

Full Length Research Paper

Evaluation of membrane stabilizing, anthelmintic, antioxidant activity with phytochemical screening of methanolic extract of *Neolamarckia cadamba* fruits

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The current study was directed on methanolic extract of *Neolamarckia cadamba* fruits, belonging to the family Rubiaceae, to reveal the possible phytochemicals existence and also to evaluate the membrane stabilizing, anthelmintic, antioxidant properties. To estimate the membrane stabilizing activity, both heat and hypotonic solution induced haemolysis techniques were used. The anthelmintic test was conducted on earthworm *Pheritima phosthuma* using five different concentrations (10, 20, 40, 60, 80 mg/ml) of the extract and albendazole as standard drug (concentration 10 mg/ml). To investigate antioxidant property, two potential tests namely total phenolic content determination and the 2,2-diphenylpicrylhydrazyl (DPPH) free radical scavenging assay were conducted. Phytochemical screening was carried out using different chemical group tests. The extract revealed good membrane stabilizing activity inhibiting both hypotonic solution and heat induced haemolysis in comparison to inhibition by standard acetyl salicylic acid. The methanolic extract showed potent anthelmintic activity at the highest concentration as it required less time for paralysis and death compared to the standard drug albendazole. The fruit extract showed potential antioxidant property. The analysis of phytochemicals reveals the presence of carbohydrate, phenol, phytosterol, protein and amino acid, terpene and glycoside. The results of the study showed that the plant extract has potential membrane stabilizing, anthelmintic, antioxidant activities along with the presence of significant phytochemicals.

Key words: *Neolamarckia cadamba*, membrane stabilizing activity, anthelmintic activity, antioxidant, phytochemicals.

INTRODUCTION

Neolamarckia Cadamba (Roxb.) Bosser is a well-known evergreen tropical flowering plant of Bangladesh, locally called "Kadam", belonging to the Rubiaceae family. It is

native to Bangladesh, Nepal, India, Myanmar, Sri Lanka, Philippines, Indonesia and Papua New Guinea (Uddin et al., 2013; Ganjewalaa et al., 2013). Since the prehistoric

time, different parts of this plant have been used as anti-diuretic, anti-pyretic, in the treatment of anemia, tumor as well as for the improvement of semen quality (Ahmed et al., 2011; Umachigi et al., 2007). Some previous studies evidenced that the plant also possesses antimicrobial, antioxidant, and wound healing (Umachigi et al., 2007) as well as anti-diarrheal properties (Alam et al., 2008). The plant is sometimes used in the treatment of various ailments like diabetes mellitus, inflammation, haemoptysis, cough, vomiting, ulcers and debility (Dubey et al., 2011). As the fruit is edible, its juice is given to children for the remedy of gastric irritability. Furthermore, its timber is used for making pulp and paper, boxes and furniture while its wood is used as fuel (Mishra, 2011).

A stabilized membrane is required to prevent oxidative damage and related inflammatory actions caused by free radicals produced within the body. Erythrocyte membrane stability test is an extensive study which highlights the effect of synthetic and herbal anti-inflammatory agents on erythrocyte membrane that is exposed to hypotonic solution and heat.

Due to the similarity of erythrocyte membrane with the lysosomal membrane, the effect of drug on erythrocyte stabilization can be compared to the lysosomal membrane stabilization (Sikder et al., 2010). To treat the consequences of oxidation and inflammation, there are many anti-inflammatory agents or drugs like nonsteroidal anti-inflammatory drugs (NSAIDs) available in the market. As these drugs are responsible for intestinal side effects and mucosal erosions, researchers have focused on medicinal plants for finding natural anti-inflammatory drugs with reduced side effects (Richard et al., 2011).

