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PHYTOCHEMICAL SCREENING, TOXICITY, LARVICIDAL & ANTIDIABETIC ACTIVITY OF AQUEOUS EXTRACT OF MICROCOS PANICULATA LEAVES

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Abstract

The main objectives of this study were to qualitatively evaluate the profile of phytochemical constituents present in aqueous extract of Microcos paniculata leaves (LWE), as well as to evaluate its toxicity, larvicidal & antidiabetic activity. Phytochemical constituents of LWE were determined by different qualitative tests such as Molisch's test, Fehling's test, alkaloid test, frothing test, $FeCl_3$ test, alkali test, Salkowski's test and Baljet test. Toxicity, larvicidal & antidiabetic activity of LWE were evaluated through brine shrimp lethality bioassay (BSLB), standard WHO protocol with slight modification and α-amylase inhibitory assay. LWE revealed the presence of carbohydrates, alkaloids, saponins, flavonoids, triterpenoids and glycosides. In BSLB, the extract was found to safe with 17889.19 µg/mL of LC_{50} value. Moreover, in larvicidal bioassay, LWE displayed LC_{50} value as 695.2186 PPM. Again, the extract demonstrated significant ($P<0.05$) α-amylase inhibition with an IC_{50} value of 3425.89±438.61 µg/mL. The results obtained in the present study point out that LWE can be possible source of larvicidal and antidiabetic agents.

Key Words: Phytochemical, toxicity, larvicidal, antidiabetic, Microcos paniculata.

Introduction

Microcos paniculata L. of Tiliaceae family is locally known as 'Kathgua' or 'Fattashi' in Bangladesh. It has the growth form of a shrub or small tree, grows wildly and is cultivated throughout Bangladesh. Traditionally the plant is used to treat fever, diarrhea, dyspepsia, heat stroke, colds, hepatitis, wounds, for its activity in the digestive system and to kill insects. A review of the literature showed that M. paniculata has been found to have a wide range of activities, including neuropharmacological, larvicidal, insecticidal, free radical scavenging, antimicrobial, brine shrimp lethality, antidiarrheal, analgesic, anti-inflammatory, antipyretic, α-glucosidase inhibition, cytotoxic and nicotinic receptor antagonistic activities, as well as preventative effects for coronary heart disease and angina pectoris. Moreover, acute toxicity study of the methanolic extract of M. paniculata fruits were conducted also [1, 2]. Therefore, the present study was designed to identify phytoconstituents contained in, and to evaluate the toxicity, larvicidal and antidiabetic activity of aqueous extract of Microcos paniculata leaves (LWE).

Materials and Methods

Collection and Identification of the Plant

Leaves of M. paniculata were collected from the Jahangirnagar University campus, Savar, Dhaka, Bangladesh in November, 2012. Species identification was verified by Sarder Nasir Uddin, Principal Scientific Officer at the Bangladesh National Herbarium. Dried specimens were deposited in the herbarium for future references.

Extraction

Aqueous extraction was carried out on 200 g of powdered leaves of M. paniculata. Leaves were rinsed 3–4 times successively with running water and once with sterile distilled water that were then dried in the shade for a period of 7 d. The dried plant part was then ground by using a laboratory grinding mill (Model 2000 LAB Eriez®) and passed through a 40-mesh sieve to get fine powder. Powdered leaves of M. paniculata (200 g) were extracted in 2 L of water, using a soxhlet apparatus and a hot extraction procedure. Whatman No.1 filter papers were used to filter the liquid extract. The filtrate was then dried in a hot air oven at 40^0C. The extraction yield of leaves of M. paniculata was 8.72% (w/w). Extract was stored at 4^0C for additional studies.

Phytochemical Screening

LWE was subjected to different qualitative tests according to Aziz [2].

