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EVALUATION OF THE CYTOTOXIC, ANTIMICROBIAL, ANTIOXIDANT, ANTHELMINTIC AND CNS DEPRESSANT ACTIVITIES OF *MANILKARA ZAPOTA* LEAF (SAPOTACEAE)

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ABSTRACT

Manilkara zapota (Sapotaceae) is an important medicinal plant having application in cancer, oxidative stress, infectious diseases and various other disorders. The aim of this study was to evaluate the cytotoxic, antimicrobial, antioxidant, anthelmintic and CNS depressant properties of the leaf of *M. zapota*. The leaf part of the plant *M. zapota* was dried, extracted using ethanol and was partitioned into different fractions. The ethanolic extract and ethyl acetate fraction showed significant cytotoxicity with LC_{50} value of 1.92 µg/ml and 5.04 µg/ml respectively compared to the value obtained by standard vincristine sulfate (LC_{50} 0.544 µg/ml) in brine shrimp lethality bioassay. Antimicrobial screening by disc diffusion method showed that, the crude extract and ethyl acetate fraction had mild inhibitory effects (zone of inhibition is 10-11 mm) against some gram (+) and gram (-) strain of bacteria and fungi. In DPPH free radical scavenging assay, the ethyl acetate fraction exhibited potent antioxidant activity with IC_{50} value of 8.28 µg/ml. The ethanolic extract also had good free radical scavenging ability (IC_{50} 32.16 µg/ml) compared to that obtained by standard Butylated hydroxyl toluene (IC_{50} 15.46 µg/ml). The extract didn't possess any kind of anthelmintic property against adult earthworm *Pheretima posthuma*. The ethanolic extract of *M. zapota* also showed mild CNS depressant activity by prolonging duration of sleeping time. The findings of the studies demonstrated potent cytotoxic, antioxidant, antimicrobial and mild CNS depressant potential of the leaves of *M. zapota* which could be the therapeutic option against cancer, tumor, oxidative stress and infectious diseases.

KEYWORDS: *Manilkara zapota*, Brine shrimp lethality bioassay, DPPH free radical scavenging, Disc diffusion.

INTRODUCTION

The use of natural products to prevent cancer is becoming increasingly popular now a days. Many plant derived natural products are used as anti-cancer agents like vincristine and vinblastine. Plants also represent a rich source of antimicrobial agent [1] and natural antioxidants. [2] Many of the plant materials used in traditional medicines are readily available in rural areas at relatively cheaper than modern medicines. [3] Plants generally produce many secondary metabolites which constitute an important source of microbicides, antioxidants. Many natural substances having antioxidant and antimicrobial properties have been used in health foods for medicinal and preservative purposes.[4]

Manilkara zapota (Family: Sapotaceae) is an important medicinal plant having various ethnopharmacological uses. It is commonly known as Sofeda or Sobeda in Bengali, Sapota or Chikku in Hindi, Simaiyiluppai in Tamil, Sapotasima in Telugu, Sapotille or Sapodilla in French and American bully in English. It is cultivated throughout Bangladesh and India, though it is native to Mexico and Central America. [5] The major constituents isolated from leaves of *M. zapota* are lupeol acetate, oleanolic acid, apigenin-7-O-α-L-rhamnoside, myricetin-3-O-α-L-rhamnoside and caffeic acid. [6] In traditional system of remedies the leaves of the plant are used to treat cough, cold and diarrhea. [7] The leaves also have analgesic potential [8] and antihyperglycemic, hypocholesterolemic activity. [6] Bark is used as tonic and the decoction is given in diarrhea, dysentery and peludism. [5, 7] The bark of the *M. zapota* is also traditionally used for the treatment of gastrointestinal disorder, fever and pain [9] and also against inflammatory condition. [10]

Although *M. zapota* (Family: Sapotaceae) has traditionally been used in the treatment of infectious diseases, oxidative stress, no scientific data are available to validate these uses as the plant yet has not been undergone any extensive chemical or pharmacological study. Therefore, the present study was designed to investigate the cytotoxic, antimicrobial, antioxidant, anthelmintic and CNS depressant activities of the crude ethanolic extract and its fractions of the leaves of *M. zapota* and we, here in, report the results of our investigation.

MATERIALS AND METHODS

Collection and identification of plant material: Fresh leaves of *M. zapota* used in this study were collected from Curzon Hall, Dhaka University campus, Bangladesh during the month of February 2012 at the flowering stage. The plant samples were identified and authenticated by experts in the Bangladesh National herbarium Mirpur, Dhaka. A voucher specimen (accession No: DACB 37661) was deposited there for future reference. The leaves of *M. zapota* were freed from any of the foreign materials. The plant parts, after cutting into small pieces, were sun dried for several days. The plant materials were then oven dried for 24 h for better grinding. The dried cutting pieces were pulverized by a mechanical grinder and stored into an air tight container.

