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Phytochemical Screening and Biological Activity Evaluation of the Methanolic Crude Extract of *Ixora chinensis* (Family: Rubiaceae)

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Abstract

Natural compounds have long been employed as medicines throughout the course of human history. Traditional medicine has employed and still uses natural materials, especially plant treatments, based on practical observations rather than pharmaceutical research. *Ixora chinensis* (Lam) plant has several important medicinal properties which have not been studied extensively in Bangladesh. The present work has been carried out to evaluate diverse biological actions of the plant extract and identification of pure compounds. The crude powder of leaves and barks of the plant was extracted using absolute methanol. The methanolic extract was subjected to GC-MS to reveal chemical entities present in the extracts and from GC-MS 1,2,3,5-Cyclohexanetetrol was identified. In the present study, methanolic extract of *I. chinensis* was exposed to investigate antioxidant activity and antimicrobial screening. The antimicrobial screening was performed against gram-positive and gram-negative bacteria including fungus by using disc diffusion technique. The extract of *I. chinensis* showed mild antimicrobial activity in reference to standard kanamycin and ketoconazole. The results of the present study exhibited high phenolic content (498.8 mg/g) in the methanolic extract compared to gallic acid. Total phenolic content (TPC) of a sample indicates potency of antioxidant. TPC value (224.81 mg GAE/g of sample) of the present study exhibits higher total flavonoid content. The results indicate that the extracts of *I. chinensis* have lower antioxidant property in respect of total flavonoids content. Antioxidant activity was also measured using DPPH free radical scavenging activity. IC_{50} value of the methanolic crude extract was found to be 129.128 µg/ml. The data suggested that the crude methanolic extracts possess mild anti-bacterial and antifungal activities with moderate antioxidant activity.

Key words*: Ixora chinensis*, Rubiaceae, antioxidant activity, DPPH, phytochemical screening, biological activities.

Introduction

Traditional herbal treatments have been used to treat illnesses for a long time, and they still play a significant role in daily health care, especially in rural areas of most endemic countries where there are few modern health facilities. More than 70.0 % of people living in underdeveloped countries today rely on traditional medicine, also known as complementary or alternative medicine (Azazieh *et al.*, 2010).

The World Health Organization (WHO) has reported that the usage of herbal medicine is two to three times greater compared with traditional medications worldwide (Evans, 1994). Plants having medicinal properties were used for therapeutic purposes since the dawn of time and a great deal of contemporary medicine stems from this legacy. The popular of the few effective treatments from a century ago were plant-based. Therefore, many standard drugs such as morphine (Opium poppy),

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quinine (Cinchona bark), digoxin (Foxglove) and aspirin (Willow bark) (Vickers, A. and Zollman, C. 1999) have been originated from the plant sources. *I. chinensis* also referred to as *Chinese ixora*, is a kind of plant in the *Ixora* genus. A family of flowering plants called Rubiaceae includes the genus *Ixora*. There are 500 species of these tropical evergreen trees and shrubs, with tropical Asia serving as the region with the greatest diversity. One of the most common native species is *I. chinensis* was identified by its almost stalkless leaves and red flowers in the southern China. *Ixora* is a plant that is frequently found in subtropical regions of the United States (i.e., Florida). *I. chinensis* is a dense, multi-branched, evergreen shrub that typically grows to a height of 4-6 feet (1.2 to 2.0 m) but can grow as high as 12 feet (3.6 m). It is widespread in south-east Asian region and used to treat various disorders such as rheumatism and wounds (Khare, 2007). The roots used in Asian medicine to relieve from stomach problems. For example, the Malays used decoction of the root after childbirth. While the Indonesians used flower decoction for amenorrhea and hypertension and root decoction for the bronchial ailments (Uy M. M. *et al.*, 2015). In the Philippines an infusion of the fresh flowers used as a remedy against incipient tuberculosis and hemorrhage. In Vietnam roots, stems, leaves and flowers are used for acne, high blood pressure, hemoptysis, irregular menses, rheumatism and tuberculosis. Numerous phytochemicals were isolated in previous time from the different parts of *I. chinensis* are Ixoroside, Ixoside, Geniposidic Acid, Lupeol, Betulin, B-Sitosterol, Stigmasterol, D-Mannitol, Stearic Acid, Ixoric Acid, Azelaic Acid, and 1,5-Cyclooctadiene (Kharet *et al.*, 2013; Takeda *et al.*, 1975; Hui *et al.*, 1968; Rafe M. R. 2017). Ethnobotanical research has been increased recently in greater range in national and international level. A significant gap between scientific validation of ethnomedicine and their uses was found from a number of literatures review (Singleton *et al.*, 1965). There is not enough literature found regarding the chemical and biological study of *I. chinensis*. So, it is worthwhile to screen out the alcoholic extract of *I. chinensis* barks and leaves for the presence of phytochemicals to identify and characterize phytoconstituents in its various crude extracts for chemical profiling by gas chromatography-mass spectrometric (GC-MS) performance and to evaluate its antioxidant potential by using *in vitro* methods to correlate with the phenolic and flavonoid content.