Test for Carbohydrates

Molisch's Test for Carbohydrates

Approximately 500 mg of crude extract was dissolved in 5 mL of distilled water and later filtered. A few drops of Molisch's reagent (α-naphthol 10% (w/v) in 90% ethanol) were added to the filtrate. Then 1 mL of concentrated H_2SO_4 was poured carefully along the side of the test tube. Two minutes later, 5 mL of distilled water was added. A positive test, indicating the presence of carbohydrates, was confirmed with formation of dull violet or red color at the interphase of the two layers.

Fehling's Test for Reducing Sugars

Crude extract (2 mg) was dissolved in 1 mL of distilled water and filtered. Next, 1 mL mixture of Fehling's solutions A and B (a ratio of 1:1) was added to the filtrate, which was heated in a water bath for a few minutes. Formation of brick-red precipitate confirmed the presence of reducing sugars.

Tests for Alkaloids

Aqueous HCL (5 mL, 1% v/v) was used to dissolve 50 mg extract and later filtered. Mayer's, Wagner's and Dragendorff's reagents were used to test the filtrate for the presence of alkaloids.

Mayer's Test

One or two drops of 0.35 mol/L Mayer's reagent (potassium-mercuric iodide solution, 1.36 g mercuric chloride and 5 g of potassium iodide, dissolved in 100 mL distilled H_2O) were added to 2 mL filtrate along the side of the test tube. A positive test, demonstrating the presence of alkaloids, was indicated by a white creamy precipitate.

Wagner's Test

A few drops of 0.44 mol/L Wagner's reagent (solution of iodine in potassium iodide, 2 g of iodine and 6 g of potassium iodide were dissolved in 100 mL distilled water) were added to 2 mL filtrate along the side of the test tube; a positive test, demonstrating the presence of alkaloids, was indicated by the formation of reddish-brown precipitate.

Dragendorff's Test

Dragendorff's reagent was made of two solutions. Solution A contained 1.7 g basic bismuth nitrate in 100 mL water/ glacial acetic acid (80 mL water and 20 mL glacial acetic acid in a 4:1 ratio), and solution B

contained 40.0 g potassium iodide in 100 mL of water. Both solutions were mixed in following manner to produce 100 mL Dragendorff's reagent (5 mL solution A + 5 mL solution B + 20 mL glacial acetic acid + 70 mL water). Dragendorff's reagent at 0.136 mol/L was added (1–2 mL) to 2 Ml filtrate solution. The formation of an orange-red precipitate indicated the presence of alkaloids.

Frothing Test for Saponins

Crude extract (100 mg) was dissolved in 10 mL methanol for making stock solution. This stock solution was diluted to 0.5 mg/mL by the addition of 20 mL distilled water. Test tube containing the dilution was then shaken for 15 min. Formation of foam on the top of the test tube indicated the presence of saponins.

$FeCl_3$ Test for Tannins

Crude extract (50 mg) was dissolved in 5 mL distilled water, followed by the addition of a few drops of 5% $FeCl_3$. Tannin was confirmed by the development of a bluish-black color.

Alkali Test for Flavonoids

A few drops of 5% NaOH solution were added to 1 mL filtered stock solution (100 mg extract/10 mL of methanol), which produced a deep-yellow color. The color was lost in the presence of dilute HCl and confirmed flavonoids.

Salkowski's Test for Triterpenoids

Plant extract (2 mg) was shaken in 1 mL $CHCl_3$. A few drops of concentrated H_2SO_4 were added to this solution along the side of the test tube. Development of a red-brown color at the interface indicated the presence of triterpenoids.

Baljet Test for Glycosides

Filtered stock solution (1 mL) of the plant extract was added to 1 mL sodium picrate solution. And the sodium picrate solution was immediately made before use by the addition of 95 mL 1% (w/v) aqueous picric acid with 5 mL 10% (w/v) aqueous NaOH and filtered through Whatman No.1 filter paper. That filtrate was used as Baljet reagent. The transformation of the sodium picrate solution's yellow color to orange confirmed the presence of cardiac glycosides.

