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Biochemical investigation and biological evaluation of the methanolic extract of the leaves of *Nyctanthes arbortristis in vitro*

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ABSTRACT

Objective: *Nyctanthes arbortristis* is a common plant in Bangladesh. The objective of our research was to biochemical and biological analysis of the methanolic extract of the dried leaves of *Nyctanthes arbortristis* found in Bangladesh. **Methods:** We investigated the presence of polyphenols, flavonoids and other types of compounds by thin layer chromatography, infrared spectroscopy, and UV spectroscopy analysis. We performed antioxidant assay by colorimetric methods. We investigated antibacterial assay by disk diffusion method. Cell surface receptor binding assay was performed by hemagglutination inhibition assay and hemolysis assay. **Results:** Methanolic extract of the leaves of *Nyctanthes arbortristis* contains flavonoids and other biologically active compounds. The extract showed antioxidant, peroxide scavenging and total reducing activity. The extract also showed antibacterial activities against several strains of bacteria. It also showed hemagglutination inhibition activities and hydrogen peroxide induced hemolysis inhibition activity in human blood cells. **Conclusions:** Therefore, *Nyctanthes arbortristis* may be considered as a plant of various health benefits.

1. Introduction

Nyctanthes arbortristis Linn. is a valuable medicinal plant which belongs to the family Oleaceae. The plant generally grows in tropical and subtropical region. The plant has numerous medicinal values. In ayurveda the leaves of this plant are used in chronic fever, obstinate sciatica [1], coughs [2], malaria, constipation, intestinal worms and excessive diuresis [3]. Leaves of *N. arbortristis* are responsible for some CNS activities like hypnotic, tranquilizing and local anesthetics [4–6] and antiasthmatic activity [7]. Fresh juice of leaves is antimalarial [8,9]. Antifungal activity of the leaves was established against *Alrenergaria alternate* [10]. Aqueous extract of leaves is proved to be hepatoprotective [10,11] isolated an alkaloidal principle named nyctanthin from the leaves. Iridoid glucosides were isolated from the plant and have antileishmanial activity [12]. A new iridoid glycoside along with nyctanthic acid, oleanolic acid, friedelin, 6- β -hydroxy-loganin and arbortristoside A has

been isolated from the plant [13]. A minor iridoid glucoside, arborside D and its acetyl derivatives were identified from the plant [14]. Traditionally the plant is used in the treatment of asthma and cough. Histamine, present in mast cells is responsible for asthma hence present work was undertaken to check mast cell stabilizing property and bronchodilatory property of the extracts of *N. arbortristis* bark and in this way to check its application in the treatment of asthma. It is also reported that β -sitosterol in the plants leaves is responsible for analgesic and anti-inflammatory activity [15]. The ethanolic extracts, various fractions and two pure compounds arbortristoside A and arbortristoside C isolated from the plant were tested against Encephalomyocarditis virus and Semliki Forest virus [16].

Here we examined the antioxidant, antibacterial, hemagglutination inhibition activity and hemolysis inhibitory effect of the methanolic extract of the leaves of *Nyctanthes arbortristis* plant collected from Bangladesh.

2. Materials and methods

2.1. Plant collection and identification

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The fresh leaves of the plant were collected from the surrounding of Dhaka, Bangladesh during January, 2010 and identified by the taxonomist of the Bangladesh National Herbarium, Mirpur, Dhaka. A voucher specimen of the plant has been deposited (Accession No.: DACB 37509) in the herbarium for further reference.

2.2. Extraction of the plant material

Sun-dried and powdered plant material (750 g) was extracted with methanol by cold extraction process. The extracts were concentrated with a rotary evaporator (IKA, Germany) at low temperature (40–50 °C) and reduced pressure. The extracts (5 g) were stored at 4 °C until used.

2.3. Phytochemical screening

The freshly prepared crude extracts were qualitatively tested for the presence of Alkaloids (Hager's test), Flavonoids (Modified Ammonia test), Steroids (Salkowski test), Terpenoids (Modified Salkowski test), Reducing sugars (Fehling's test), Saponins (Frothing test), Tannins (FeCl₃ test), Cardiac glycosides (Killer–Killani's test) and Anthraquinones (Chloroform layer test) [17].