Helminthiasis is a macro parasitic disease that is very common among the developing countries all over the world including Bangladesh. Parasitic worms like Roundworms (Nematodes), Tapeworms (Cestodes) or Flukes (Trematodes) are responsible for this disease. According to the World Health Organization (WHO), about 2 billion people are affected by parasitic worm infection throughout the world because of poor management practices and insufficient control measures (Gaikwad et al., 2011). Oxidative stress occurs due to the increased formation of free radicals. It is a chain reaction that damages cell component like proteins, lipids and nucleic acids leading to cell death (Elmastas et al., 2007). Antioxidants have the ability to inhibit or delay the oxidation of an oxidizable substrate in a chain reaction. There are a number of synthetic antioxidants in the market which cause serious adverse effects on the body (Lobo et al., 2010). This is why finding natural antioxidants

without any adverse effects has gained importance. Phytochemicals are naturally occurring components in the medicinal plants that have various defence mechanisms and can protect us from various diseases. Phytochemical constituents present in medicinal plants can be useful in healing and assessing human diseases (Wadood et al., 2013).

Traditionally whole medicinal plant or different parts are used in the treatment of all kinds of diseases, and people prefer mostly traditional medicine because of its availability, cost effectiveness, non-toxic nature and high percentage of cure rate with single therapeutic dose (Rastogi et al., 2009). There are some evidences of various studies performed on different parts of *N. cadamba* plant but reports on fruits of the plant are very few.

Thus, our present study was designed to evaluate the membrane stabilizing, anthelmintic, antioxidant activity and also to identify the presence of phytochemicals in methanolic extract of *N. cadamba* fruits with the aim of developing new drugs.

METHODOLOGY

Plant collection and authentication

For this current investigation, the fresh fruits of the plant *N. cadamba* (Family: Rubiaceae) were collected from the surrounding campus of Noakhali Science and Technology University, Sonapur, Noakhali - 3814, Bangladesh in August, 2013 and identified by an expert botanist of the Bangladesh National Herbarium, Mirpur, Dhaka (DACB: Accession number: 38770).

Preparation of plant materials

The collected plant parts (fruits) were separated from undesirable materials of plants or plant parts. They were sun-dried for one week. The fruits were grounded into a coarse powder with the help of suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

Extraction procedure

550 g of dried powdered sample was submerged in 2500 ml of 90% methanol (Merck KGaA, Darmstadt, Germany) with sporadic shaking. After 15 days, the solvent was decanted and filtered using sterile cotton and Whatman® filter paper No. 1 (Sargent-Welch, USA) and then evaporated using rotary evaporator and freeze-dried (yield 28 g deep orange gummy extract) (Raju et al. 2013).

Membrane stabilizing activity

Membrane stabilizing activity was assessed by using hypotonic-

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solution and heat induced haemolysis of human erythrocyte by the method developed by Omale and Okafor (2008).

Collection of blood samples

Human red blood cells (RBCs) were collected for the study. 2 ml of blood was collected from each of the healthy Bangladeshi male human volunteers (aged 20 to 23 years) without a history of oral contraceptive or anticoagulant therapy and free from diseases (using a protocol approved by Institutional Ethics Committee). The collected RBCs were kept in a test tube with an anticoagulant Ethylenediaminetetraacetic acid (EDTA) under standard conditions temperature ($23 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 10\%$).

Preparation of erythrocyte suspension

To prepare the erythrocyte suspension, 2 ml of blood was obtained using syringes (containing anticoagulant EDTA) from male volunteers through puncture of the anti-cubital vein. The blood was centrifuged using centrifugal machine for 10 min at 3000 g and then it was washed three times using isotonic solution (0.9% saline). The volume of saline was measured and reconstituted as a 40% (v/v) suspension with isotonic buffer solution (pH 7.4) which contained 1 L of distilled water: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.26 g; Na_2HPO_4 , 1.15 g; NaCl, 9 g (10 mM sodium phosphate buffer). Thus, the suspension finally collected was the stock erythrocyte (RBC) suspension.

