Preliminary phytochemical, cytotoxic, thrombolytic and antioxidant activities of the methanol extract of Murraya exotica *Linn. leaves*

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RESEARCH ARTICLE

Preliminary phytochemical, cytotoxic, thrombolytic and antioxidant activities of the methanol extract of *Murraya exotica* Linn. leaves

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Abstract The methanol extract of leaves of traditionally used medicinal plant *Murraya exotica* Linn. (Family: Rutaceae) was evaluated for possible cytotoxic, thrombolytic and antioxidant activities in this study. The extract was tested for phytochemical group test by standard method using chromogenic reagents, in vivo brine shrimp lethality assay using *Artemia salina*, thrombolytic effect by using the standard streptokinase and antioxidant activity by using 2,2-diphenyl-1-picryl-hydrazyl (DPPH). The study revealed the presence of reducing sugars, tannins, saponins and alkaloids. The extract showed statistically significant ($p<0.01$) potent cytotoxic effect in brine shrimp lethality bioassay where the value of LC_{50} and LC_{90} were 1.27 μg/ml and 5.09 μg/ml respectively compared with the standard vincristine sulphate with the value of LC_{50} and LC_{90} 0.09 μg/ml and 4.83 μg/ml respectively after 24 h. The study gave a significant indication to the use of the plant extract as a potential source for cytotoxic compounds. The extract showed mild thrombolytic effect of 15.78 % thrombolytic activity whereas the standard streptokinase showed 76.50±0.82 %. In the antioxidant activity study, the extract showed free radical scavenging activity where IC_{50}= 1.25 μg/ml and IC_{90}=4.4 μg/ml, compared with the standard ascorbic acid showing IC_{50}=0.01 μg/ml and IC_{90}=3.58 μg/ml. Based on the findings of phytochemical, toxicological and anti-oxidative activity, it is evident that the plant may contain some novel compounds that possess potent anti-mutagenic and anti-oxidative activities. The obtained results support the use of this plant in traditional medicine.

Keywords *Murraya exotica* · Cytotoxic · *Artemia salina* · Thrombolytic · Antioxidant · DPPH free-radical scavenging

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Introduction

Murraya exotica Linn. Mantiss. (Family: Rutaceae), synonym *Murraya paniculata* (Linn.) Jack locally known as Kamini; Chinese Box in English, is a small evergreen tree with smooth and slender barks having glossy green foliage and large clusters of fragrant flowers (Little et al. 1974; Kirtikar and Basu 2006). This plant has been used in ethnomedicine. The plants have stimulant and astringent, abortive properties and are used to treat diarrhea, dysentery, cuts, joints pain, body aches, venereal diseases (Parrotta 2001; Kinoshuta and Fireman 1996; Xiao and Wang 1991). Infusion of the leaves and flowers of *M. exotica* is tonic and stomachic. It is said to be aromatic, refrigerant, digestive, and beneficial in rheumatic fever, coughs, giddiness, hysteria, thirst, and burning of the skin (Jorge et al. 2011). The plant has been reported for its antioxidant using butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), carvacrol and timol, anti-inflammatory, antinociceptive and insecticidal activity (Jorge et al. 2011; Wu et al. 2010; Li et al. 2010). Some compounds like coumarins bismurrangatin and murramarin A (Negi et al. 2005), murraxocin (Sharma et al. 2006),

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essential oils like bicyclogermacrene, beta-caryophyllene, alpha-caryophyllene, delta-cadinene, spathulenol, trans-alpha-bergamotene, germacrene D, beta-bisabolene, ar-curcumene (Jiang et al. 2009); glycosides like murrayain (Kirtikar and Basu 2006), sitosterol-beta-D-galactoside (Ahmad et al. 1987); polymethoxylated flavonoids (Zhang et al. 2014) and other compounds like aurantiamide acetate (Kong et al. 1987) have been reported to obtain from the plant.

Materials and methods

Collection and identification of plant material

The plant *Murraya exotica* was collected from Satkhira, Bangladesh in May 2012. The samples of the plant were mounted on paper and the species was taxonomically confirmed by Sarder Nasir Uddin, Principle Scientific Officer, Bangladesh National Herbarium (BNH), Mirpur, Dhaka. The voucher specimen of the plant has been deposited and preserved in BNH library for further collection and reference and an accession no was provided as DACB-32,808.