2.4. TLC analysis

The extracts were analyzed by performing TLC to determine the composition of each extract. TLC was done under three different solvent systems including nonpolar basic solvent, intermediate polar basic solvent, and polar basic solvent. Successively the polarity of the solvent systems was increased to get a clear graph of all the possible compounds present in the extract. In nonpolar basic solvent benzene was used to elute the non polar compounds present in the extract. Again, since Alkaloid and terpenoid type compounds are basic in nature, ammonium base is used in the solvent system to help them run over TLC plate. The resulting nonpolar mobile phase consisted of benzene, ethanol, and ammonium hydroxide (9:1:0.1). Intermediate polar basic solvent consisted of chloroform, ethyl acetate, and formic acid (5:4:1). Polar basic solvent consisted of ethyl acetate, ethanol, and water (8:1.2:0.8).

After completion of TLC, the plates were exposed to UV light for compound detection and identification. For charring the plates were exposed to 10% sulphuric acid solution, dried and then heated to 80–90 °C for charring purpose. This helped in the fixation of spot and allowed it to be prominently visible. For detection of flavanoids the plates were dipped into 0.04% DPPH solution and dried while keeping in a dark place. For detection of polyphenols the plates were washed with Folin & ciocalteu reagent and dried.

2.5. Chemical analysis of the extract

Ultraviolet (UV) spectroscopy of the extract was performed within 200 nm to 400 nm using a Lambda UV spectrometer (Shimadzu, Japan) [18]. The infrared (IR) spectra of the extract were recorded within 4000 cm⁻¹ to 400 cm⁻¹ using a Fourier–transform IR (Shimadzu, Japan) instrument with KBr pellets [19].

2.6. Determination of total phenolic content

The total phenolic content of extracts was determined using Folin–Ciocalteu method using gallic acid as standard [20]. The extracts were oxidized with 10% Folin–Ciocalteu reagent (Merck, Germany), and were neutralized with 700 mM sodium carbonate solution. The absorbance of the resulting blue color was measured at 765 nm after 60 min. The total phenolic contents were determined using a standard curve prepared with gallic acid. The estimation of the phenolic compounds was carried out in triplicate. The results were mean ± standard deviations and expressed as milligram of gallic acid equivalent/g of extract.

2.7. Total Flavonoid Assay

The total flavonoid compounds in each extract were determined as previously described by Jothy *et al.* 2011 [20]. An aliquot (1.5 mL) of methanolic extract was added to 6ml of deionized water and then 0.45 mL 5% (w/v) NaNO₂ and incubated for 6 min. 0.45 mL 10% (w/v) AlCl₃ and 6 ml 4%(w/v) NaOH was added and the total volume was made up to 15 ml with distilled water. The absorbance was measured at 510 nm by using visible spectrophotometer. The results were expressed as mg rutin equivalents/g. The experiments were performed in three times.

2.8. DPPH radical scavenging activity

The free-radical scavenging activity of the extract were measured by decrease in the absorbance of methanolic solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl) [21]. The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the extracts. Different concentrations of the plant extract (2, 4, 6, 8 & 10 µg/mL, in methanol) were added at an equal volume (10ml) to methanol solution of DPPH (400 µg/mL). Different concentrations of L-Ascorbic acid (2–10 mg/mL) were used as the standard antioxidant. After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and converted into the percentage antioxidant activity using the following equation: DPPH antiradical scavenging capacity (%) = (Absorbance of sample – Absorbance of blank) × 100/Absorbance of blank. Methanol plus different concentration of plant extract solution was used as a blank, while DPPH solution plus methanol was used as a control. IC₅₀ values denote the

concentration of the sample required to scavenge 50% of DPPH radicals.

2.9. Hydrogen peroxide scavenging assay

A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM, pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (0 – 20 μ g/mL) in phosphate buffer is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. L-ascorbic acid was used for comparison. The percentage of hydrogen peroxide scavenging is calculated as follows: % Scavenged (H₂O₂) = (A₀ – A₁ / A₀) X 100 Where; A₀ is the absorbance of control and A₁ is the absorbance of test. Ascorbic acid was used as a positive control.

