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Antimicrobial Investigation on *Manilkara zapota* (L.) P. Royen

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Abstract

The present study was designed to screen antimicrobial activity of *Manilkara zapota* (L.). Bioassays for antimicrobial activities were carried out using ethyl acetate extracts of both stem bark and leaves of *Manilkara zapota* against some pathogenic bacteria and fungi. TLC (Thin layer chromatography) profile of isolated extracts showed the presence of terpenoids, glycosides and flavoniods type compounds. The ethyl acetate extract of stem bark exhibited antimicrobial activity against all the pathogenic bacteria used in this study and also showed activity against *Aspergillus flavus*, *Vasianfactum* sp and *Fusarium* sp. with inhibition zones in the range of 08-16 mm. Ethyl acetate extract of leaves possessed mild activity against *Bacillus subtilis*, *Bacillus megaterium*, *Sarcina lutea*, *Escherichia coli* and *Salmonella typhi*. The minimum inhibitory concentrations (MICs) of the extracts were found to be in the range of 256 ~ 512 µg/ml. The cytotoxicity (LC_{50}) against brine shrimp nauplii (*Artemia salina*) were also evaluated and found to be 16.17 µg/ml for ethyl acetate extract of leaves, 50.26 µg/ml for ethyl acetate extract of stem bark and 12.38 µg/ml for ampicillin trihydrate.

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Introduction

Natural products either as pure compounds or as standardized plant extracts provide unlimited opportunities for new drug [1]. Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in

many developing countries [2]. To promote the proper use and to determine their potential as sources for new drugs, it is essential to study the medicinal plants. Therefore, researchers are increasingly turning their attention to folk medicine to develop better drugs against microbial infections [3].

Manilkara zapota (L.) P. Royen, rarely found in Bangladesh as sofeda, is a small to medium evergreen tree of slow growth. This plant has antioxidative property [4] and its fruit is preventive against biliousness and attacks of fever whereas seeds are diuretic [5]. The major constituents isolated from fruits of *Manilkara zapota* are polyphenols (methyl chlorogenate, dihydromyricetin, quercitrin, myricitrin, (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and gallic acid) [6]. From perusal of literature it was found that the leaf and stem bark of *Manilkara zapota* had not been subjected to screening for antibacterial and antifungal properties so far. The present study was conducted to evaluate the antimicrobial activity and cytotoxic effect of ethyl acetate extract of leaves and stem bark of *Manilkara zapota* against microorganisms and brine shrimp nauplii (*Artemia salina*), respectively.

Materials and Methods

Plant materials

Leaves and stem bark of *Manilkara zapota* (Family: Sapotaceae) were collected in the month of October, 2009 from Rajshahi district of Bangladesh. The plant material was taxonomically identified by Professor A.T.M Naderuzzaman, Department of Botany, University of Rajshahi and a voucher specimen was deposited under the accession number DACB-23801 at the Bangladesh National Herbarium.

Extraction

The collected leaves and stem bark were cleaned and shade-dried. The dried leaves and stem bark were then pulverized into a coarse powder by a grinding machine (FFC-15, China). The powdered leaves (150 gm) and stem bark (290 gm) was extracted with ethyl acetate at room temperature. These two extracts were then filtered through filter papers and filtrates were evaporated under reduced pressure at 40^{o}C using a rotary evaporator to have 4.1 and 3.5 g ethyl acetate extracts of stem bark and leaves, respectively.

TLC Screening

Both extracts were run on pre-coated silica gel plate using the mixture of n-hexane, petroleum ether and ethyl acetate in different proportion as the mobile phase and vanillin-H_2SO_4 reagent was used as spray reagent. R_f values were also calculated for every spots [7].

