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Phytochemical analysis and bioactivities of *Argemone mexicana* Linn. leaves

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Summary

The aim of the study was to evaluate the *in vitro* antimicrobial, antioxidant, cytotoxic and thrombolytic activities of n-hexane, ethyl acetate and methanol extracts of Argemone mexicana leaves. Phytochemicals were also identified to establish the possible causes of the pharmacological activities. The leaves extracts were screened for major phytochemicals using established procedures. Antimicrobial and cytotoxic studies of the leaves extracts were conducted using disc diffusion and brine shrimp lethality bioassay methods, respectively while an *in vitro* thrombolytic model was used to assess the clot lysis effect of the extracts using streptokinase as positive control. Antioxidant activity was evaluated by free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) assay as well as total phenolic content. The results showed that the leaves extracts were a rich source of phytochemicals. Extracts showed partial antimicrobial activity (zone of inhibition was ranges between 9 to 13 mm) against the tested microorganisms at the 1000 µg/disc compared to the standard, ciprofloxacin. Ethyl acetate and methanol extracts of the leaves showed higher antioxidant property compared to n-hexane extract. Median inhibitory concentration, IC_{50}, of ethyl acetate extract was 39.91 µg/ml for DPPH free radical which was much lower than standard ascorbic acid (IC_{50}: 76.11 µg/ml). Although the extracts were not cytotoxic, n-hexane and ethyl acetate extracts showed significant clot lysis activity ($p<0.001$) with reference to negative control and % clot lysis of the extracts was around 13. The present findings suggest that the plant widely available in Bangladesh could be a prominent source of medicinally important natural compounds.

Key words: *Argemone mexicana*, Antimicrobial, Antioxidant, Cytotoxic, Phytochemical screening, Thrombolytic

Introduction

Argemone is a genus of flowering plants in the family Papaveraceae. It contains 30-32 species, commonly known as 'Prickly Poppies' all with prickly stems, leaves and capsules [1]. Among the species, *Argemone mexicana* Linn. (Papaveraceae), commonly known as 'Mexican Poppy' is used in rural areas of Mexico as a medicinal plant. It is an herbaceous plant with latex, which has naturalized widely in many tropical and subtropical regions although it's a native of tropical America [2]. It is a very common annual weed in agricultural and wastelands found all parts of Bangladesh where it is popular as 'Shialkanta' (Bengali) [3].

Argemone mexicana (*A. mexicana*) is widely used in folk medicine to alleviate several ailments especially for its analgesic, antibacterial, antimalarial, antispasmodic, sedative and narcotic effects [4]. Seeds are useful in cough and asthma. It is used traditionally as an antidote to various poisons [5]. The fresh yellow, milky, seed extract contains protein dissolving substances which are effective in the treatment of warts, cold sores, cutaneous infections, skin diseases, itches and also in dropsy and jaundice [4]. The root has an anthelmintic activity. Leaves of *A. mexicana* are also traditionally used as antiasthamatic [5]. The wide variety of the traditional uses of the plant may be due to the presence of various phytochemicals like alkaloids [6,7], amino acids [8], phenolics [9] and fatty acids [10].

To establish its traditional uses, *A. mexicana* has been investigated for its analgesic, anthelmintic, antiallergic, anti-cataleptic, anticancer, antifungal, antimalarial, antimicrobial, antimutagenic, anti-stress, anti-HIV, antioxidant, anxiolytic, hepatoprotective and sedative activity [11-15].

The investigations were carried out to identify the phytochemicals and *in vitro* antimicrobial, antioxidant, cytotoxic and thrombolytic activities of *n*-hexane, ethyl acetate and methanol extracts of leaves of *A. mexicana* available in Bangladesh.

Materials and Methods

Plant collection and identification

The leaves of *A. mexicana* were collected in the month of February, 2011 from Tangail, Bangladesh. Collected plant was identified by a taxonomist (Dr. Bushra Khan, Principal Scientific Officer) from Bangladesh National Herbarium, Mirpur (Dhaka) and a duplicate specimen (Accession no. DACB-35574) has been deposited for future reference.