Preparation of methanol extract

The collected leaves of the plant were separated from undesirable materials. They were dried in open air for 2 weeks. The shade dried leaves were ground into a coarse powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powder of the leaves was stored in an airtight container and kept in a cool, dark and dry place until the analysis commenced. About 310 g of powered material was taken in a clean, flat-bottomed glass container and soaked in 1,200 ml of methanol. The container along with its contents was sealed and kept for a period of 10 days with occasional shaking or stirring (Nayeem et al. 2011). The whole mixture then underwent a coarse filtration by cotton and whatman filter paper (Bibby RE200, Sterilin Ltd., UK). The filtrate was concentrated under air. It rendered a 30 g concentrate (9.677 % yield) extract which was designated as crude methanol extract.

Chemicals and reagents

Standard chromogenic reagents used for chemical group test were of reagent grade and purchased from Sigma-Aldrich Co. LLC, Missouri, United States. Vincristine sulphate, used as a standard drug in the cytotoxic assay and ascorbic acid, used as a standard drug in the antioxidant assay were collected from the Techno Drugs Limited, Bangladesh. Methanol supplied by Laboratory Patterson Scientific, U.K. was used as solvent. Dimethyl sulfoxide (DMSO, ≥99.9 %, BioReagent, for molecular biology; Sigma-Aldrich, India) was used as solvent to dissolve the extracts. Laboratory reagent grade phosphate buffered saline (PBS) used as buffering ingredients was purchased from Fisher Scientific, U.K. Commercially available lyophilized streptokinase vial (Shanghai SIPI Pharmaceutical Co. Ltd., China) was used in thrombolytic activity assay. 2,2-diphenyl-1-picryl-hydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO.).

Table 1 Results of phytochemical screening of *Murraya exotica* L. extract

Test for phytochemical group	Reagent	Results of the extract of *Murraya exotica*
Reducing sugar	Fehling's test	+
	Benedict's test	+
Alkaloid	Hager's test	+
	Wagner's test	+
	Dragendorff's test	+
Steroid	Salkowski's test	−
Tannin	Ferric chloride test	+
	Lead acetate test	+
	Potassium dichromate test	+
Gum	Molisch's test	−
Flavonoid	Shinoda test	−
	Alkaline reagent test	−
Saponin	Frothing test	+

+: Positive result; −: Negative result

Instruments and equipment

pH Meter (pHep-HI 98107, Hanna Instruments, Romania), double beam Analykjena UV/Visible spectrophotometer (model- Shimadzu, UV-1800, Japan), electronic balance (serial no.- 1508, OHAUS, Germany), vortex mixer (VM-2000, 220 V, Digisystem Laboratory Instruments Inc.

Table 2 Brine shrimp lethality bioassay of *Murraya exotica* L. extract

Concentration (μg/ml)	% mortality of-	
	Methanol extract of *Murraya exotica*	Vincristine sulfate
10	46.67	60.33
20	60	63.67
40	63.33	73.33
80	83.33	83.33
160	86.67	93.67
320	100	100
LC_{50}	1.27	0.09
LC_{90}	5.09	4.83

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Taiwan), centrifuge machine (Model 800, 50 W, 4,000 rpm, China), Hot plate (Serial no.- SWT.550010W, Gallenkamp, England) were used for this study.

Test for different chemical groups

The preliminary phytochemical screening of the crude methanol extract was carried out by using standard chromogenic reagents- lead acetate, potassium dichromate, ferric chloride, hydrochloric acid, sulphuric acid, Mayer's reagent, Dragendorff's reagent, Wagner's reagent, Hager's reagent, Molisch reagent, Benedict's reagent and Fehling's solutions were used to detect steroids, alkaloids, gums, flavonoids, saponins, tannins, and reducing sugars using standard protocol (Khatun et al. 2013). The colour intensity or the precipitate formation was used as analytical responses to these qualitative tests. 10 % (w/v) solution of the extract in methanol was used for each of the above test.

Test for cytotoxic activity

Cytotoxicity assay was performed on brine shrimp nauplii using the method of Meyer et al. (1982). Brine shrimp nauplii were obtained by hatching brine shrimp eggs (Carolina Biological Supply Company, Burlington, NC, USA) in artificial sea-water (3.8 % NaCl solution) for 24 h. Dissolution of 30 mg of extract was performed in 3 ml of artificial sea water containing 20 % DMSO to give concentration of 10 μg/μl. From this solution 10, 20, 40, 80, 160 and 320 μl were transferred to each 10 ml vial and using artificial sea water volume was adjusted to 10 ml by artificial sea water to give concentrations of compound of 10, 20, 40, 80, 160 and 320 μg/ml respectively. Brine shrimp nauplii were grown in these solutions and observed their mortality after 24 h. The resulting data were transformed to probit analysis software (LdP Line software, USA) (Uddin et al. 2011; Finney 1971) for determination of LC_{50} values of the extract. Artificial seawater medium containing DMSO used for the analysis was employed as negative control. Vincristine sulfate was used as standard in this assay.