Brine Shrimp Lethality Bioassay

Toxicity of the plant extract can be determined through brine shrimp lethality bioassay of Meyer et al [3]. Seawater was used for performing the hatching of Artemia salina Leach (brine shrimp eggs) into matured nauplii (Larvae) within 48 h at 25^0C. The seawater contained 10 napulii, where the test solution was added that were diluted serially. Then the number of alive larvae was counted after 24 h incubation period that was carried out at 25^0C. In this bioassay, the positive control was vincristine sulfate (VS).

Larvicidal Bioassay

WHO protocol was modified to some extent for carrying out the larvicidal assay [4]. The 4th instar larvae of Culex quinquefasciatus was utilized for determining the larvicidal activity of LWE. In this experiment, a series of glass beakers, each having 200 mL capacity were used and 100mL tap water was kept in these beakers separately. Then for obtaining exact concentration of the extract, sufficient quantity of the stock solution was added into each beaker. Only 100 mL tap water was used as control medium. The larval mortalities of 4th instar larvae of C. quinquefasciatus were observed separately in control, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 ppm concentrations. Insertion of ten 4th instar larvae of C. quinquefasciatus were done individually into the control and different concentrations of the extract. At the end of 24 h, the number of alive and dead larvae, response %, corrected response %, linear response %, linear probit, LC_{50}, LC_{90}, lower limit & upper limit and chi-square values were documented.

In Vitro Antidiabetic Study

α-Amylase Inhibitory Activity

A modified starch iodine protocol was used for determining *in vitro* antidiabetic activity [5]. Pre-marked test tubes were filled with 1 mL standard (Acarbose) or plant extract having distinct concentrations (2, 1, 0.5 mg/mL). Then, the addition of α-amylase (20 µL) into each test was completed and incubation was done at 37°C for 10 min. Later, 1% (w/v) starch solution (200 µL) was poured into each test tube. Again, the incubation temperature for the test tubes was set at 37°C and maintained for 1 h. After that each test tube was filled with 1% (w/v) iodine solution (200 µL) and distilled water (10 mL) was flowed subsequently. The wavelength was set at 565 nm for taking the absorbance of the mixture of each test tube. However, identical conditions were applied for getting the absorbance of α-amylase, sample and substrate blank. Half inhibitory concentration (IC_{50}) values were calculated taking the average of triplicate values.

α-amylase inhibition (%) = [1-(SA-SBB)-SMB/AAB] X 100

SA=Sample absorbance, SBB=Substrate blank, SMB=Sample blank, and AAB=α-Amylase blank.

Statistical Analysis

LdP Line software (trial version, Ehab Mostafa Bakr, Dokki, Cairo, Egypt) was used for calculating the LC_{50}, LC_{90}, chi-square values and other statistics at 95 percent fiducial limits of upper confidence limit and lower confidence limit from duplicate experiments [6]. All results were expressed as mean ± S.E. (Standard Error). Statistical analysis for % α-amylase inhibition activity was evaluated by one way analysis of variance (ANOVA) followed by Post hoc Tukey's HSD test through SPSS software (version 16; IBM Corporation, New York, USA). IC_{50} values were calculated by linear regression equations through the usage of Microsoft Excel 2013 (Microsoft, Redmond, Washington, USA).

Results

Phytochemical Screening

During the evaluation of candidate plant materials for pharmacological activities, the characterization of their chemical nature is essential. Phytochemical screening of LWE showed the presence of several primary and secondary metabolites, or phytoconstituents, which are summarized in Table 1. LWE exhibited the presence of almost all of the phytoconstituents like carbohydrates, alkaloids, saponins, flavonoids, triterpenoids and glycosides that were tested here. However, carbohydrate content in LWE was indicated by Molisch's test, but not by Fehling's test. Tannins were absent in LWE.