2.10. Total reducing assay

The reducing power of the extracts of night jasmine leaves was measured using the potassium ferricyanide reduction method. Various amount of extracts (0–200 mg) and L-ascorbic acid (0–1000 mg) were taken in different test tubes as previously described by Oyaizu [22]. Then 2.5 mL of distilled water and 2.5 mL of potassium ferricyanide [K₃Fe₃(CN)₆] solution were added in all test tubes and mixed well. After incubation at 50 °C for 20 min, 2.5 mL of trichloro acetic acid(TCA)[10% w/v] was added in all test tubes and centrifuged at 3000 rpm for 10 min. Afterwards, upper layer of solution(5ml) was mixed with 5 mL distilled water. Then 1ml of FeCl₃ was added each test tube. Then from each test tube we collect 1ml of solution and mixed it with 9 mL of distilled water. Then the solution was incubated at 35 °C for 10 min. the formation of perls prussian color was measured at 700 nm in a spectrometer. Increased absorbance of the reaction mixture indicate increasing reducing power. L-ascorbic acid was used as a standard. The analysis was performed in twice.

2.11. Antibacterial assay

Five different bacterial strains were used to carry out this assay. These are *V. mimicus*, *S. typhi*, *S. dysentery*, *S. aureus* & *B. serus*. Nutrient agar was used as the culture media. Stocks of these bacterial solutions were revived in nutrient agar by incubating at 37 °C for 24 h. A single disk diffusion method was used to assess the presence of antimicrobial activities of the methanolic extract of night jasmine leaves. Whatman's filter paper was punched, and 6mm disks were collected in a beaker. The beaker was covered with foil paper and autoclaved. 20 μ L of different concentration extracts (1 mg/mL, 10 mg/mL) were loaded was pipette per disk. The revived test organisms were plated onto nutrient agar plates. The disks were then placed equidistant on all

plates for all extracts. Standard disc (Himedia, India) of Azithromycin (30 μ g/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. After incubation at 37 °C for 24 h, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm.

2.12. Hemagglutination inhibition assay

Hemagglutination activity of the crude extract and fractions was tested against human erythrocyte blood groups ABO as previously described by Saha *et al.* [23]. Stock solution of the test samples was prepared at concentration of 5 mg/ml and each solution was serially diluted. Fresh blood was collected from healthy persons, centrifuged and the erythrocytes were separated. 4% erythrocyte suspension was prepared in phosphate buffer (pH 7.4) of all blood groups. 1 mL of the test sample dilution was taken with 1 ml of 4% erythrocyte and incubated at 4 °C. After incubation, the results were noted. Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity. The intensity of hemagglutination was determined from the extent of deposition.

2.13. Anti hemolytic assay

Inhibition of H₂O₂ induced red blood cell (RBC) hemolysis of methanolic extract was examined by the in vitro method described previously by Tavazzi *et al.* [24]. The erythrocytes from human blood were separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4) until the supernatant was colorless. The erythrocytes were then diluted with saline or phosphate buffer to give a 4% suspension. Varying amounts of sample (0–1 mg/mL) with saline or buffer were added to 2 mL of the suspension of erythrocytes and the volume was made up to 3.5 mL with saline or buffer. This mixture was preincubated for 120 min and then 0.5 mL H₂O₂ solutions of appropriate concentration in saline or buffer were added. The concentration of H₂O₂ in the reaction mixture was adjusted so as to bring 90% hemolysis of blood cells after 120 min incubation. Incubation was concluded after these time intervals by centrifugation during 5 min at $\times 1000$ g and the extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation. Antihemolytic activity was expressed as the inhibition percentage and was calculated using the following formula: Antihemolytic activity (%) = (Control 540 nm – Sample 540 nm) \times 100/ Control 540 nm, where, Sample 540 nm was the absorbance of the sample and Control 540 nm was the absorbance of the control.

3. Results

3.1. Phytochemical screening

Preliminary phytochemical screening showed (Table 1) the presence or absence of alkaloids, flavonoids, steroids, terpenoids, reducing sugars, saponins, tannins, cardiac glycosides, anthraquinones in varying amount in the extracts.