Preparation and extraction of plant material

The dried, coarsely powdered plant material (500 gm) was extracted by maceration (1 L) over 72 hours period with *n*-hexane at room temperature. Similar extraction method of the plant materials was carried out using ethyl acetate and methanol. The extracts were concentrated with a rotary evaporator (IKA, Germany) at low temperature (40-50 °C) and reduced pressure to get the dried *n*-hexane (AMHE), ethyl acetate (AMEA) and methanol (AMME) extracts.

Phytochemical screening

Phytochemical screening of leaves extracts of *A. mexicana* was carried out qualitatively for the presence of alkaloids (Hager's test), anthraquinones (chloroform layer test), cardiac glycosides (Killer-Killani's test), flavonoids (modified ammonia test), reducing sugars (Fehling's test), saponins (frothing test), steroids (Salkowski test), tannins (ferric chloride test) and terpenoids (modified Salkowski test) [3].

Antimicrobial screening

The Kirby-Bauer disc diffusion method [16] was used to evaluate the antimicrobial potential of *A. mexicana* extracts on Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Shigella dysenteriae*) and fungus (*Candida albicans*). Sterile 6 mm filter paper discs (Whatman no. 1) were impregnated with 500 and 1000 µg of the extracts of each

solvent system and dried in open air to evaporate the residual solvent. Standard ciprofloxacin discs (5 µg/disc) were used as positive control. Two sample discs of two different concentrations, standard antibiotic disc and blank disc (impregnated with solvents followed by evaporation) were placed gently on previously marked zones in the agar plates pre-inoculated with the test bacteria and fungus. After incubation of bacteria plates at 37 °C for 24 h and fungus plate at 25 °C for 48 h, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm [16]. The screening of antimicrobial activity was carried out in triplicate.

Antioxidant activity

Estimation of total phenolic content

The amount of total phenolic compounds in the leaves extracts was measured as a way of determining antioxidant activity according to Folin-Ciocalteu procedure [17]. The plant extracts (20 mg) and 2 ml 99.8 % (v/v) methanol were taken in a test tube and incubated at room temperature for 48 h in the dark; 2 ml of the solution was transferred to a centrifuge tube and centrifuged at 5000 rpm for 5 min after which 300 µl of the supernatant was mixed with 600 µl of 10 % Folin-Ciocalteu reagent (Merck, Germany) and 2.4 ml of 700 mM sodium carbonate in a test tube and the mixture was incubated for 2 h at room temperature and the absorbance was measured at 765 nm in a UV-VIS spectrophotometer (Shimadzu, Japan). Total phenolic content was determined (as gallic acid equivalent) in triplicate from a standard curve prepared with gallic acid.

Determination of DPPH radical scavenging activity [18]

The free-radical scavenging activity of *A. mexicana* leaves extracts was measured by the decrease in the absorbance of the methanol solution of a stable free radical DPPH (2,2-Diphenyl-1-picrylhydrazyl). A stock solution of DPPH (Sigma-Aldrich, USA, 400 µg/ml) was prepared in 99.8% (v/v) methanol and 100 µl of this stock solution was added to 5 ml of a methanol solution of *A. mexicana* extracts of varying concentrations (20 - 100 µg/ml). The solutions were mixed properly, kept in the dark for 20 min at room temperature and the absorbance measured at 517 nm against blank (methanol). Scavenging activity was calculated as: Inhibition (%) = $\{(A_c - A_s)/A_c\}$ x 100; where A_c is the absorbance of control (DPPH in methanol; 8 µg/ml) and A_s the absorbance of the test sample. For each concentration, a separate blank sample was used for background subtraction. Inhibition (%) was plotted against the respective extract concentrations used and IC_{50} values were extrapolated from the plot. Ascorbic acid, an antioxidant, was used as positive control.