Extraction of the plant material and sample preparation

About 1.0 kg of the powdered sample was taken in a clean, round bottomed flask (5 L) and soaked in 4 L of 95% ethanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 39ºC with a Heidolph rotary evaporator. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from the *M. zapota* was 50 g. The crude ethanolic extract was partitioned successfully by three solvents of different polarity such as petroleum ether, carbon tetra chloride and ethyl acetate respectively by the modified kupchan partition method. [11]

Chemicals and reagents

Vincristine sulfate, used as a standard drug in cytotoxicity assay was collected from the Techno Drugs Limited, Bangladesh. Ethanol supplied by Laboratory Patterson Scientific, U.K. was used as solvent. Dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich, India was used as solvent to dissolve the extracts. Sodium Chloride Crystal GR from Merck Ltd., Mumbai, India was used to prepare sea water in brine shrimp lethality bioassay. 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) was from Nacalai Tesque Inc. Kyoto, Japan.

Phytochemical screening: The freshly prepared crude ethanolic extracts of leaves were qualitatively tested for the presence of alkaloids, phenols, tannins, reducing sugar, flavonoids, steroids, terpenoids and saponins by using standard phytochemical procedures. [12, 13]

Test for Cytotoxic activity: The cytotoxicity assay was conducted using brine shrimp lethality test according to the method described by Meyer *et al*. [14] Ten brine shrimp matured shrimps were applied to each of all experimental vials and control vial. The mortality of brine shrimp was observed after 24 h of treatment for each of the concentrations. An approximate linear correlation was observed**,** when logarithm of concentration versus percentage of mortality was plotted and the values of LC_{50} were calculated by Microsoft Excel program. Vincristine sulfate was used as positive control.

Test for Antimicrobial activity: Antimicrobial screening was performed using disc diffusion method. **[15]** 8 mg of samples from different extract were dissolved in methanol to obtain desired concentration in aseptic condition. Sterilized filter paper discs were taken in a blank petridis under laminar hood. Then discs were soaked with solutions of test samples and dried. Standard Ciprofloxacin (30 µg/disc) discs were used as positive control and blank discs were used as negative control. The sample discs, standard antibiotic discs and control discs were placed gently on marked zones in the agar plate's pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4ºC for about 24 h to allow sufficient diffusion of materials from discs to surrounding agar medium. The plates were then inverted and kept in an incubator at 37ºC for 24 h. The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. Both gram positive and gram negative organisms were taken for the test.

Test for Antioxidant activity

DPPH free radical scavenging activity. The ability of ethanolic extracts and different fractions of *M. zapota* to scavenge 1, 1-diphenyl-2- picryl-hydrazyl (DPPH) free radicals was estimated by the Brand-Williams method. **[16]** Butylated hydroxytoluene (BHT) was used as reference standards. The DPPH solution without sample solution was used as control and 95% methanol was used as blank. Inhibition of free radical DPPH in percent (I %) was calculated as follows: $(I\ \%) = (1- A_{sample}/A_{blank}) \times 100$

Where A_{blank} is the absorbance of control reaction (containing all reagents except the test material) and A_{sample} is the absorbance of sample material.

Lower absorbance values show higher free radical scavenging activity. The 50% inhibitory concentration value (IC_{50}) was indicated as the effective concentration of the sample that was required to scavenge 50% of the DPPH free radicals.

Test for Anthelmintic activity: The anthelmintic activity was performed according to the method of Ghosh *et al* on the adult earthworm *Pheritima posthuma*. [17, 18] Albendazole, the standard drug, with concentration 10 mg/ml was poured into petri dishes. Ethanolic extracts of plants were diluted with normal saline to obtain 25, 50 and 100 mg/ml concentrations. Normal saline (0.9% NaCl) alone served as the negative control. All these dilutions were poured into the petri dishes accordingly. Six groups of earthworms (n = 6) were taken for the study. Earthworms, nearly equal sizes (about 8 cm), were placed in each petri dish at room temperature. Time for paralysis was noted down when no movement of any sort could be observed, except when the worms were shaken vigorously. Time of death for worms was recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water (50°C). The paralysis time and lethal time were recorded in terms of min.