In vitro thrombolytic activity

In vitro thrombolytic activity was carried out according to the method of Rahman et al. (Rahman et al. 2013). 5 ml of phosphate buffered saline was added to the commercially available lyophilized streptokinase vial (15,00,000 I.U.) and mixed properly. This suspension was used as a stock from which appropriate dilutions were made to observe the thrombolytic activity. In brief, 2 ml venous blood drawn from healthy volunteers was distributed in three different pre weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37 °C for 45 min. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube—weight of tube alone). To each microcentrifuge tube containing pre-weighed clot, 100 μl of methanol extract (10 mg/ml) of was added. As a positive control, 100 μl of streptokinase and as a negative non thrombolytic control, 100 μl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37 °C for 90 min and observed for clot lyses. After incubation, released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lyses was expressed as percentage of clot lyses.

Test for antioxidant activity

The antioxidant activity of plant extract and the standard antioxidant were assessed on the basis of free radical scavenging effect of the Stable 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical (Sadhu et al. 2007). Stock solution (10 mg/mL) of the methanol extract of *M. exotica* was prepared in respective solvent systems from which serial dilutions were carried out to obtain concentrations of 1, 5, 10, 50,

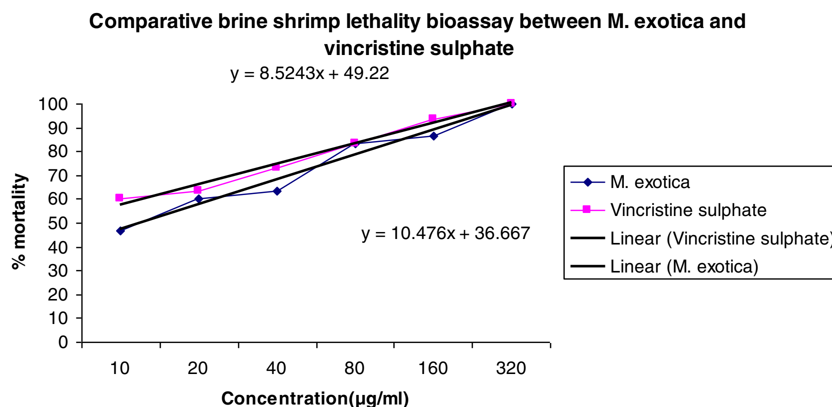

Fig. 1 Comparative brine shrimp lethality bioassay between *Murraya exotica* L. extract and vincristine sulphate

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Table 3 In vitro thrombolytic activity of Murraya *exotica* L. extract

Sample	% clot lyses
Methanol extract of *M. exotica* for volunteer 1	17.79±0.82
Methanol extract of *M. exotica* for volunteer 2	15.97±1.90
Methanol extract of *M. exotica* for volunteer 3	13.57±1.03
Average	15.78
Streptokinase	76.50±0.82

Data are presented as Mean ± SD

100 μg/mL. In this assay, an equal amount of sample solution was added to an equal amount of 0.1 mM methanolic DPPH solution, vortexed and allowed to stand at the dark place at 25 °C for 30 min for the reaction to occur. After 30 min of incubation period, the absorbance was read against a blank at 517 nm with Analykjena UV/Visible spectrophotometer. The radical scavenging activity was expressed as the percentage of inhibition (I%) and calculated as per the equation:

$$I(\%) = \left(A_{blank} - A_{sample}/A_{blank}\right) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound with all reagents. IC_{50} value is the concentration of sample required to scavenge 50 % DPPH free radical and was calculated from the plot of inhibition (%) against extract concentration. All the tests were carried out in triplicate and average of the absorptions was noted. Ascorbic acid was used as positive control standard for this study.

Statistical analysis

Data were presented as mean ± S.D. Statistical differences between control and treated groups were tested by Student's "*t*"-test. The differences were considered significant at $P<0.05$. Obtained results were interpreted using Microsoft Excel 2007.

Results

Chemical group test Results of different chemical tests on the methanol extract of *Murraya exotica* showed the presence of alkaloids, reducing sugar, saponin and tannin (Table 1).