Hypotonic solution-induced haemolysis

The test sample which consisted of stock erythrocyte (RBC) suspension (0.50 ml) was mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (2.0 mg/ml) or acetyl salicylic acid (0.1 mg/ml). The control sample which consisted of 0.5 ml of RBCs was mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer (Biswas et al., 2013). The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ Inhibition of haemolysis} = 100 \times (\text{OD1} - \text{OD2} / \text{OD1})$$

Where,

OD1 = Optical density of hypotonic-buffered saline solution alone (control) and

OD2 = Optical density of test sample in hypotonic solution

Heat-induced haemolysis

Aliquots (5 ml) of the isotonic buffer, containing 2.0 mg/ml of extract of the plant were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 μl) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54°C for 20 min in a water bath. The other pair was maintained at 0 to 5°C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer (Biswas et al., 2013). The percentage inhibition or acceleration of haemolysis in tests was calculated using the following equation:

$$\% \text{ Inhibition of haemolysis} = 100 \times [1 - (\text{OD2} - \text{OD1} / \text{OD3} - \text{OD1})]$$

Where, OD1 = test sample unheated; OD2 = test sample heated

and OD3 = control sample heated

In vitro anthelmintic activity

The anthelmintic test was carried out according to the method reported in Ajaiyeoba et al. (2001) with some requisite amendments. Adult earth worm (*Phertima posthuma*) was used to perform the test because of its anatomical and physiological resemblance with intestinal round worm parasite (Vidyarthi, 1967; Lakshmi et al., 2012). The worms were collected from the moist soil of Noakhali Science and Technology University area. Methanolic extract of *N. cadamba* fruit was taken at different concentrations (10, 20, 40, 60 and 80 mg/ml) separately. 100 mg of albendazole was dissolved in 10 ml water to prepare a concentration of 10 mg/ml which was referred as standard. A control group was established with distilled water for the test validation. Earthworms were placed into seven petri dishes in 7 groups, each containing five earthworms where five dishes were used for the five concentrations of methanolic extract of *N. cadamba* and one for the reference standard and another for the control group. The paralyzing time was counted only when there was no movement observed except that the worm was shaken vigorously. After ascertaining that the worms moved neither when vigorously shaken nor when dipped in warm water (50°C), the death time was recorded (Raju et al., 2013).

In vitro antioxidant activity

The in-vitro antioxidant activity test was done using two methods:

Determination of total phenolic content: The amount of total phenolic content present in plant extract was determined by using Folin-Ciocalteu reagent. As gallic Acid was used as standard, the total phenolic contents were expressed as mg/g of gallic acid equivalents (GAE). Concentration of 6.25, 12.5, 25, 50, and 100 mg/ml of gallic acid and concentration of 2 mg/ml of plant extract were also prepared in methanol. Then 0.5 ml of sample was introduced into test tubes and mixed with 2.5 ml of a 10 fold dilute Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The tubes were enclosed with para-film and allowed to stand for 30 min at room temperature and the absorbance was measured at 760 nm spectrophotometrically (UV-1800, Shimadzu, Japan). Total phenolic content was determined as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve (Raju et al., 2013).

Free radical scavenging activity by DPPH method: The free radical scavenging activity of *N. cadamba* fruit extract was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (Diphenylpicrylhydrazyl) by using the standard method (Alam et al., 2008) at different concentrations (6.25, 12.5, 25, 50, 100 mg/ml), 2 ml of methanolic solution of sample (extract/standard) was mixed with 3.0 ml of a DPPH methanol solution (20 mg/ml). The mixture was kept in a dark place at room temperature for 30 min and later absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ Scavenging Activity} = (1 - A_{\text{test sample}} / A_{\text{control}}) \times 100.$$

Table 1. Effect of *N. cadamba* fruit extract on hypotonic solution and heat induced haemolysis of erythrocyte membrane.