Test for Central nervous system depressant activity: The ethanolic extract of the leaves of *M. zapota* was assessed for effect on the CNS using phenobarbitone-induced sleeping time test in mice. [19] Thirty min after the oral administration of crude ethanolic extract, petroleum ether, ethyl acetate fraction (200 mg/kg), vehicle control (1% Tween-80 solution in saline, 5 mL/kg) and intraperitoneal injection of diazepam (1 mg/kg), all mice were injected with phenobarbitone (40 mg/kg, i.p.). The animals were observed for the latent period (time between phenobarbitone administration to loss of righting reflex) and duration of sleep (time between the loss and recovery of righting reflex).

Statistical analysis: All values were expressed as the mean ± standard error of the mean (SEM) and the results were analyzed statistically by one way analysis of variance (ANOVA) followed by Dunnett's "t" test by using SPSS Ver.16. $P<0.05$ was considered to be statistically significant.

RESULTS

Phytochemical screening: In preliminary phytochemical screening, the ethanol extract of leaves of *M. zapota* demonstrated the presence of alkaloids, flavonoids, tannins, saponins and glycosides. (Table 1)

Table 1: Results of different chemical group tests of the ethanolic extract of *M. zapota* leaves.

Plant Extract	Alkaloid	Flavonoids	Tannins	Saponins	Quinones	Anthocyanins	Glycosides
EE	+	+	+	+	–	–	+

EE: Ethanolic extract of *M. zapota*; +: Positive result; –: Negative result

Cytotoxic activity

In Brine shrimp lethality assay, the LC_{50} values of the crude ethanolic extract, the petroleum ether, carbon tetrachloride and ethyl acetate soluble fraction of the ethanolic extract were found to be 1.92 g/ml, 10.25 g/ml, 12.65 g/ml and 5.04 g/ml respectively compared to the value exhibited by Vincristine sulfate (LC_{50} 0.544 µg/ ml) (Fig. 1).

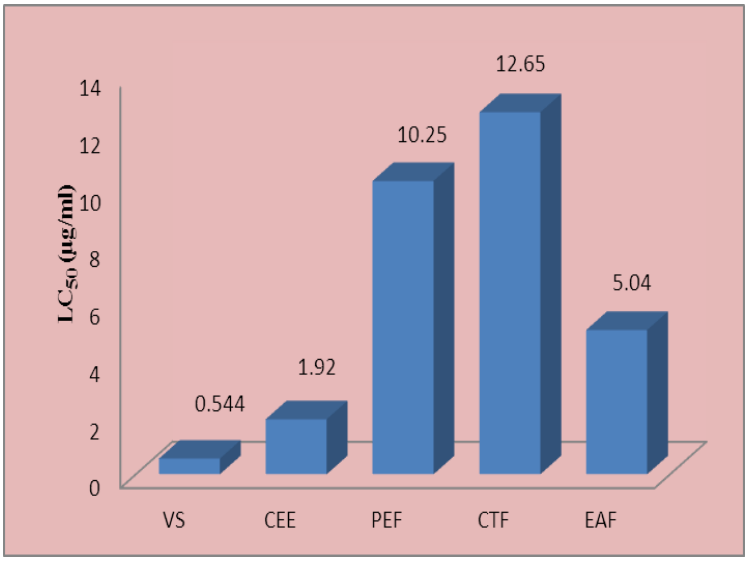

Fig. 1: LC_{50} values of the different extractives of *M. zapota* leaves. VS: Vincristine sulfate, CEE: Crude ethanolic extract, PEF: Petroleum ether fraction, CTF: Carbon tetrachloride fraction, EAF: Ethyl acetate fraction

Antimicrobial activity: In antimicrobial activity screening by disc diffusion method, crude ethanolic extract (CEE) and ethyl acetate fractions (EAF) showed promising inhibitory activity (zone of inhibition is 10-11 mm) against microbial growth. Here, Ciprofloxacin was used as a standard. The result is shown in Table 2.

Table 2: Measurement of zone of inhibition for different experimental samples.

Test Microorganism	Diameter of zone of inhibition				
	CEE	PEF	CTF	EAF	Ciprofloxacin
Gram positive bacteria					
Bacillus cereus	9	-	-	9	40
Bacillus megaterium	9	-	-	9	45
Bacillus subtilis	8	-	-	9	42
Sarcina lutea	8	-	-	10	42
Staphylococcus aureus	8	-	-	10	42
Gram negative bacteria					
Escherichia coli	9	-	-	10	46
Pseudomonas aeruginosa	9	-	-	11	43
Salmonella paratyphi	8	-	-	10	41
Salmonella typhi	8	-	-	9	45
Vibrio parahemolyticus	9	-	-	9	42
Shigella boydii	8	-	-	10	42
Shigella dysenteriae	8	-	-	10	46
Vibrio mimicus	8	-	-	10	45
Fungi					
Aspergillus niger	8	-	-	9	45
Candida albicans	8	-	-	9	45
Sacharomyces cerevacae	8	-	-	9	46