Materials and Methods

Preparation of plant materials: In December 09, 2022, the entire plant of *Ixora chinensis* was collected from a local nursery (Sabuj Bangla Nursery) at Agargoan in Dhaka, Bangladesh. When a voucher specimen for this collection was placed at the Bangladesh National Herbarium in Dhaka and the plant was recognized with an assigned accession number (DACB 88221). The collected leaves of the plants were properly washed and dried under shade for around 20 days. After proper drying, the leaves and barks of the plant were ground to fine powder by using a high- capacity grinding machine. The dried powder was weighed and found to be 157.0 g. In a clean, cylindrical, amber-colored container, 157 g of powdered *I. chinensis* material was soaked in 900 ml of laboratory grade solvent (Methanol). The flasks and their contents were preserved for 11 days (January 18, 2023 - January 29, 2023). During this period, the container was occasionally shaken and stirred for proper extraction of components into the solvent. On January 29, 2023, the methanolic extract was filtered using a vacuum filter at the Pharmacy lab, Jagannath University. A rotary evaporator (RE-200, Thomas Scientific) was used to decrease the volume of the filtrate at 47 ^{0}C and the rpm was 121. The weight of the crude extract was found to be 12.44 g.

Analysis of GC-MS: GC-MS examination was performed with SHIMADZU GC-2010 Plus (Japan) using a column (SH-5MS, 30 m × 0.25 mm) with 0.25 µm film with a column flow of 0.54 mL/min. Pure helium gas (99.99 %) was used as transporter. The sample (1.0 µL) was studied in electron ionization approach. The ion source temperature and

interface temperature of mass spectrometer was 200 ^{0}C and 250 ^{0}C respectively.

Antimicrobial examination: The disc diffusion method was employed for the evaluation of the antibacterial and antifungal activities. Here the tested samples were evaluated against two gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), two gram-negative bacteria (*Salmonella typhi* and *Escherichia coli*) and two fungal strains *(Aspergillus niger* and *Aspergillus flavus*). The test samples were dissolved in DCM and MeOH separately. The discs with treated sample were placed in nutrient agar medium inoculated with the potato dextrose agar medium along with bacteria and fungi. The plates were then incubated at 37 °C for 24.0 h and 48.0 h at 28 °C for bacteria and fungi respectively. The diameter of inhibitory zones was measured around the sample treated disc in millimeters (mm). Accordingly, the sensitivities of the microorganism to the samples (100 µg/disc) were determined whereas the kanamycin (30 µg/disc) and ketoconazole (30 µg/disc) were used as standard for the antibacterial and antifungal activities.