Nitric oxide scavenging assay [19]

Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with various concentrations of the extracts (5 - 200 µg/ml) dissolved in 99.8% (v/v) ethanol and incubated in the dark at room temperature for 2 h. The solution (2 ml) was mixed with 1.2 ml of Griess reagent and the absorbance measured spectrophotometrically at 546 nm against blank (phosphate buffered saline). Ascorbic acid was used as a positive control and was treated in the same way as the extract. Nitric oxide (NO) scavenging activity was computed as: Inhibition (%) = $\{(A_c - A_s)/A_c\}$ x 100; where A_c is the absorbance of control (solution contained every reagent except extract) and A_s the absorbance of the test sample. For each concentration, a separate blank sample was used for background subtraction. Inhibition (%) was plotted against the respective extract concentrations used and IC_{50} values were extrapolated from the plot.

Brine shrimp lethality bioassay

A. mexicana leaves extracts were screened

for their cytotoxic activity using brine shrimp lethality bioassay [20]. For the experiment, 200 mg each of the extracts was dissolved in dimethylsulfoxide (DMSO) and solutions of varying concentrations were obtained by serial dilution using simulated sea water. The solutions were taken in each pre-marked test tubes containing 5 ml simulated seawater and 10 shrimp nauplii. After 24 h, the numbers of survivors were counted and mortality (%) was calculated for each dilution as well as for control. Percent mortality was corrected and then converted to Probit mortality as describe by other researcher [21]. The LC_{50} values (concentration of sample required to kill 50 % of brine shrimp) were calculated (at the confidence interval level of 95%) using Microsoft Excel 2007 by a plot of Probit mortality against the logarithm of the sample concentrations. Simulated seawater containing varying quantities of DMSO were used as control. Potassium permanganate (KMN) was used as the positive control.

In vitro thrombolytic study

Median cubital venous blood (3 ml) was drawn from 10 healthy volunteers (5 males and females each) who had not taken any oral contraceptives or anticoagulants in the preceding two weeks. The blood was transferred to 5 pre-weighed sterile Eppendorf tubes (500 µl/tube) and incubated at 37 °C for 45 min. After clot formation, serum was completely removed without disturbing the clot formed. Each tube having clot was again weighed to determine clot weight. The extract (100 mg/ml, 100 µl) was added to the tube [22]. As positive control, 100 µl of streptokinase (CSL Behring GmbH, Germany, 3000,000 IU/ml) was used, whereas, 100 µl of normal saline served as negative control. The tubes were then incubated at 37 °C for 90 min and observed for clot lysis. The released fluid was removed and the tubes again weighed to compute the difference in weight after clot disruption. The percentage difference in weight before and after clot lysis was expressed as clot lysis (%).

Statistical analysis

Statistical comparisons were performed with Student's '*t*' tests using Microsoft Excel 2007. A *p* value of 0.05 and 0.001 or less was considered to be significant. Mean ± SEM was calculated for the parameters where applicable.

Results

Phytochemical screening of the extracts of leaves of *A. mexicana* revealed the presence of various bioactive components of which alkaloids, anthraquinones flavonoids, saponins and steroids were the most prominent (Table 1).

see Table 1.

Antimicrobial activities (Table 2) of the *A. mexicana* extracts were tested against three pathogenic organisms. AMEA was found to create clear zone around the disc at both the lower and higher concentrations. In case of bacteria and fungus, the zone of inhibition created by the plant extracts were ranges between 7-12 mm and 7-13 mm respectively.

see Table 2.

The results of both the total phenolic content and free radical scavenging activity (DPPH and NO) of the crude extracts (Table 3) showed promising antioxidant activity. The ethyl acetate extract exhibited the highest total phenolic content as well as high radical scavenging activity, comparable to that of ascorbic acid (AA).

In brine shrimp lethality bioassay, % mortality increased gradually with the increase in concentration of the test samples. In comparison to positive control (KMN: Potassium permanga-

nate), the cytotoxic potentiality exhibited by *A. mexicana* leaves extracts were very negligible (Table 3).

see Table 3.

Maximum clot lysis was visually observed when streptokinase (100 µl) was added to the clots. The clot lysis data are shown in Figure 1. With normal saline, 4.71 % clot lysis was seen, while *n*-hexane (AMHE), ethyl acetate (AMEA) and methanol (AMME) extracts produced 10.40, 13.86 and 3.16 % clot lysis, respectively.