Cytotoxic activity The methanol extract of *M. exotica* leaves was tested for Brine shrimp lethality bioassay using brine shrimp nauplii and DMSO as a solvent. Control was used to see whether DMSO had any effect on brine shrimp lethality. The control group of brine shrimp nauplii with and without DMSO exhibited no mortality. For the extract, the number of nauplii died and percent mortality was counted. In the test, the extract showed LC_{50} and LC_{90} of 1.27 μg/ml and 5.09 μg/ml after 24 h respectively. In contrast, the standard vincristine sulphate showed LC_{50} and LC_{90} of 0.09 μg/ml and 4.83 μg/ml after 24 h respectively. No mortality was found in the control group. The result is shown in Table 2. An approximate linear correlation was observed when concentrations versus percentages of mortality was plotted on graph paper (Fig. 1).

Thrombolytic activity Table 3 shows the effect of the methanol extract on clot lyses activity. The percentage (%) clot lyses was statistically significant ($p<0.001$) when compared with vehicle control. The plant extract showed mild colt lyses activity (17.79±0.82 %, 15.97±1. 90 and 13.57±1.03for volunteer 1, 2 and 3, respectively whereas standard streptokinase showed 76.50±0.82 % clot lyses activity) (Fig. 2).

Antioxidant activity Free radical scavenging activity of the methanol extract of *M. exotica* leaves, was measured by DPPH assay and shown in Table 4 and Fig. 3. DPPH free radical scavenging capacity was found to be increased with

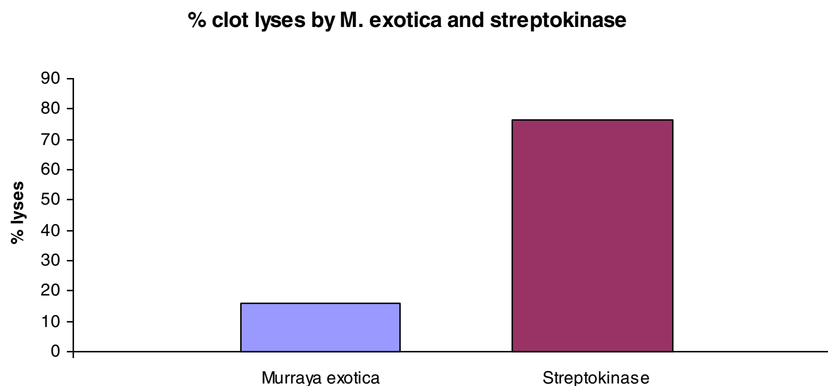

Fig. 2 Comparative clot lyses by *Murraya exotica* L. extract and streptokinase

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Table 4 Anti-oxidant activity of *Murraya exotica* L. extract

Concentration (μg/ml)	% inhibition of-	
	Methanol extract of *Murraya exotica*	Ascorbic acid
1	27.27	41.5
5	80.11	92.14
10	79.2	92.71
50	86.93	94.8
100	87.27	96.18
IC_{50}	1.25	0.01
IC_{90}	4.4	3.58

the increase of concentration of the extract. The extract showed significant antioxidant activity where the IC_{50}= 1.25 μg/ml and IC_{90}=4.4 μg/ml compared with the standard ascorbic acid that showed IC_{50} at 0.01 μg/ml and IC_{90} at 3.58 μg/ml.

Discussion

The methanol extract of *Murraya exotica* demonstrated the presence of alkaloids, reducing sugar, saponin and tannin as secondary metabolites with potential biological activities. Usually, plants combines different end products of metabolites and toxic substances in the form of secondary metabolite. These are stored and used for protection from insects, herbivorous animals, pathogenic organisms and source of different essential elements. The secondary metabolites were further exploited as antibiotics, anthelminthic agents, anticoagulants, antitumour substances and carcinogens, cardio-excitatory substances, growth substances and hormones, haemaglutinins and lectin-type agglutinins, hypotensives, insecticides, toxins and vitamins. Many of the aforementioned materials are directly and synthetically modified used in medicine (Evans 1989). The Further studies should be going on fractionation and identification of bioactive constituent which may led to identify the responsible alkaloids, reducing sugar, saponin and tannin for cytotoxic, thrombolytic and antioxidant activities.