Determination of Phytoconstituents

Determination of total phenolics: The method followed to determine the total phenolic content of *I. chinensis* was that recorded by Singleton and his co-workers (Bauer *et al.,* 1996). For the colorimetric testing of polyphenolic and phenolic antioxidants, the Folin-Ciocalteu, also known as Folin Denis reagent or Folin's phenol reagent was used (Pham *et al.,* 2020). It is performed by determining the level of the substance under test necessary to inhibit the reagent from oxidizing. First, the standard solution at different concentrations was mixed well with Folin–Ciocalteu reagent solution (5.0 ml) and sodium carbonate solution (4.0 ml). The same procedure was followed for plant extract (1.0 ml) also. Then the test tubes containing reaction mixture were incubated for 30 minutes at 20 °C to complete the reaction and the absorbance of the solution was measured at 765.0 nm. Gallic acid used as the standard to prepare the standard curve (y = 0.007x + 0.124 and R^2 = 0.994) against which the total content of phenolic compounds of our studied plant extracts was compared.

Determination of total flavonoids: Wang and Jiao's aluminum chloride colorimetric method was used to calculate total flavonoid content where quercetin was employed as a reference (Zhishen *et al.,* 1999). Accordingly, individual fraction of plant extracts (1.0 ml) and different concentrations of standard were mixed with methanol (3.0 ml), 10.0 % aluminum chloride (2.0 ml), potassium acetate (1.0 M and 0.2 ml), and distilled water (5.6 ml) respectively and incubated for 30.0 minutes. The absorbance was measured at 415.0 nm after the incubation. A standard curve was plotted (y = 0.013x + 0.093 and R^2 = 0.993) to compare the result with dried extractives as a quercetin gram equivalent.

Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity: A method established by Baliyan (Bdiyan *et al.,* 2022) was used to determine the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. Methanol solution of DPPH (2.0 ml) was added into a test tube with plant extract (1.0 ml) and then incubated at RT for 30.0 minutes in a dark place to complete the reaction. Lastly, the absorbance of the solution was measured at 517.0 nm using a spectrophotometer against a blank solution.

Results and Discussion

Determination of compound: According to the GC-MS examination of the crude extract of *I. chinensis*, one compound was determined as 1,2,3,5-cyclohexanetetrol having molecular weight of 148.0 g, similarity 65.0 %, cas number: 53585-08-3, and chemical formula $C_6H_{12}O_4$ (Figures 1-2).

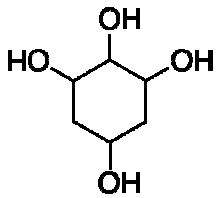

Figure 1. 1,2,3,5-Cyclohexanetetrol.

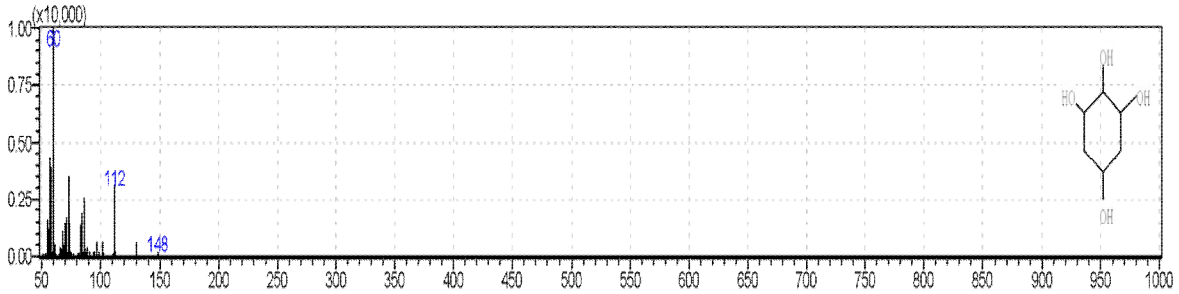

Figure 2. GC-MS chromatograph of 1,2,3,5-Cyclohexanetetrol.