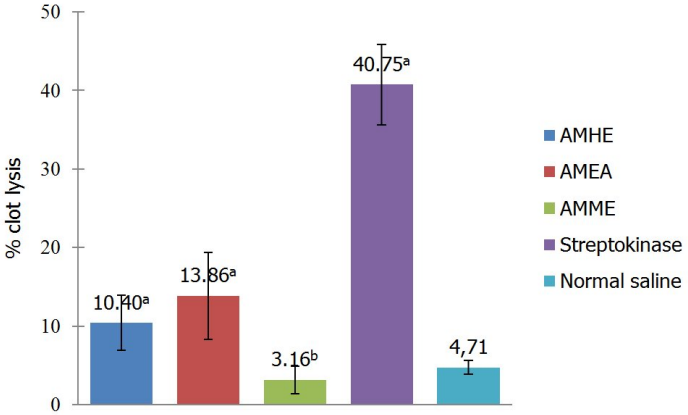

Figure 1: Clot lysis of blood samples of normal subjects by AMHE, AMEA, and AMME extracts of *A. mexicana* leaves, streptokinase and normal saline (n = 10, $^{a}p < 0.001$, $^{b}p < 0.05$ compared with negative control).

Discussion

The easiest and simplest method for the identification of various bioactive secondary metabolites in plant is the phytochemical screening. The phytochemical analysis of *A. mexicana* leaves extracts reveal that alkaloids, anthraquinones, flavonoids, saponins and steroids were present in the *n*-hexane (AMHE), ethyl acetate (AMEA) and methanol (AMME) extracts in varying quantities (Table 1). Anthraquinones and flavonoids were abundantly present in all the extracts whereas AMEA extract was found to be rich in alkaloids and saponins. Presence of the similar types of compounds in *A. mexicana* were also reported by the other researchers [4,14]. Phytochemicals present in *A. mexicana* leaves extracts may be responsible for numerous pharmacological activities.

The antimicrobial activity of the extracts of *A. mexicana* leaves tested against Gram positive, Gram negative bacteria and *Candida* species did not show significant inhibition of growth (Table 2). Although the plant extracts were inactive (zone of inhibition less than 9 mm) at the lower dose, the extracts were found to be partially active (zone of inhibition between 9-12 mm) at the higher dose [23]. Among the extracts, AMEA extract showed inhibitory activity against all the tested microorganism at both the doses; which may due to the presence of higher quantities of phytochemicals like alkaloids, anthraquinones and flavonoids (Table 2). *A. mexicana* extracts showed antifungal activities may be due the presence of anthraquinones. Anthraquinone and anthraquinone derivatives reported to have antifungal activities [24]. Similar results are also reported by the other researchers [15,25].

Among the extracts of *A. mexicana* leaves, high total phenolic content values (Table 3) were found in ethyl acetate and methanol extracts (106.65 and 70.19 mg/g, gallic acid equivalent respectively). Plant phenolics, in general, are highly effective free radical scavengers and antioxidants [26]. Due to the presence of phenolic compounds, ethyl acetate (AMEA) and methanol (AMME) extracts showed higher scavenging ability of the free radicals (on the basis of median inhibitory concentration, IC_{50} of DPPH, NO free radicals) compared to the *n*-hexane (AMHE) extract (Table 3).

DPPH is one of the compounds that has a proton free radical with a characteristic absorption at 517 nm, which decreases significantly on exposure to proton radical scavengers. It is well accepted that the DPPH radical scavenging by antioxidants is attributable to their hydrogen donating ability [27]. The nitric oxide scavenging assay is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH (7.2) spontaneously generates NO free radical, which interacts with oxygen to produce nitrate and nitrite ions that can be estimated by the use of Griess reagent.

Antioxidants (scavengers of NO free radical) compete with oxygen leading to reduced production of nitrate and nitrite ions and a pink color chromophore is formed [19].

