sample solution in the first test tube was 400 µg/ml. Then a series of sample solutions of lower concentrations were prepared by consecutive dilution. In each case 100 µL sample was added to each test tube containing 5 ml of brine solution with 10 living nauplii and fresh 100 µL DMSO was added into the mother solution. Finally, the prepared sample concentrations in each test tube were 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391 and 0.195 µg/ml. After 24 hours, test tubes were inspected using a magnifying glass and the number of survived nauplii of each test tube was counted visually. The mortality percentages of the nauplii at different concentrations were plotted against the logarithm of particular sample concentration to achieve LC_{50} value (the concentration when 50% of brine shrimp nauplii died). The LC_{50} values were calculated by windows Microsoft Excel 2007 software. Standard vincristine sulfate was used as a positive control to compare the results obtained for test samples.

Antibacterial study

Antibacterial assay was determined by disc diffusion method (Bauer *et al.,* 1966; Barry, 1980). Four gram-positive bacteria (*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus* and *Enterococcus fecalis*) and four gram-negative bacteria (*Salmonella typhi, Escherichia coli* 12079, *Salmonella enteritis and Pseudomonas*) were taken for the analysis. Crude extracts of n-hexane, dichloromethane, ethyl acetate and methanol were taken for antimicrobial screening. Standard ciprofloxacin and tetracycline were used as positive control. Each sample and standards were weighed accurately, then dissolved in their required volume of specific solvent (used DMSO for all samples as well as standards). The diluted extracts were applied to sterile discs at a concentration of 400 µg /disc. Standard ciprofloxacin and tetracycline containing doses were 5 and 30 µg/disc respectively. The sample and control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. Then the plates were incubated at 37 °C for 24 h. After incubation, the antimicrobial activity for each test material was determined by measuring the diameter of the zone of inhibition in millimeter and compared with the results obtained for positive control.

Antioxidant activity

The antioxidant activity analysis by DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay test was carried out according to the method reported by Brand-Williams (Brand-Williams *et al.,* 1995).

Ascorbic acid was used as a positive control. The absorbance was measured at 517 nm against methanol as blank (zero absorbance) by UV-VIS spectrophotometer.

Inhibition of free radical DPPH in percent was calculated as follows:

$$\text{Inhibition (\%)} = [1 - (ABS_{sample} / ABS_{blank})] \text{ X } 100$$

Where,

ABS_{sample} = the absorbance of particular test sample, and

ABS_{blank} = the absorbance of the control reaction (containing all reagents except test sample).

IC_{50} values were calculated as the concentration of each sample required to give 50% DPPH radical scavenging

Table I. Fatty acid composition in the flower of *Nyctanthes arbor-tristis* L.

Serial no.	Name of the fatty acid	Area	Relative percentage (%)
1	Palmitic acid	362861	44.15
2	Stearic acid	158927	19.34
3	Arachidic acid	123773	15.06
4	Behenic acid	80312	9.77
5	Lignoceric acid	96056	11.69
	Total	821929	100

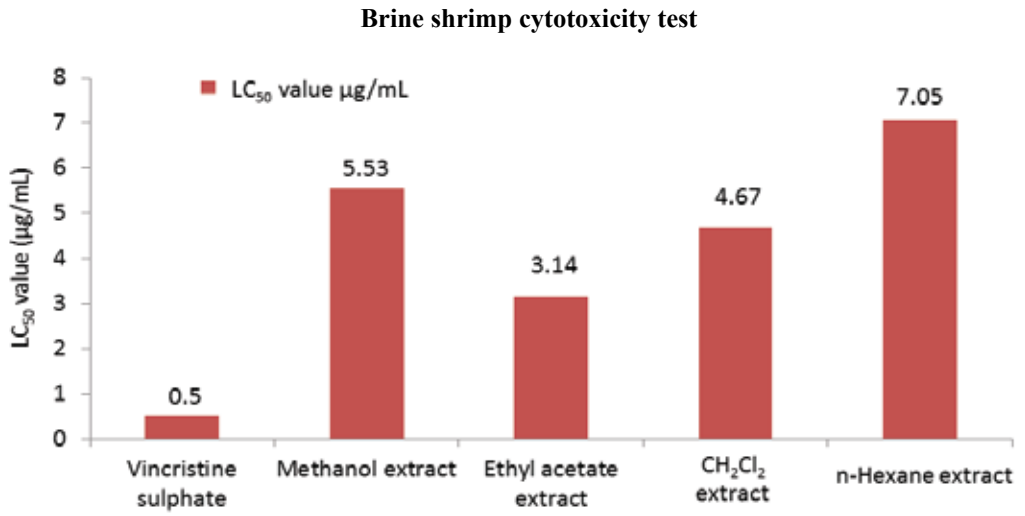

Fig. 1. Cytotoxicity test results (LC_{50} values) of different extracts of *Nyctanthes arbor-tristis* L. flower

activity from the graph (linear regression curve) by excel 2007 Office software.

Results and discussion

Fatty acid compositions

A total of 5 (five) fatty acids was identified as their methyl esters. The relative percentages of the individual acids were found to be (Table I) palmitic acid (44.15%), stearic acid (19.34%), arachidic acid (15.06%), behenic acid (9.77%) and lignoceric acid (11.69%). The percentage of palmitic acid was the major fatty acid while stearic, arachidic, behenic and lignoceric acids were the minor fatty acids. All fatty acids found in *Nyctanthes arbor-tristis* L. flower are saturated fatty acids.