Antimicrobial activity screen screening: The methanolic crude extract of *I. chinensis* was exposed to antimicrobial screening with a concentration of 400 µg/disc in every case to measure the zone of inhibition in mm. Antimicrobial screening for the crude extract of *I. chinensis* was carried out against two gram-positive (*Bacillus subtilis and Staphylococcus aureus*), two gram-negative bacteria (*Salmonella typhi* and *Escherichia coli*) and two fungi (*Aspergillus flavus* and *Aspergillus niger*). Both gram positive and gram-negative bacteria showed mild activity in reference to standard antibiotic kanamycin. The sample didn't show any sensitivity to *A. flavus* but showed mild activity against *A. nigar* (7.0 mm) compared to standard ketoconazole. The results for anti-bacterial activity and anti-fungal activity are given in table 1.

Table 1. Anti-bacterial and anti-fungal activities of crude extract of *Ixora chinensis.*

Test organisms	Zone of inhibition (mm)		
		Kanamycin	Ketoconazole
Staphylococcus aureus	7	26	---
Escherichia coli	7	40	---
Salmonella typhi	7	25	---
Bacillus subtilis	7	32	---
Aspergillus flavus	---	---	36
Aspergillus niger	7	---	13

Determination of phytoconstituents

Total phenolic content (TPC): TPC of different partitionists were measured and calculated using the method of Folin−Ciocalteu reagent where gallic acid was used as standard (Table 2 and Figure 3). It was expressed as equivalent of gallic acid in mg per dried partitionists. Total phenolic contents of the methanol extracts of *I. chinensis* were found to be 498.8 mg/gm (Table 3). The high amount of phenolic content indicates good antioxidant activity. The TPC was calculated using the linear regression equation obtained from the standard plot of gallic acid (y = 0.015x–0.0302, and R^2 =0.9984). here y is absorbance and x is the amount of gallic acid in microgram (µg).

Determination of total flavonoids contents (TFC): The flavonoids content of extracts was measured by using colorimetric method with $AlCl_3$. The total flavonoids of our observed fractions were measured by comparing them with gram equivalent of quercetin (standard). Results were denoted as QE/g, which means quercetin equivalent with per

gram of dried sample extracts. The total flavonoids content of the methanolic crude extract of *I. chinensis* was found to be 224.81 mg/gm (Table 4 and Figure 4) which indicates the presence of polyphenolic secondary metabolites in the plant. The TFC were calculated using the linear regression equation obtained from the standard plot of quercetin (y = 0.0079x-0.0646, and R^2 = 0.9986). Where y is absorbance and x is the amount of quercetin in microgram (µg).

Table 2. Absorbance found with different concentrations of gallic acid and *I. chinensis* extract.

	Conc. (µg/ml)	Abs-I	Abs-II	Abs-III	Mean	SD	RSD (%)
Gallic acid	100	1.81	1.23	1.43	1.49	0.29	19.74
	50	0.87	0.53	0.69	0.69	0.17	23.77
	25	0.40	0.21	0.35	0.32	0.097	30.35
	12.5	0.18	0.11	0.20	0.164	0.05	29.16
	6.25	0.11	0.05	0.11	0.09	0.04	39.49
IC	**100**	**0.704**	**0.732**	**0.718**	**0.718**	**0.014**	**1.95**

Abs: Absorbance, SD: Standard deviation, RSD: Relative standard deviation and IC: Ixora chinensis

Table 3. Total phenolic content of the methanolic crude extract of *I. chinensis.*

Sample solution (µg/ml)	Weight of dry extract (gm/ml)	Absorbance	GA conc. (mg/ml)	TPC = (C×V)/m (mg/gm)
100	0.0001	0.718	0.0498	498.8