Brine Shrimp Lethality Bioassay (BSLB)

For determining the toxicity of LWE by BSLB, eight different concentrations (1, 5, 10, 20, 50, 100, 200 and 500 µg/mL) were used. Same response percentages (35 % and 45 %) i.e., mortality percentages of brine shrimp napulii were found by LWE for the concentrations of 1, 5, 10, 20, 50 µg/mL and 100, 200, 500 µg/mL respectively. Moreover, LC_{50} and calculated chi square (χ^2) values of LWE were 17889.19 µg/mL and 0.60 separately (Table 2). For the above data (Table 2), lower limit & upper limit of LC_{50} & LC_{90} couldn't be calculated by LdP Line software as 'g' value was greater than 0.40. During fiducial limit calculations 'g' is used as a factor [6]. 'g' value will be markedly lesser than 0.10, and rarely higher than 0.40 in case of nearly all suitable sets of data [7]. VS showed 95% mortality rate at 12.5 and 25 µg/mL separately. Again, the LC_{50} value of VS was 1.83 µg/mL with lower limit 1.17 µg/mL and upper limit 2.98 µg/mL. Besides, the calculated chi square (χ^2) value of VS was 5.90 (Table 3).

Larvicidal Bioassay

At the end of 24 h, LWE was monitored for its larvicidal activity against the 4th instar larvae of Culex quinquefasciatus and the results are represented in table 4. The 24 h LC_{50} value of LWE for C. quinquefasciatus was found to 695.22 PPM with lower limit 494.26 PPM and upper limit 1655.11 PPM. In addition to, the calculated chi square (χ^2) value of LWE was 8.53. However, LWE showed 55% mortality rate at 500 PPM (Table 4).

In vitro Antidiabetic Study

α-Amylase Inhibitory Assay

In the present study, LWE was found to possess inhibitory effects on starch break down shown in table 5. Most importantly, the effect was dose dependent. 3425.89±438.61 µg/mL was found as the IC_{50} value of LWE, along with 37.67±1.45 % α-amylase inhibition at 2000µg/mL (table 5 & 6). But the standard drug acarbose showed the IC_{50} value as 814.41±85.04 µg/mL (Table 6).

Discussion

Phytochemical components are identified as bioactive compounds of plant extracts and may be responsible for the diverse activities when herbs are used medicinally. Secondary herbal metabolites have influence on the medicinal and pharmacological actions of medicinal herbs. Primary metabolites (e.g., amino acids, monosaccharides, nucleic acids, polysaccharides, proteins, lipids) are present in almost all plant species, whereas secondary metabolites are found in fewer plant species; in the plant, these compounds provide defences against herbivores and pathogens, attract pollinators and fruit dispersers, give mechanical support, absorb harmful ultraviolet radiation and reduce the growth of nearby competing plants. Alkaloids, phenolics (simple phenolics and flavonoids), terpenoids, fatty acids, glycosides, waxes and their derivatives are examples of secondary metabolites that can have medicinal properties. In the field of drug discovery and development, the preliminary screening of secondary metabolites facilitates the recognition of bioactive compounds [2]. Bioactive compounds can be bio assayed with brine shrimp lethality bioassay (BSLB). Plant extract undergone BSLB can be used for inferring diverse pharmacological effects such as pesticidal effects, antifungal effects, teratogenic

effects etc. Toxicity of the plant extracts can be decided by BSLB. LC_{50} values of the plant extracts lower than 1000 µg/mL by BSLB will determine the bioactivity of them [8, 9, 10]. As an uncomplicated and inexpensive technique, BSLB is adopted during drug exploration process for screening wide range of extracts. BSLB technique is advantageous as it needs reduced amount of sample. Moreover, researchers can quickly test higher amount of samples along with dilutions than applying the initial test vials. Link exist between human solid tumor cell lines with BSLB of plant extracts comprising toxic compounds [11]. Though BSLB is used for evaluating the bioactivity of the plant extracts, but it is insufficient concerning the explanation of the mechanism of action of these extracts [12]. Preliminary phytochemical screening of LWE revealed the presence of alkaloids and triterpenoids (table 1).