Table 1
Phytochemical compositions of *Nyctanthes arbortristis* extracts.

Phytoconstituents	Name of the test	Result
Alkaloids	Hager's test	+++
Anthraquinones	Chloroform layer test	–
Cardiac glycosides	Killer–Killani's test	+
Flavonoids	Ammonia test (modified)	++
Reducing sugars	Fehling's test	–
Saponins	Frothing test	+++
Steroids	Salkowski test	++
Tannins	FeCl ₃ test	–
Terpenoids	Salkowski test (modified)	–

Key: +++ = highly present; ++ = moderately present; + = slightly present; – = absent

TLC analysis also showed the presence of various bioactive components in the extract as shown in Figure 1–3. The separation performed by nonpolar mobile phase consisted of benzene, ethanol, and ammonium hydroxide (9:1:0.1) is shown here in Figure 1.

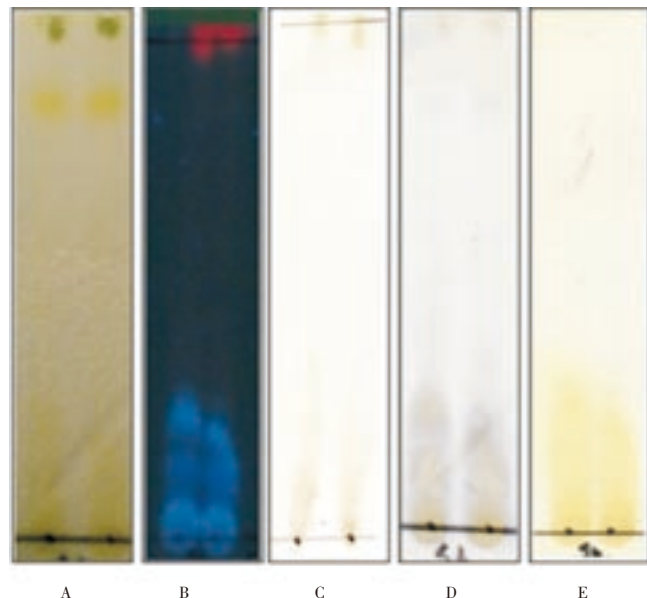


Figure 1. Separation of methanolic extract of *Nyctanthes arbortristis* using benzene, ethanol, and ammonium hydroxide (9:1:0.1) solvent system. Key: A= Normal view; B= Ultra–Violet view; C= Charring view; D= FC reagent staining view; E= DPPH staining view.

TLC plates were seen under UV light and found some compounds were separated at the bottom of the plates. Charring with H₂SO₄ in high temperature the separated compounds transformed into black color. Staining the plate with FC–reagent the color of the separated

compounds changed into bluish and after staining the plate with DPPH solution the color of the separated compounds changed into yellow color. Such a result indicated the presence of flavanoids in the separated fractions of the extract in the nonpolar mobile phase. Intermediate polar basic solvent consisted of chloroform, ethyl acetate, and formic acid (5:4:1). The separation performed by the intermediate polar basic solvent is shown here in Figure 2.

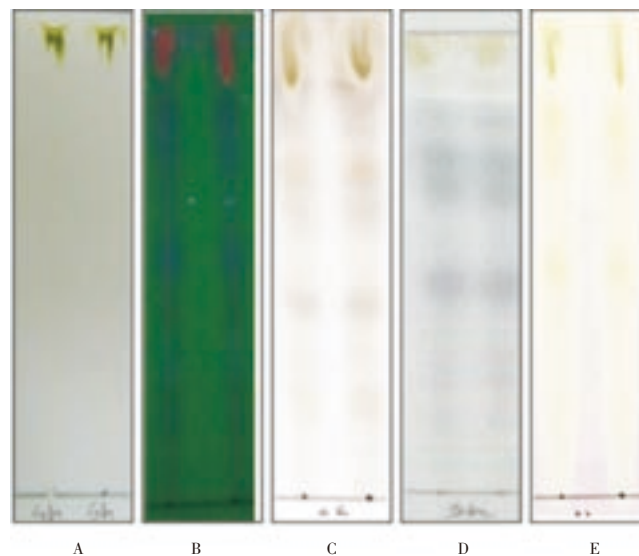


Figure 2. Separation of methanolic extract of *Nyctanthes arbortristis* using chloroform, ethyl acetate, and formic acid (5:4:1) solvent system. Key: A= Normal view; B= Ultra–Violet view; C= Charring view; D= FC reagent staining view; E= DPPH staining view.