Sample/Standard	% Inhibition on haemolysis	
	Hypotonic solution induced	Heat induced
-		
ME	34.36±0.31*	21.28±0.15*
ASA	52.22±0.54	40.02±0.37

ME= Methanolic extract, ASA= Acetyl salicylic Acid, Each value is presented as mean ± standard deviation (n = 5). Data are found to be significant by testing through one way ANOVA at 5% level of significance * P<0.05 when compared to the standard.

Here, A stands for Absorbance.

The graph plotted with inhibition percentage against extract/standard concentration; extract concentration providing 50% inhibition (IC₅₀) was calculated (Sikder et al., 2010).

Phytochemical screening

The freshly prepared crude extract was qualitatively tested to determine the presence of chemical constituents. Phytochemical screening of the fruit extract was performed using the following reagents and chemicals: Dragendorff's reagent for Alkaloid test, Mg and HCl for Flavonoid test, Ferric chloride and potassium dichromate solutions for Tannin and saponins with ability to produce stable foam and Libermann- Burchard reagent for Steroid test, Carbohydrates with Benedict's reagent. These chemical constituents were identified by characteristic colour changes using standard procedures (Howlader et al., 2012).

Statistical analysis

All data were presented as mean ± standard deviation (SD) and were analysed by One- way analysis of variance (ANOVA) (SPSS for windows, version 18.0, IBM corporation, NY, USA) and MS Excel for windows version 2010[®]. The values were considered significantly different at p<0.05.

RESULTS

Membrane stabilizing activity

The crude methanolic extract of fruits of *N. cadamba* was subjected to assays for membrane stabilizing activities by following standard protocols and the obtained results were statistically presented in Table 1. The results showed that the extracts (at concentration 2 mg/ml) were significantly (p<0.05) potent on human erythrocyte, adequately protecting it against hypotonic solution and heat induced lyses, when compared to the standard drug acetyl salicylic acid (0.10 mg/ml). In hypotonic solution and heat induced conditions, the extract was found to inhibit 34.36±0.31% and 21.28±0.15% haemolysis of erythrocyte membrane respectively, while in the same

conditions, acetyl salicylic acid inhibited 52.22±0.54 and 40.02±0.37% haemolysis of erythrocyte.

Anthelmintic activity

From the data in Table 2, it is observed that the gradual increase of sample concentration of methanolic extract of *N. cadamba* demonstrates paralysis as well as death of worms in fewer times. At the concentration of 80 mg/ml and 60 mg/ml, the methanolic extract showed paralysis time of 5.67±1.53 min, 10.00±1.00 min and death time of 8.67±1.53 min, 14.00±1.00 min respectively. These results were compared to that of the standard albendazole for which paralysis time was found as 8.66±0.58 min and death time 36.67±1.53 min at a concentration of 10 mg/ml.

Antioxidant activity

Determination of total phenolic content

Table 3 shows the total phenolic contents of methanolic extracts of *N. cadamba* fruits. Total phenolic compounds were reported as gallic acid equivalents by reference to a standard curve ($y=0.0125x+0.0521$; $R^2 = 0.9978$). The results showed that the total phenol content of methanolic extract was found to be 91.19±0.14 mg of GAE/g. of extract. The results of total phenolic contents suggest that the plant may possess good antioxidant activity.

Free radical scavenging activity by DPPH method

In this investigation, the crude methanolic extract of *N. cadamba* fruits showed the free radical scavenging activity with IC₅₀ value of 1.01±0.01 mg/ml and the maximum inhibition was found as 92.68%. On the other hand, the standard ascorbic acid showed maximum inhibition of 95.86 and 50% inhibitory concentration (IC₅₀) was found as 1.53±0.02 mg/ml. Figure 1 shows the scavenging activity of fruit extract in a good way.

Table 2. Anthelmintic activity of crude methanolic extract of fruits of *N. cadamba* against *Pheretima posthuma*.