On the basis of the scavenging ability of the free radicals, DPPH and NO, the highest antioxidant activity (lowest IC_{50} values, Table 3) was found in AMEA (IC_{50} 39.91 µg/ml) and AMME (IC_{50} 65.56 µg/ml) respectively among the extracts. On the other hand, the antioxidant activity of standard (Ascorbic Acid) was with IC_{50} value of 76.11 µg/ml for DPPH and IC_{50} value of 34.06 µg/ml for NO radical scavenging ability. Although some scientists have reported that antioxidant activity of natural extracts depend on the assay [28], based on results obtained from the present study, it was obtained that there was a pattern of the antioxidant properties exhibited by extracts of *A. mexicana* in DPPH and NO assays.

From the results of brine shrimp lethality bioassay (Table 3), it can be concluded that the *n*-hexane (AMHE), ethyl acetate (AMEA) and methanol (AMME) extracts of *A. mexicana* leaves did not show any apparent *in vitro* toxicity compared to positive control as the extracts showed higher LC_{50} values than 100 µg/ml, [29]. Large amount of phytochemicals present in the plant are basically non-carcinogenic.

In the present study, it has been observed that AMHE and AMEA extracts showed statistically highly significant ($p < 0.001$) clot lysis activity compared to negative control. The phytochemical analysis of the extracts showed that the extracts were a rich source of alkaloids, anthraquinones, flavonoids and saponins which could be responsible for the clot lysis activity [30]. Methanol extract of *A. mexicana* showed blood clot lysis activity of 3.16 % which was less than that of normal saline (4.71 %).

Conclusion

Finally, it can be concluded from the study that the pharmacological activities showed by the extracts of *A. mexicana* leaves may be due to the presence of different chemical compounds which works through the specific and non-specific mechanisms. However, extensive studies are needed to evaluate the precise mechanism(s), active principles, and the safety profile of the plant as a remedy for different disease conditions.

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Phytochemicals	Test	AMHE	AMEA	AMME
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)	+	-	-

Table 1: Preliminary phytochemical screening of leaves extracts of *A. mexicana*

Key: +++ = remarkably present; ++ = moderately present; + = slightly present; - = absent; AMHE, AMEA and AMME indicate n-hexane, ethyl acetate and methanol extracts respectively.

Micro-organisms	Type	Negative control	AMHE		AMEA		AMME		CP (5 µg/disc)
			500 µg/disc	1000 µg/disc	500 µg/disc	1000 µg/disc	500 µg/disc	1000 µg/disc	
			Zone of inhibition (mm)						
Staphylococcus aureus	Gram+ve bacteria	-	-	9.0±0.1	8.1±0.4	12±1.2	-	8.1±0.1	42.7±1.3
Shigella dysenteriae	Gram-ve bacteria	-	-	10.0±0.8	7.2±0.3	10.2±0.7	7.0±0.3	9.1±0.5	38.7±1.5
Candida albicans	Fungus	-	9.1±0.6	13.0±1.2	7.3±0.4	11.1±0.4	-	8.0±0.3	49.3±0.9

Table 2: Antimicrobial activity of *n*-hexane (AMHE), ethyl acetate (AMEA), methanol (AMME) extracts of *A. mexicana* leaves, positive control ciprofloxacin (CP) and negative control (respective solvents).

Key: Zone of inhibition (mm): <9, inactive; 9–12, partially active; 13–18, active; >18, very active [23]; *- = no zone of inhibition.*

Extract	Total phenol (in mg/g, gallic acid equivalent) (*n*=3)	DPPH radical scavenging activity IC_{50} (µg/ml)	Nitric oxide scavenging assay IC_{50} (µg/ml)	Extract	LC_{50} (µg/ml) (*n*=3)	R^2	95% CI	
							Upper limit	*Lower limit*
AMHE	35.59±1.49	73.73	152.73	AMHE	245.25±3.19	0.869	239.01	251.49
AMEA	106.65±1.39	39.91	69.61	AMEA	152.15±2.43	0.913	147.39	156.91
AMME	70.19±2.10	54.32	65.56	AMME	201.20±2.60	0.774	196.10	206.29
AA*	---	76.11	34.06	KMN*	11.27±0.90	0.790	9.51	13.03

Table 3: Antioxidant and cytotoxic activities of extracts of *A. mexicana* leaves.

**Positive control*