Brine shrimp lethality bioassay is an easy and straight forward bench top screening method for predicting important pharmacological activities like enzyme inhibition, ion channel interference, antimicrobial and cytotoxic activity (Anderson et al. 1991). *M. exotica* was reported for insecticidal activity (Negi et al. 2005) and another species of the plant *M. koenigii* was reported for anti-tumour effect (Mohan et al. 2013; Noolu et al. 2013; Kaplan 2012), apoptosis of HL-60 leukemia cells induced by carbazole alkaloids isolated from *M. euchrestifolia* (Ito et al. 2012). The present study revealed that, the extract has significantly strong cytotoxic activity and showed LC_{50} at a low concentration. This indicates clearly the presence of potent bioactive principles in these crude extracts which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents (Meyer et al. 1982). Ideally, any agent useful in the treatment of cancer should not be toxic to normal cell. It is necessary to test this extract against various cancer cell lines as well as normal cell lines to justify the potential to further investigate this plant for anticancer activity. Further investigation is required to find the responsible compound(s) for the cytotoxic activity observed for *Murraya exotica*. Each cytotoxic agent exerts its effect by disrupting one or more phases of the cell cycle (Gibaldi and Perrier 2007). The toxicity of cytotoxic compounds is often not specific to diseased tissue and cancerous tissues. Cancer cells are more susceptible to these compounds, but the therapeutic index can be low. For this reason, the distribution of these compounds between tissues containing target and non-target cells in the patient is of high interest. Physiologically based pharmacokinetic (PBPK) modelling can be used to predict this (Ekins 2006).

The plant *M. exotica* was reported for antioxidant activities done by various process other than DPPH free radical scavenging capacity (Jorge et al. 2011). Other species of *Murraya*

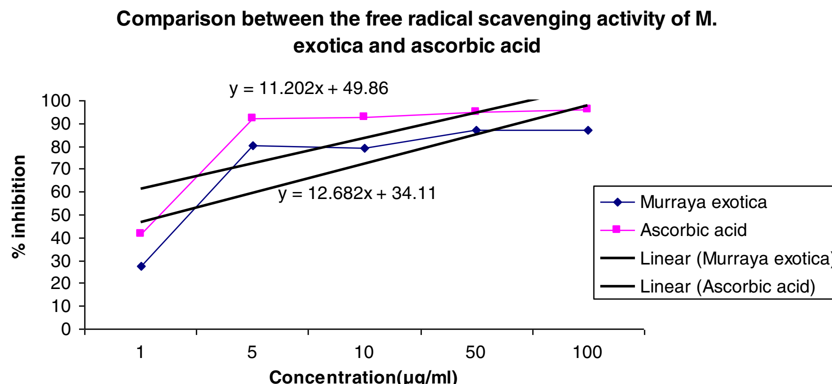

Fig. 3 Comparison between the free radical scavenging activity of *Murraya exotica* L. extract and ascorbic acid

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were also reported for anti-oxidant activity (Zahin et al. 2013). The plant is reported to possess many polyhydroxy, Flavonoids and polyphenolic compounds (Negi et al. 2005; Jiang et al. 2009; Zhang et al. 2013). These compounds may be responsible for anti-oxidant property. In this experiment the significant antioxidant activity justifies the previous findings. Further research is needed on the determination of the correlation between the antioxidant capacity and the chemical composition of the plants.

In human body or any biological system, oxidative stress consequences from the overproduction of reactive oxygen species (ROS) or decrease in antioxidant potential. These are capable of chemically altering all major biomolecules including lipids, proteins and nucleic acids by changing the structure and function. Human and animals have developed mechanism to protect these biomolecules from damage of free radicals by endogenous antioxidants including enzymes like Superoxide Dismutase, gluthalion peroxidase and Catalase and non enzymes like vitamins, uric acid, albumin and seroloplasmin (Abdollahi et al. 2004). In vivo evaluation on biological model could be carried out to elucidate the antioxidant potential of *M. exotica*.

The thrombolytic bioassay was done as an available routine test. The extract showed mild to moderate activity on human blood specimen. Thrombolytics dissolve blood clots and are used in treating myocardial infarction (heart attack), angina, cerebral vascular accident (CVA) or stroke, hyper/hypotension (high/low blood pressure), congestive heart failure (CHF), coronary artery disease (CAD), arrhythmias, high cholesterol, unwanted blood clots, and arteriosclerosis. (American Pharmacist Association 2010). However, possible mechanism could be sketched out using computer guided simulation techniques (Ekins 2006) as well as animal model.

Present study is based on the report of preliminary phytochemical and biological screening of *M. exotica* extract. The results are quite promising; support the use of this plant in traditional medicine and also previous investigation. Advanced studies including Liquid chromatography–mass spectrometry (LC-MS) can be carried out to get a bigger picture of the chemical constituents present in the plant. Screening methods applying various cell lines or bacterial enzymes can be carried out to find the underlying mechanism for the observed biological activities. On the basis of the results from above studies, bioassay guided approach can be undertaken to isolate and identify the active component(s). However, further studies comprising of thorough phytochemical investigations of the used plant to find out the active principles and evaluation for these activities using other models are essential to confirm its pharmacological properties.

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Conflict of Interest The authors declare that they have no conflict of interest.

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