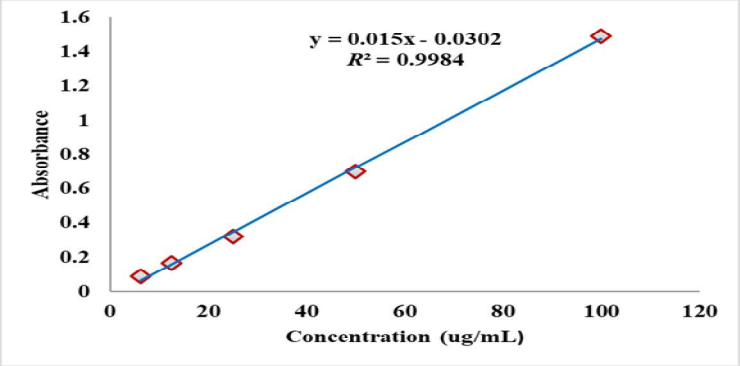

Figure 3. Standard calibration curve of gallic acid.

Table 4. Total flavonoids content of the methanolic crude extract of *I. chinensis.*

Sample solution (µg/ml)	Weight of dry extract (gm/ml)	Absorbance	QE conc. (mg/ml)	TFC as QE, A = (C × V)/m (mg/gm)
100	0.0001	0.113	0.0225	224.81

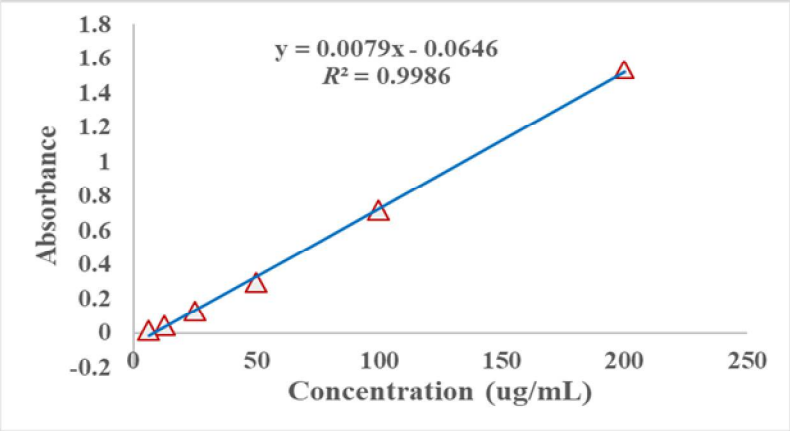

Figure 4. Calibration curve of quercetin.

DPPH free radical scavenging activity: The IC_{50} value of methanolic crude extract of *I. chinensis* was calculated from the linear regression curve (y = 0.2282x + 20.533 and R^2 = 0.9179) (Figures 5-6) and found as 129.13 µg/ml. The values of percentage of inhibition of ascorbic acid and the crude extract of *Ixora chinensis* are given in tables 5-6. Figures 5-6 showed the DPPH free radical scavenging assay of ascorbic acid and *Ixora chinensis* respectively.

% Inhibition = {1-(Absorbance of sample/Absorbance of control)} × 100

Table 5. The inhibition (%) scavenging activity of ascorbic acid.

Conc (ug/ml)	Absorbance (Control)				Absorbance (Sample)				Inhibition (%)
	I	II	III	Mean	I	II	III	Mean	
6.25	0.78	0.81	0.84	0.81	0.68	0.79	0.71	0.70	12.92
12.5	0.78	0.81	0.84	0.81	0.45	0.56	0.52	0.51	36.69
25	0.78	0.81	0.84	0.81	0.19	0.15	0.16	0.17	79.19
50	0.78	0.81	0.84	0.81	0.06	0.06	0.02	0.05	94.10
100	0.78	0.81	0.84	0.81	0.04	0.02	0.03	0.03	96.12
200	0.78	0.81	0.83	0.81	0.03	0.03	0.02	0.02	97.15

Table 6. The inhibition (%) scavenging activity of methanolic extract of *Ixora chinensis.*

Conc. (ug/ml)	Absorbance (Control)	Absorbance (Sample)	Inhibition (%)
6.25	0.807	0.79	2.11
12.5	0.807	0.72	10.78
25	0.807	0.64	20.69
501	0.807	0.58	28.13
100	0.807	0.49	39.28
200	0.807	0.34	57.87