Tests for Antimicrobial Activity

Four Gram positive (*Bacillus subtilis* BTCC19, *Bacillus megaterium* BTCC18, *Bacillus cereus* ATCC258 and *Sarcina lutea* ATCC27803), five Gram negative (*Escherichia coli* ATCC25922, *Shigella sonnei* ATTC8992, *Shigella shiga* ATCC27853, *Shigella dysenteriae* ATCC561 and *Salmonella typhi* ATCC14228) pathogenic bacterial strains and five fungal strains (*Aspergillus flavus* ACCT10558, *Aspergillus fumigatus* ATTC10231, *Candida albicans* ATTC25889, *Vasianfactum* sp ATTC235561, and *Fusarium* sp ACCT56390) were collected from the Institute of Biological Science (IBSC), University of Rajshahi, Bangladesh. The ethyl acetate extracts of leaves and stem bark of *Manilkara zapota* were tested separately for antibacterial activity by disc diffusion assay method [8]. *Kanamycin* disc (30 µg/disc) and *Fluconazole* disc (100 µg/disc) were used as positive antibacterial and antifungal control, respectively. Blank disc impregnated with the respective solvent was used as negative control. The antibacterial and antifungal activities of each extract were tested against each microorganism at concentrations of 300 µg/disc, 600 µg/disc and 900µg/disc. Antibacterial assay plates were

incubated at 37±1°C for 24h, whereas antifungal assay plates were incubated at 37±1°C for 48h. Each experiment was carried out in triplicates and diameter of the zone of inhibition surrounding each disc was recorded. The minimum inhibitory concentration for the extracts having antimicrobial activity, were also determined by serial dilution technique [9].

Brine Shrimp Lethality Bioassay

The experiment was carried out using the method described by Meyer *et al*. [10]. In brief, *Artemia salina* Leach (brine shrimp eggs) was allowed to hatch and mature as nauplii (Larvae) in seawater for 48 h at 25°C. Serially diluted test solutions (80 µL in DMSO from a stock solution of 5 mg/mL DMSO) were added to the seawater (5 mL), containing 10 nauplii. After incubation for 24 h at 25°C, the number of survivors was counted. The LC_{50} (50% lethal concentration, µg/ml) was determined from triplicate experiments using Probit analysis as described by Finney [11]. Ampicillin trihydrate was used as positive control.

Results and Discussion

Phytochemical screening of the leaves and stem bark of *Manilkara zapota* (L.) revealed that the plant contain terpenoids, flavonoids and glycosides (Table 1).

In antibacterial study, the efficacy of ethyl acetate extract of stem bark and leaves of *Manilkara zapota* (L.) to inhibit the growth of four gram (+) positive bacteria and five gram (–) negative bacteria is shown in table 2. Ethyl acetate extract of stem bark showed moderate activity against all the pathogenic bacteria used in this study and produced inhibition zone ranging from 08 to 15 mm. Ethyl acetate extract of leaves showed mild activity against only *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* with inhibition zones in the range of 06-09 mm. Minimum inhibitory concentration (MIC) values were also evaluated against four Gram positive and four Gram negative bacteria. The lowest MIC values were 256 µg/ml and 512 µg/ml for ethyl acetate extract of stem bark and leaves (Table 4).

In antifungal activity test, ethyl acetate extract of stem bark produced zone of inhibition between 08 to 13 mm against *Aspergillus flavus*, *Fusarium* sp and *Vasianfactum* sp (Table 3). Ethyl acetate extract of leaves had no antifungal activity.

The pattern of above results indicated that ethyl acetate extract of stem bark and leaves contained antibacterial constituents at very low concentrations and so both of them showed antibacterial activity at high doses (300, 600 & 900 µg/disc doses). The similar type of results has been reported by Kumaraswamy et al. [12] when they evaluated antimicrobial activity of *Woodferdia fruticosa*.

In literature, it has been found that the presence of terpenoids, flavonoids and glycosides like chemicals in crude extract plays an important role for producing antimicrobial activity [13-15]. The presence of terpenes flavonoids and glycosides as predicted by TLC profile may be the cause of antimicrobial activity of ethyl acetate extract of stem bark and leaves of *Manilkara zapota* (L.).