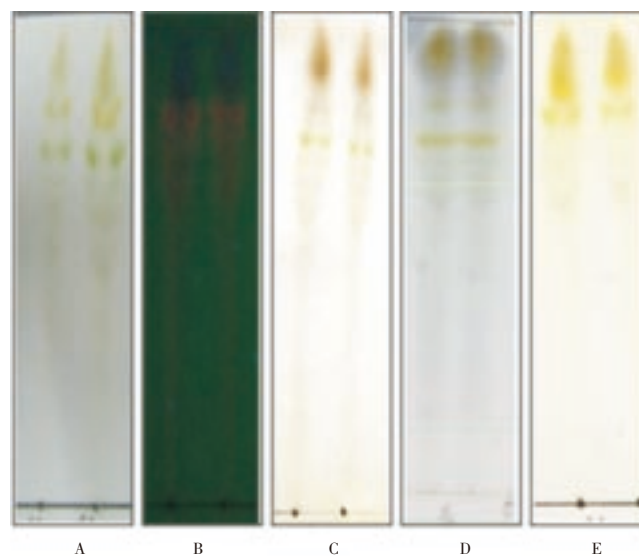


Figure 3. Separation of methanolic extract of *Nyctanthes arbortristis* using ethyl acetate, ethanol, and water (8:1.2:0.8) solvent system. Key: A= Normal view; B= Ultra–Violet view; C= Charring view; D= FC reagent staining view; E= DPPH staining view.

TLC plates were seen under UV light and found some compounds were separated at the bottom of the plates. Charring with H₂SO₄ in high temperature the separated compounds transformed into black color. Staining the plate with FC–reagent the color of the separated

compounds changed into bluish and after staining the plate with DPPH solution the color of the separated compounds changed into yellow color. Such a result indicated the presence of flavanoids in the separated fractions of the extract in the intermediate polar mobile phase. The separation efficiency of the extract in intermediate polar solvents was better than that of the non polar solvent system.

Polar basic solvent consisted of ethyl acetate, ethanol, and water (8:1.2:0.8). The separation performed by the polar basic solvent is shown here in Figure 3.

TLC plates were seen under UV light and found some compounds were separated at the bottom of the plates. Charring with H_2SO_4 in high temperature the separated compounds transformed into black color. Staining the plate with FC-reagent the color of the separated compounds changed into bluish and after staining the plate with DPPH solution the color of the separated compounds changed into yellow color. Such a result indicated the presence of flavanoids in the separated fractions of the extract in the polar mobile phase. The separation in polar solvent system has been shifted from the bottom to the top the stationary TLC plates.

Infrared spectroscopy analysis of the methanolic extract of the leaves of *Nyctanthes arbortristis* also indicates the presence of various types of functional groups presenting compounds present in the extract as shown in Figure 4.

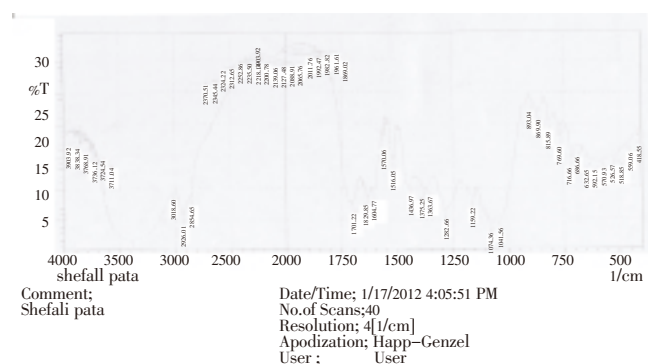


Figure 4. Infra-red spectroscopy of methanolic extract of *Nyctanthes arbortristis*.