Test Substance	Concentration (mg/ml)	Time taken for paralysis (min)	Time taken for death (min)
Control (Distilled water)	-	-	-
Standard (Albendazole)	10	8.66±0.58	36.67±1.53
Sample 01	10	33.33±1.53***	46.66±1.53***
Sample 02	20	25.67±1.53***	31.33±1.53**
Sample 03	40	18.00±1.00***	22.00±1.00***
Sample 04	60	10.00±1.00*	14.00±1.00***
Sample 05	80	5.67±1.53*	8.67±1.53***

Each value is presented as mean ± standard deviation (n=5). * = p < 0.05, ** = p < 0.01, *** = p < 0.001. Data are found to be significant by testing through one way ANOVA at 5% level of significance (p<0.05) when compared to the control. (min = minute)

Table 3. Determination of total phenolic contents of *N. cadamba* fruits.

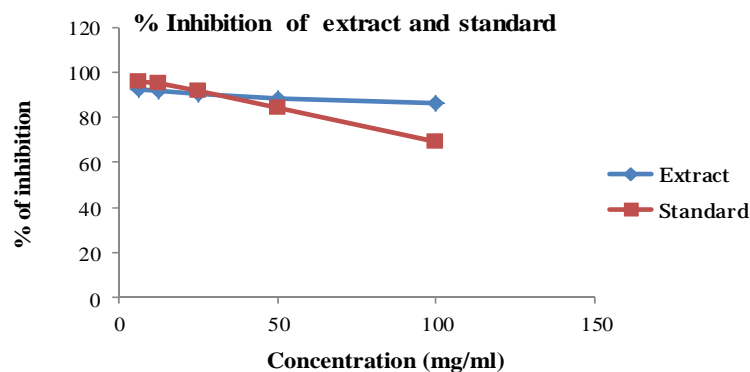
Extract	Absorbance of the sample	Average absorbance	Total phenolic content (mg of GAE/g) of Extracts
Methanol extract	2.43	2.33±0.06	91.19±0.14
-	2.34	-	-
-	2.22	-	-

Data represent mean ± standard deviation (n=3) of duplicate analysis.

Table 4. Phytochemical screening of the methanolic extract of *N. Cadamba* fruits.

Phytochemical constituents	Methanolic extract
Alkaloids	-
Carbohydrate	+
Glycosides	+
Saponins	-
Phytosterols	+
Phenols	+
Tannins	-
Flavonoids	-
Proteins and amino Acids	+
Terpenes	+

(+) = presence of constituents; (-) = absence of constituents.

**Figure 1.** DPPH free radical scavenging activity of methanol extract of *N. cadamba* fruits and standard ascorbic acid

Phytochemical Screening

The preliminary phytochemical evaluation of methanolic extract of *N. cadamba* confirmed the presence of carbohydrates, glycosides, phytosterols, diterpenes, protein, amino acids and phenol, though alkaloid, flavonoid, saponin and tannin were absent (Table 4).

DISCUSSION

The present study was an attempt of investigating several properties of methanolic extract of *N. cadamba* fruits and membrane stabilizing activity test was one of them. As we know, the vigour of cells depends on the integrity of their membranes; haemolysis of RBC on exposure to hypotonic or heated medium is an indication of its injurious membrane. It is therefore expected that membrane stabilizers should offer significant protection against hazardous substances and thereby elicit anti-inflammatory properties (Umukoro and Ashorobi, 2006). Previous reports showed that foods and fruits rich in flavonoids and other phenolic compounds have been associated with decreased risk of developing associated with decreased risk of developing inflammatory and other related diseases (Sies et al., 2005). Compounds with membrane-stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reaction namely the prevention of the release of phospholipases that trigger the formation of inflammatory mediators (Saffoon et al., 2014).