Again, the LC_{50} value of LWE was 17889.19 µg/mL. Reports exist on the role of alkaloids and triterpenoids in cytotoxic activity of plant extracts, which warrants further investigation [10, 13]. For determining the toxicity and non-toxicity of any drug or plant extract, LC_{50} value will be less than 1000 µg/mL and greater than 1000 µg/mL respectively [14]. LWE showed LC_{50} > 1000 µg/mL and according to toxic dose level it clearly indicates that the extract is nontoxic, though alkaloids and triterpenoids were present in it (table 1). This directed that the LWE are safe for further use. Filariasis, Japanese encephalitis, malaria, dengue etc. are spread by mosquitoes causing millions of deaths every year and have an immense impact on public health. Disturbances in natural biological control systems, undesirable effects on non-target organisms and development of resistance are occurring by frequent usage of synthetic insecticides for controlling mosquitoes. Consequently, the environment and human health are affected mostly. Considering these problems plants may be an alternative source of mosquito control agent [12]. Insects may die by the active ingredients of plants or drugs. These active ingredients may hinder the discharge of nervous impulses stimulating the gathering of acetylcholinesterase at the post synaptic membrane. These events lead to the decrease of oxygen uptake and become the cause of insect's death. Secondary metabolites, which have larvicidal or insecticidal activity are toxic in nature may bind with copious molecular targets as like as ion channels, enzymes, receptors, proteins, structural proteins, nucleic acids, signaling molecules, bio membranes, and additional cellular composites. Due to this binding, insect's physiology may be affected. Basically nervous system (synthesis, release, storage, reuptake and fixing of neurotransmitter, enzymes of signal transduction pathway, activation as well as role of receptor) may be affected aberrantly. Moreover, secondary metabolites may inhibit G_AB_A gated chloride channel, cellular respiration, acetylcholinsterase, sodium and potassium ion exchange for interrupting insect's physiology. Among these activities, spread of nerve impulse through synaptic pathway is ended by acetylcholinsterase. These phenomena singly or combinedly may be liable for larvicidal or insecticidal activity [15]. Many primary and secondary metabolites as like as flavonoids, alkaloids, saponins, terpenoids, phenols, carbohydrates, phytosterols, tannins are responsible for larvicidal activity of mosquitoes [16]. Outcomes of the existing phytochemical analysis revealed that LWE contained several secondary metabolites such as alkaloids, saponins, flavonoids, triterpenoids which may inhibit either chloride channel, cellular respiration, acetylcholinsterase or sodium and potassium ion exchange for interrupting insect's physiology that may be responsible for its larvicidal activity against 4th instar larvae of C. quinquefasciatus. However, primary metabolite carbohydrates were present also being accountable for larvicidal activity. Alpha amylase breaks polysaccharide into monosaccharide. Hence, diabetes can be controlled by inhibiting alpha amylase enzyme which prolongs the overall digestion period of carbohydrate and reduces the absorption of glucose from starch [17, 18]. Previous study showed that alkaloids, terpenoids, saponins, flavonoids, tannins, steroids, phlobatannins, anthraquinone might be responsible for inhibiting alpha amylase enzyme [18, 19]. LWE exerted its antidiabetic activity that may be due to its inhibitory effect on alpha amylase enzyme because of the presence of secondary metabolites such as alkaloids, saponins, flavonoids and triterpenoids.

Conclusion

The present results showed that aqueous extract of Microcos paniculata leaves may have larvicidal and antidiabetic activity. However, the extract is nontoxic as well as safe for further use determined by brine shrimp lethality bioassay. Further investigations are required to find the active component of the extract in order to confirm the mechanism of action in the development of larvicidal and antidiabetic agents. Beisdes, acute toxicity and genotoxicity study of this extract may be a promising area for the researcher.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

List of abbreviations

Conc. = Concentration.
LC_{50}= Lethal Concentration, 50%
LC_{90}= Lethal Concentration, 90%
PPM= Parts Per Million

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Table 1. Phytochemical screening of LWE.