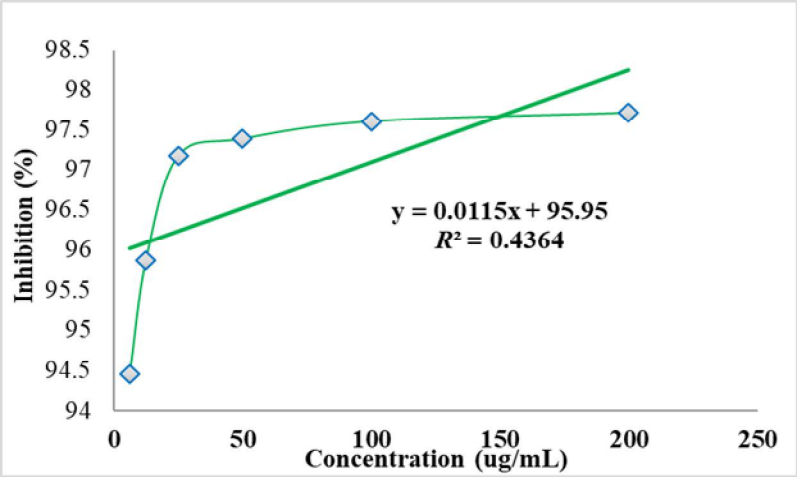

Figure 5. DPPH free radical scavenging assay of ascorbic acid.

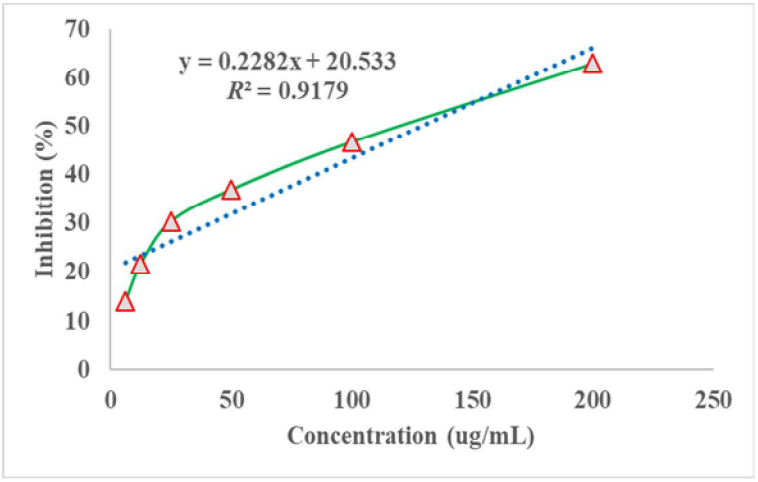

Figure 6. DPPH free radical scavenging assay of *Ixora chinensis*

Conclusion

It was found that methanolic crude extracts of *I. chinensis* consist of a good amount of phenolic and flavonoids content. Antimicrobial activity examination indicated that the extract exhibited mild anti-bacterial action against both gram positive and gram-negative bacteria and relatively low activity against fungi. Any activity against *Aspergillus flavus* was not found. One pure compound (1,2,3,5-Cyclohexanetetrol) was identified from the GC-MS analysis of the crude extract. Consequently, considering the potential bioactivity, a further study can be accomplished to elucidate the bioactive compounds and their unexplored efficacy for using as traditional medicines.

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Author's contribution: N.H. Sraboni performed the entire experiment. M.R. Rafe guided N.H.

Sraboni to collect plant and prepare crude extract. M.M. Hussain supervised the entire research work. N.H. Sraboni wrote the whole draft of the manuscript. M.M. Hussain corrected the manuscript. M.M. Hussain and N.H. Sraboni revised the manuscript. At last, all authors approved the revised manuscript to publish in this journal.

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