Base values for absorption bands were found at various wavelengths including 3000, 2250, 2150, 1715, 1650, and 1100 cm^{-1} indicates the presence of C-H, C \equiv N, C \equiv C, C=O, C=C, and C-O. Therefore, the results indicate the possibility of the presence of flavanoids or flavanoid-conjugated compounds in the extract.

UV spectroscopic analysis of the methanolic extract of *Nyctanthes arbortristis* leaves showed the maximum absorbance values at 670, 630, 500, 430, 370, 350, 320 and 310 nm (Figure 5). Such an absorbance values in UV spectroscopic analysis indicates the presence of various types of biomolecules including flavanoids, tannins, flavanone glycosides, chlorophyll etc.

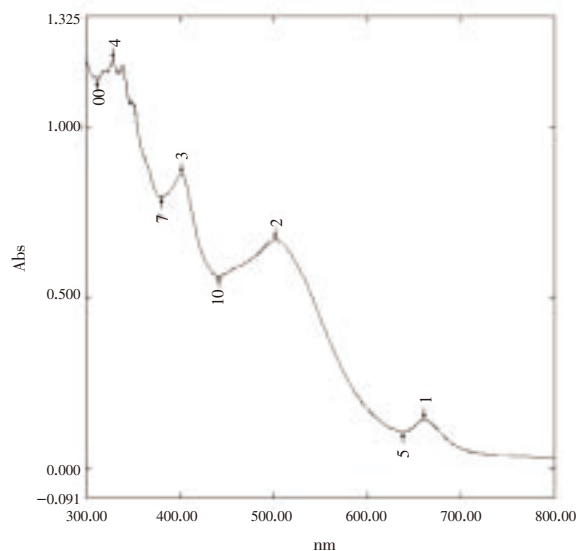


Figure 5. UV spectroscopy of methanolic extract of *Nyctanthes arbortristis*.

3.2. Total phenolic content

Quantitative analysis of polyphenols and flavanoids were performed as described previously. In case of polyphenols quantification as standard curve was used in Mongkolsilp *et al.* [25], where the equation is $y = 1.4456X - 0.0186$, $R^2 = 0.999$. From the standard curve, the Total phenolic compounds as galic acid equivalent (GAE) of the extract was 1.37 ± 0.11 mg/100 mg sample. In case of flavanoid quantification a standard curve was used Jothy *et al.* [20] where the equation is $y = 0.0071X + 0.1139$, $R^2 = 0.9927$. From the standard curve the amount of catechin present in the extract is 7.74 ± 0.15 μ g/mg extract.

3.3. DPPH radical scavenging activity

From the analyses of figure 6, we can conclude that the scavenging effect of *N. arbortristis* extracts increase as the concentration increases. Therefore, methanolic extract of the plant showed stronger antioxidant activity than that of vitamin C. The 50% inhibitory effect of the extract was calculated from the curve and it was 0.039 mg/mL.

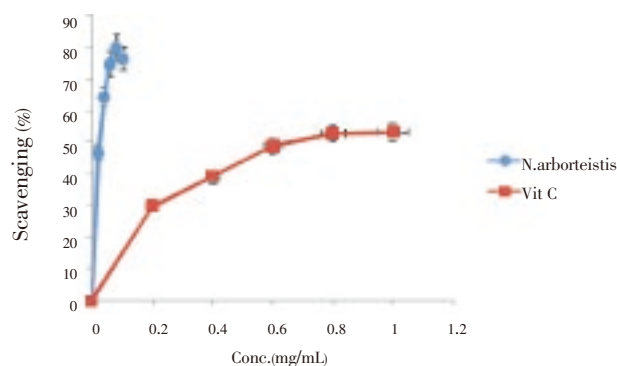


Figure 6. DPPH scavenging activity of the methanolic extracts of *N. arbortristis*

3.4. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of the extract was measured in compared with ascorbic acid as shown in Figure 7. Their rate of hydrogen peroxide scavenging activity was measured comparing with ascorbic acid. It was found that methanolic extract showed stronger scavenging activity than that of ascorbic acid.