Phytoconstituents	Test name	Observation of LWE
Carbohydrates	Molisch's test	+
	Fehling's test	-
Alkaloids	Mayer's test	+
	Wagner's test	+
	Dragendorff's test	+
Saponins	Frothing test	+
Tannins	Lead acetate test	-
Flavonoids	Alkali test	+
Triterpenoids	Salkowski's test	+
Glycosides	Baljet test	+

+: presence of specific phytoconstituents; -: absence of specific phytoconstituents.

Table 2. Probit analysis, $LC_{50,}$ LC_{90} & calculated chi square (χ^2) values for LWE.

Conc. (µg/ml)	Treated	Response %	Corrected response %	Linear response %	Linear probit	LC_{50}(µg/ml)	LC_{90}(µg/ml)	χ^2
1	20	35	31.58	28.48	4.43	LC_{50}=17889.19	LC_{90}=68631616936175.40	0.60
5	20	35	31.58	31.73	4.52			
10	20	35	31.58	33.18	4.57			
20	20	35	31.58	34.65	4.61			
50	20	35	31.58	36.64	4.66			
100	20	45	42.11	38.16	4.70			
200	20	45	42.11	39.70	4.74			
500	20	45	42.11	41.77	4.79			

Table 3. Probit analysis, $LC_{50,}$ LC_{90} & χ^2 values for VS.

Conc. (µg/ml)	Treated	Response %	Corrected response %	Linear response %	Linear probit	LC_{50}(µg/ml)	LC_{90}(µg/ml)	χ^2
0.06	20	10	5.26	3.29	3.16	LC_{50}=1.83 Lower limit=1.17 Upper limit=2.98	LC_{90}=19.63 Lower limit=10.02 Upper limit=54.92	5.90
0.125	20	15	10.53	7.41	3.55			
0.25	20	15	10.53	14.17	3.93			
0.5	20	25	21.05	24.23	4.30			
1	20	55	42.11	37.26	4.68			
5	20	65	52.63	70.66	5.54			
12.5	20	95	94.74	85.03	6.04			
25	20	95	94.74	92.10	6.41			

Table 4. Response %, corrected response %, linear response % and linear probit of 4th instar larvae of C. quinquefasciatus exposed for 24 h to different concentrations of LWE.

Conc. (PPM)	Treated	Response %	Corrected response %	Linear response %	Linear probit	LC_{50}(PPM)	LC_{90}(PPM)	χ^2
50	20	10	10.5	5.263	2.6735	LC_{50}=695.2186 Lower limit=494.2591 Upper limit=1655.1069	LC_{90}=2963.769 Lower limit=1363.0353 Upper limit=26979.3026	8.5263
100	20	10	15.8	5.263	3.2861			
150	20	10	26.3	5.263	3.6445			
200	20	15	31.6	10.526	3.8987			
250	20	25	42.1	10.526	4.0959			
300	20	25	42.1	21.053	4.2571			
350	20	20	52.6	15.789	4.3935			
400	20	35	52.2	31.579	4.5115			
450	20	45	73.7	42.105	4.6155			
500	20	55	79.0	52.632	4.7087			

Table 5. % of α-amylase inhibition of LWE.

Group	%of α-amylase inhibition (µg/mL)		
	500	1000	2000
Standard	41.33±1.76^{a}	56.00±3.06^{c}	75.33±0.67^{ab}
LWE	23.67±0.88^{bc}	32.33±1.20^{a}	37.67±1.45^{a}

Values are presented as mean±S.E.; n=3. LSD test was performed for the pair wise mean comparison. Values along the same column and row with different superscripts a, c, ab, & bc are significantly different from one another

Table 6. IC_{50} of standard and LWE.

Group	IC_{50} (µg/mL)
Standard	814.41±85.04
LWE	3425.89±438.61

Values are presented as mean±S.E.; n=3.