Table 2: Diameter of zone of inhibition of the different extractives of *M. zapota* leaves. CEE: Crude ethanolic extract, PEF: Petroleum ether fraction, CTF: Carbon tetrachloride fraction, EAF: Ethyl acetate fraction. '-' means no zone of inhibition

Antioxidant activity

The antioxidant activity of the different fractions of *M. zapota* was measured on the basis of its DPPH scavenging activity. In this experiment, highest scavenging was observed with ethyl acetate fraction of crude extract with an IC_{50} value of 8.28 µg/ml as opposed to the IC_{50} value of BHT (15.46 µg/ml), which is a well-known antioxidant. Scavenging of DPPH radical was found to rise with increasing concentration of the extracts.

The IC_{50} values of the ethanolic extract and its partitionates have been furnished in the Fig. 2.

Anthelmintic activity: The effect of ethanolic extract of *M. zapota* leaves at different conc. (mg/ml) to paralyze and cause death to earthworm to evaluate in vitro anthelmintic activity were observed as follows is shown in Table 3. Here, the extract didn't show any promising anthelmintic property.

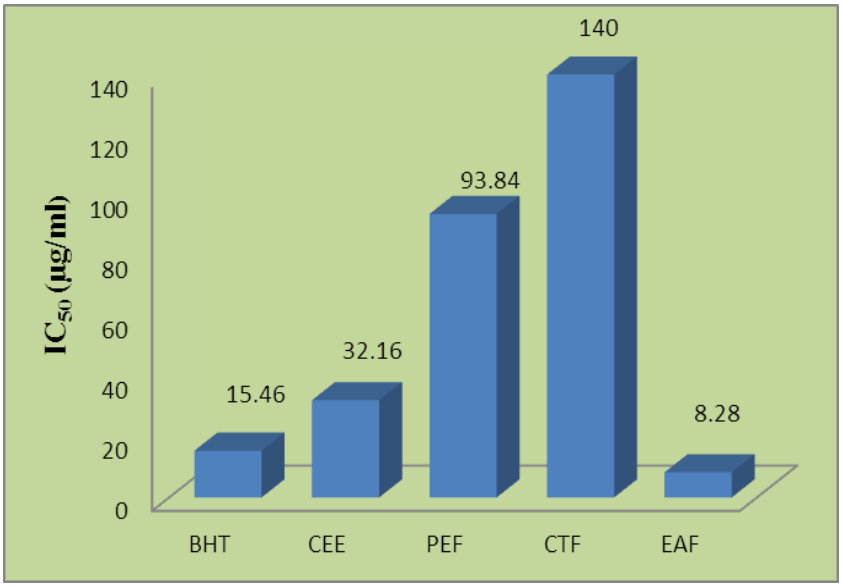

Fig 2: IC_{50} values of the standard BHT and different extracts of *Manilkara zapota* leaves. BHT: Butylated hydroxyl toluene, CEE: Crude ethanolic extract, PEF: Petroleum ether fraction, CTF: Carbon tetrachloride fraction, EAF: Ethyl acetate fraction

Table 3:

Test sample	Conc. (mg/ml)	Time taken for paralysis (min)	Time taken for death (min)
Ethanolic extract	25	77.17± 1.046	88.17±1.28
	50	67.50±1.176	80.00±1.77
	100	63.00±1.713	73.17±2.37
Albendazole	10	4.297± 0.234	6.49±0.31
Normal saline (Control)	-	-	-

Results are expressed as Mean ± SEM (n=6). **P<0.001, *P<0.05 compared to standard (albendazole). Control worms were alive up to 24 h of observation.

CNS depressant activity

In Phenobarbitone induced sleeping time test, the extract at a dose of 200 mg/kg significantly ($p<0.001$) prolonged the duration of sleeping time (93.0 min) in test animals as compared to control (33.2 min) which was comparable to that of standard drug diazepam (101.6 min; $p<0.001$). Petroleum ether and Ethyl acetate fraction also increased duration of sleep. But the extract did not significantly reduce the sleep latency at the same dose. The overall result of CNS depressant property of *M. zapota* is shown in Fig 3.