Ethyl acetate extracts of both stem bark and leaves showed cytotoxicity against brine shrimp nauplii (*Artemia salina*) (Table 5). Among the samples, ethyl acetate extract of leaves (LC_{50}: 16.17 µg/ml) showed the highest toxicity, whereas ethyl acetate extract of stem bark (LC_{50}: 50.26 µg/ml) exhibited mild activity in comparison with ampicillin trihydrate (LC_{50}: 12.38 µg/ml). The mortality was not shown in the negative control experiment. Several studies have shown that brine shrimp bioassay has been an excellent method to screen the cytotoxic property of medicinal plants and for the isolation of a great variety of biologically active compounds [16]

The results of this study therefore, offer a scientific basis for isolation and purification of bioactive

principles from stem bark and leaves of *Manilkara zapota*. Our future studies to isolate these active phytochemicals and determine their activities against microorganisms, are in progress.

Table 1: TLC profile of crude ethyl acetate extracts of stem bark and leaves of *Manilkara zapota*

Sample	Solvent system	Number of spots	R_f value	Color with vanillin-H_2SO_4	Possible compound
Ethyl acetate extract of stem bark	n-Hexane: Petroleum ether (9:1)	4	0.22	Violet	Terpenoids
			0.40	Violet	Terpenoids
			0.58	Violet	Terpenoids
			0.77	Violet	Terpenoids
Ethyl acetate extract of leaves	n-Hexane: Ethyl acetate (9:1)	5	0.21	Black	Glycosides
			0.40	Black	Glycosides
			0.58	Black	Glycosides
			0.74	Yellow	Flavonoids
			0.86	Yellow	Flavonoids

Table 2: *In vitro* antibacterial activity of ethyl acetate extracts of stem bark and leaves of *Manilkara zapota* and *Kanamycin*:

Micro Organisms	Zone of inhibition (in mm)						
	Ethyl acetate extract of stem bark			Ethyl acetate extract of leaves			*Kanamycin*
	Dose (µg/disc)						
	300	600	900	300	600	900	30
Bacillus subtilis	09	12	13	-	08	10	28
Bacillus cereus	09	11	13	-	-	-	29
Bacillus megaterium	11	12	14	-	09	11	30
Sarcina lutea	09	11	14	-	08	09	28
Escherchia coli	10	13	16	-	08	10	30
Salmonella typhi	09	10	12	-	08	09	28
Shigella dysenteriae	08	10	11	-	-	-	29
Shigella sonnei	09	11	13	-	-	-	27
Shigella shiga	08	10	13	-	-	-	29

Table 3: *In vitro* antifungal activity of ethyl acetate extracts of stem bark and leaves of *Manilkara zapota* and *Fluconazole*

Microorganisms	Zone of inhibition (in mm)						
	Ethyl acetate extract of stem bark			Ethyl acetate extract of leaves			*Fluconazole*
	Dose (µg/disc)						
	300	600	900	300	600	900	100
Aspergillus flavus	09	12	13	-	-	-	20
Fusarium species	08	11	12	-	-	-	21
Aspergillus fumigatius	R	R	R	-	-	-	20
Candida albicans	R	R	R	-	-	-	19
Vasianfactum sp	08	11	13	-	-	-	20

Table 4: MIC values of ethyl acetate extracts of stem bark and leaves of *Manilkara zapota*

Test organism	Ethyl acetate extract of stem bark (µg/ml)	Ethyl acetate extract of leaves (µg/ml)
Bacillus subtilis	256	512
Bacillus megaterium	256	-
Bacillus cereus	256	512
Sarcina lutea	256	512
Escherichia coli	256	512
Salmonella typhi	256	512
Shigella dysenteriae	256	-
Shigella sonnei	256	-
Shigella shiga	256	-

Table 5: Cytotoxicity of ethyl acetate extracts of stem bark and leaves of *Manilkara zapota*

Sample	LC_{50} (µg/ml)
Ampicillin trihydrate	12.38
Ethyl acetate extract of stem bark	50.26
Ethyl acetate extract of leaves	16.17

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