Brine shrimp lethality bioassay

Bioactive compounds (natural or synthetic origin) are almost always toxic to living bodies in higher doses. These compounds are often toxic to the Artemia salina (Brine shrimp) nauplii. Thus, *in vivo* lethality to brine shrimp nauplii can be used as a simple, rapid and favorable monitor for screening and fractionation in the discovery of new bioactive natural products (McLaughlin *et al.,* 1998). All the extracts showed significant cytotoxicity towards brine shrimps within 24 h. The results of LC_{50} values for different extracts and standard vincristine sulfate (positive control) are shown in Fig. 1. The LC_{50} values for n-hexane, dichloromethane, ethyl acetate and methanol extracts of *Nyctanthes arbor-tristis* L. flower as well as for standard vincristine sulphate were found to be 7.05, 4.67, 3.14, 5.53 and 0.50 µg/ml respectively. The best cytotoxicity was found for the ethyl acetate extract (3.14 µg/ml). In comparison to the positive control (standard vincristine sulfate), it appeared that all the test samples were lethal to brine shrimp nauplii. However, ethyl acetate, dichloromethane and methanol extracts demonstrated more potent activity in brine shrimp lethality bioassay than n-hexane extract.

The brine shrimp lethality bioassay test is considered to be very useful in determining various biological activities such as pesticidal, phototoxic, trypanocidal, cytotoxic, ion regulation and enzyme inhibition activities (Ramamoorthy *et al.,* 2012). So the results of different extracts suggested that they might have one or more of this kind of biological activities.

Antibacterial study

The results of antibacterial study of different extracts were compared with that of standard antibiotic ciprofloxacin and tetracycline by measuring the zone of inhibition diameter in millimeter. The results are presented in Table II. The

Table II. Antibacterial activity of different extracts of the flowers of *Nyctanthes arbor-tristis* L. and standards

Name of the bacteria	Zone of inhibition in mm					
	n-Hexane	CH_2Cl_2	Ethyl acetate	Methanol	CP	TE
	400 µg/disc				5 µg/disc	30 µg/disc
Gram -positive bacteria						
Bacillus subtilis	7	9	11	6	27	24
Bacillus cereus	-	6	15	9	29	21
Staphylococcus aureus	-	10	10	-	25	26
Enterococcus fecalis	-	-	-	-	21	16
Gram -negative bacteria						
Salmonella typhi	-	-	-	-	35	25
Escherichia coli 12079	-	-	7	-	25	9
Salmonella enteritis	-	-	-	-	39	23
Pseudomonas	-	6	8	10	29	16

CH_2Cl_2: Dichloromethane, CP: Standard ciprofloxacin, TE: Standard tetracycline

dichloromethane and ethyl acetate extracts showed significant antimicrobial activity against gram-positive bacteria *B. subtilies, B. cereus, S. aureus* and gram-negative bacteria *Pseudomonas.*

Antioxidant activity

The summarized results of free radical scavenging activity for different extracts along with the IC_{50} value of ascorbic acid (used as positive control) are presented in Table III. The IC_{50} values of n-hexane, dichloromethane, ethyl acetate and methanol extracts as well as ascorbic acid were found to be 291.92 mg/ml, 45.74 µg/ml, 21.86 µg/ml, 64.30 µg/ml and 3.98 µg/ml, respectively. The dichloromethane, ethyl acetate and methanol extracts showed significant free radical scavenging activity. Commercially available synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are widely used but these may possess toxic side effects on human health (Albayrak and Aksoy, 2013). So, researchers gave their attention towards the natural antioxidants, especially collected from plants. In plant, polyphenolics (e.g. tannins and flavonoids) are the active ingredients which are responsible for their antioxidant effect (Tanabe *et al.,* 2002; Albayrak and Aksoy, 2013; Bendary *et al.,* 2013). Previous phytochemical screening for the flowers of *Nyctanthes arbor-tristis* L. showed the presence of tannins, flavonoids, carbohydrates,

Table III. IC_{50} values of DPPH free radical scavenging activity obtained for different crude extracts of *Nyctanthes arbor-tristis* L. flowers

Sample	IC_{50} value
n-Hexane extract	291.92 mg/ml
Dichloromethane extract	45.74 µg/ml
Ethyl acetate extract	21.86 µg/ml
Methanol extract	64.30 µg/ml
Ascorbic acid (positive control)	3.98 µg/ml

glycosides, cardiac glycosides, reducing sugar, saponins, terpenoids and steroids (Haque *et al.,* 2019). So, the present study indicates that the flower of *Nyctanthes arbor-tristis* L. has the potential to be a good source of natural antioxidants.

Conclusions

The flowers of *Nyctanthes arbor-tristis* L. contain saturated fatty acids such as palmitic, stearic, arachidic, behenic and lignoceric acid. From the cytotoxicity test, the best cytotoxicity was found for the ethyl acetate extract (LC_{50} value =3.14 µg/ml). In comparison with the positive control (standard vincristine sulfate), it is evident that all the test samples were lethal to brine shrimp nauplii. The results of antimicrobial activity of n-hexane, dichloromethane, ethyl acetate and methanol extracts of the flower were compared with standard antibiotic ciprofloxacin and tetracycline by measuring the zone of inhibition diameter in millimeter. The dicholoromethane and ethyl acetate extracts showed significant antimicrobial activity against gram positive bacteria *B. subtilies, B. cereus, S. aureus* and gram negative bacteria *Pseudomonas.* The dichloromethane, ethyl acetate and methanol extracts showed significant DPPH free radical scavenging activity. This study demonstrates that the flowers of the plant *Nyctanthes arbor-tristis* L. grown in Bangladesh have considerable importance as herbal medicine as well as have natural antioxidants.

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