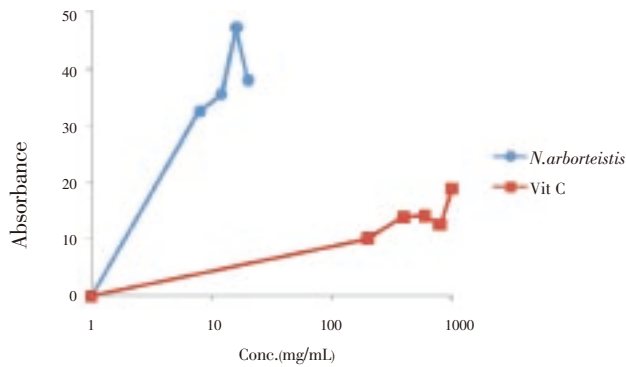


Figure 7. H₂O₂ scavenging activity of the methanolic extracts of *N. arbortristis*.

3.5. Total reducing assay

Total reducing assay of the extract was investigated in compared with ascorbic acid as shown in Figure 8. Their 50% scavenging activity was measured and found that it has stronger scavenging activity than that of ascorbic acid.

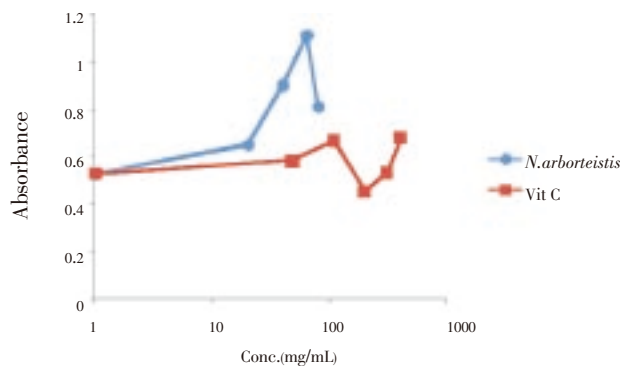


Figure 8. Total reducing activity of the methanolic extracts of *N. arbortristis*.

Table 3
Antibacterial assay of *N. arbortristis*.

Name of microorganisms	Negative control	N. arbortristis		Azithromycin
		200 µg/disc	20 µg/disc	30 µg/disc
Zone of inhibition in mm				
<i>V.mimicus</i>	–	17	7	22
<i>S.typhi</i>	–	22	6	21
<i>S.dysentry</i>	–	17	10	23
<i>S.aureus</i>	–	18	8	25
<i>B. sereus</i>	6	14	–	23

“ – ” = Indicates no zone of inhibition.

3.6. Hydrogen peroxide induced antihemolytic activity

Hemolysis caused by hydrogen peroxide was inhibited by the extract at various concentration has shown in Figure 9. 300 µ L of H₂O₂ was used for complete lysis of RBC.

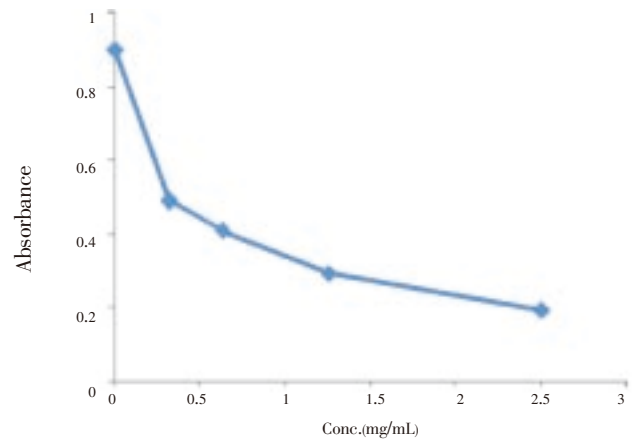


Figure 9. Antihemolytic activity of the methanolic extracts of *N. arbortristis*.

3.7. Hemagglutination inhibition assay

Various concentrations of extracts (0–5 mg/ml) were taken to investigate hemagglutination inhibition activity on different types of human blood groups. Hemagglutination inhibition activities of the *N. arbortristis* extracts were tested against four different types of human blood groups and the results are in Table 2.