In our study, we have also found the presence of phenols and terpenoids in the extract. Another research reported that phenolic compounds inhibit the activity of prostaglandin cyclooxygenase and thereby inhibit inflammatory mediators (Richter et al., 2003). The results of our investigation showed that the extract at a concentration of 2 mg/ml readily protected the lysis on human erythrocyte membrane induced by hypotonic solution as well as heat induced solution compared to the standard acetyl salicylic acid (0.1 mg/ml). This suggests that the plant extract may possess good membrane stabilizing activity.

The anthelmintic activity shown by the plant extract was dose dependant and was comparable to that of the standard drug albendazole. From the study, it was observed that the extract exhibited not only paralysis but also death of earthworms. It was also clear that the time for paralysis and the time for death of earthworms were inversely proportional to the concentrations of the extract.

Phytochemical analysis of the crude extract revealed the presence of phenols, terpenoids which are known to exhibit anthelmintic property. Previous studies showed that phenolic compounds can interfere with the energy

generation in helminthic parasites by uncoupling oxidative phosphorylation (Athnasiadou et al., 2001) and also bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite leading to death (Salhan et al., 2011). Based on the above discussion, it can be assumed that terpenoids and phenolic compounds present in the fruit extract of *N. cadamba* may be responsible for the anthelmintic activity.

In antioxidant activity test, the total phenolic content was determined considering gallic acid equivalent as reference by using the standard curve equation. Elmastas et al. (2007), reported that the phenolic compounds contain hydroxyl groups that may directly contribute to the antioxidant activity and play a critical role in scavenging free radicals. Some previous studies demonstrated that the higher the amount of total phenolic contents in a plant extract is, the higher is its antioxidant property (Madaan et al., 2011; Henríquez et al., 2010). Again it was found that absolute methanol is more effective than other solvents for extracting polyphenols from plant extracts (Lolita et al., 2012). In our study, we also found the methanolic extract of *N. cadamba* fruits rich in total phenolic components. These results are in accordance with previous reports which have shown that the fruit extract has a higher total phenolic components compared to the leaf extracts (Ganjewalaa et al., 2013) but lower than the bark extracts (Chandel et al., 2011).

Therefore, it may be said that the presence of higher total phenolic components may be responsible for demonstrating the antioxidant activity and free radical scavenging ability of the plant. We know free radicals are harmful chemical species that contain one or more uncoupled electrons which contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of different tissues and central nervous system, gastritis, cancer and AIDS (Kumar et al., 2010). For testing radical scavenging activity of various plant extracts, most commonly DPPH assay is considered as a quick method (Elmastas et al., 2007). In case of DPPH free radical scavenging activity, the extract exhibited a lower scavenging activity than the standard ascorbic acid. Figure 1 showed that methanolic extract contained significant ($p < 0.05$) antioxidant and free radical scavenging activity. The data also revealed that the percentage of free radical inhibition increased with the increasing of concentration of extract.

Therefore, the study results support the authenticity of using the plant extract as a potent antioxidant. Isolation of phytochemical constituents on the fruit extract confirmed the presence of carbohydrates, glycosides, phytosterols, diterpenes, protein, amino acids and phenols, though alkaloid, flavonoid, saponin and tannin were absent (Table 4). From the previous study, it was confirmed that phenolic compounds have anti-oxidative, antidiabetic,

anticarcinogenic, antimutagenic and anti-inflammatory activity (Arts and Hollman, 2005) and other phytochemicals present in fruit extract are also evident for having active properties against various diseases (Yadav and Agarwala, 2010)..

Conclusion

In Bangladesh, like several other countries in the world, *N. cadamba* is an indigenous flowering plant. In the context of the above discussion, it can be concluded that the *N. cadamba* fruits contain important phytochemicals and possess various biological activities. The current study has confirmed that the crude methanolic extract of *N. cadamba* fruits showed potential membrane stabilizing, anthelmintic and antioxidant properties which indicates that *N. cadamba* fruits can play an important role in drug research. Therefore, the plant is a worthy contender for further systemic, chemical and biological studies to determine the active principle.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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