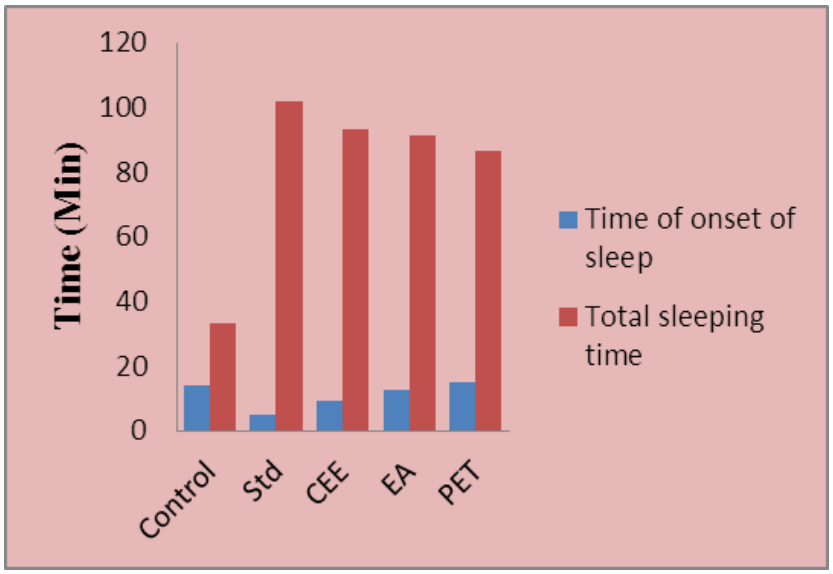

Fig 3: Phenobarbitone induced sleeping time of *M. zapota* leave extract

Control = Normal saline, Std = Standard Diazepam 1mg/kg, CEE = Crude ethanolic extract (200 mg/kg), EAF = Ethyl acetate fraction (200 mg/kg), PEF= Petroleum ether fraction (200 mg/kg).

DISCUSSION

Historically, natural products have served as a source for cancer chemotherapy agents. The brine shrimp lethality bioassay has been used routinely in the primary screening of the crude extracts to assess the toxicity towards brine shrimp. The bioassay has a good correlation with cytotoxic activity in some human solid tumors and with pesticidal activity. [20] This in-vivo lethality test has been successively employed for providing a frontline screen that can be backed up by more specific and more sophisticated bioassays once the active compounds have been isolated. Here, the crude ethanolic extract and ethyl acetate fraction demonstrated potent cytotoxic activity with LC_{50} values of 1.92 g/ml and 5.04 g/ml respectively compared to the value exhibited by Vincristine sulfate (LC_{50} 0.544 µg/ ml). The potent cytotoxic activity may be due to the presence of alkaloids, glycosides, saponins in the extract as demonstrated by phytochemical screening.

Plants are also the natural reservoir of many antimicrobial agents. In recent times, to overcome microbial resistance, the researchers are trying to investigate the antimicrobial activity of medicinal plants.[21] In antimicrobial activity evaluation by disc diffusion method, crude ethanolic extract and ethyl acetate fractions showed promising inhibitory activity (zone of inhibition 10-11 mm) against microbial growth. Though their antimicrobial actions are not so strong, the interesting matter was that they have a broad spectrum of antimicrobial activity.

So the isolation of pure compounds from the ethanolic extract and ethyl acetate fractions will lead to the development of novel antimicrobial agents.

In this study, the leaves of *M. zapota* also showed significant antioxidant activity in DPPH scavenging assay. Ethyl acetate fraction of the extract showed highest scavenging potential with IC_{50} value of 8.28 µg/ml which is even better than BHT (IC_{50} value 15.46 µg/ml), a well-known antioxidant. It has been determined that the antioxidant effect of plant products is mainly due to radical-scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins. [22] So, it can be assumed that the observed antioxidant property of *M. zapota* leaf is due to the presence of flavonoid type compounds.

This study also established the mild CNS depressant property of the leaves of *M. zapota* in phenobarbitone induced sleep method. The extract increases the duration of sleeping time in experimental animals. This action may be due to the presence of compounds which can induce sedation or hypnosis in animals by potentiating the GABA mediated postsynaptic inhibition through an allosteric modification of GABA receptors. [23]

CONCLUSION

The overall results of the present study indicate the promising cytotoxic, antimicrobial, antioxidant and CNS depressant properties of the leaves of *M. zapota* which deserves further investigation to isolate the bioactive constituents responsible for these activities and to establish the mechanism of action. Currently, phytochemical investigation to isolate bioactive pure compounds is in progress in our laboratory. The results of the present study provided a scientific support for the use of *M. zapota* in the treatment of cancer, tumor, infectious disease and oxidative stress.

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DISCLOSURE OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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