Table 2
Hemagglutination inhibition assay of *N. arbortristis*.

Blood group	5 mg/mL	2.5 mg/mL	1.25 mg/mL	Buffer only
A+	+++	++	+	---
B+	+++	++	+	---
O+	++	++	+	---
AB+	++	+	–	---

Key: +++ = no hemagglutination; ++ = moderately hemagglutination; + = slightly hemagglutination; – = hemagglutination;

3.6. Antimicrobial assay

Antimicrobial activities of the *N. arbortristis* extracts were tested against five pathogenic organisms and the results are in Table 3. In the antibacterial screening, the extracts

showed average zone of inhibition 6–22 mm at concentrations of 20–200 μ g/disc.

4. Discussion

4.1 Phytochemical screening

Previous studies have been reported that *N. arbortristis* plant contains various types of medicinal compounds. The plant leaves have potential phytochemical like nictantic acid, friedelin, beta-sitosterol and oleanolic acid and responsible for antiviral activity, [26]. Leaves of *Nyctanthes arbortristis* contain D-mannitol, β -sitosterole, Flavanol glycosides–Astragaline, Nicotiflorin, Oleanolic acid, Nyctanthic acid, tannic acid, ascorbic acid, methyl salicylate, an amorphous glycoside, an amorphous resin, trace of volatile oil, carotene, friedeline, lupeol, mannitol, Glucose, fructose, iridoid glycosides, benzoic acid derivative of kaempferol and carotene [27]. Here we also found that various types of phytochemical compounds are present in the methanolic extract of the leaves of *N. arbortristis* found in Bangladesh. (Figure 1–5 and Table 1). We also found that there is a possibility of the presence of several types of flavanoids in the leaves of the plants

4.2. Total phenolic content

Recent studies also reported that the leaves extract of *Nyctanthus arbortristis* contain total phenolic compounds and flavanoids in methanol–water extract [28]. Here we also found the similar results in the methanolic extract of the plant leaves collected from Bangladesh.

4.3 DPPH radical scavenging activity

Free radical scavenging potential of the different extracts of methanolic extract of the leaves of *Nyctanthes arbortristis*, was evaluated in vitro by using DPPH assay. In this method the antioxidants present in the plant extracts reacted with DPPH, which is a stable free radical and converted it to 1,1-diphenyl-1,2-picryl, hydrazine which is measured at 517 nm. The previous study also reported the positive result of the leaves of *Nyctanthes arbortristis* [29] which is similar to our present results.

4.4. Hydrogen peroxide scavenging activity and total reducing assay

The different solvent extracts of the dry and fresh flowers was studied for its free radical scavenging activity with different methods viz lipid peroxidation assay, reducing activity and hydrogen peroxide scavenging assay and found that it showed hydrogen peroxide scavenging activity [30]. Here we found that the methanolic leaves extract of the plant

showed hydrogen peroxide scavenging activity and total reducing assay.

4.5. Hydrogen peroxide induced antihemolytic activity and hemagglutination inhibition assay

We found that the methanolic extract of the leaves of *N. arbortristis* showed hydrogen peroxide induced antihemolytic activity. This effect is probably due to the presence of flavanoids and other types of antioxidants in the leaves. Besides, the extract may contain some types of receptor binding compounds that may affect the inhibition of erythrocytes agglutination of human blood.

4.6 Antibacterial assay

Previous study reported that the aqueous extract showed more potent antibacterial activity than that of the methanolic extract of the plants of *N. arbortristis* [31]. Here, we also found that methanolic extract of the leaves of *N. arbortristis* showed weak antibacterial activity of the freshly prepared extract.

Our present study has reported that the methanolic extract of the leaves of *Nyctanthes arbortristis* show antioxidant effect probably due to the presence of flavanoids in the leaves. Besides, the leaves extract has showed antibacterial activity and hemagglutination inhibition activity on human red blood cells. Their antibacterial activity is probably due to the presence of such compounds in the leaves of *Nyctanthes arbortristis* those may bind with bacterial cell membrane. However, further studies are required to isolate the pure compounds and identify them for specific application.

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