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Antioxidant Activity of the Ethanol Extract of *Manilkara zapota* Leaf

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Abstract

The present study evaluated the antioxidant activity of cold ethanolic extract of *Manilkara zapota* (Sapotaceae) leaves. *In vitro* antioxidant activity was determined using 1, 1-diphenyl-2-picrylhydrazyl radical, reducing power capacity, total phenol and flavonoid content. The extract demonstrated significant dose dependent antioxidant activity *in vitro* methods. In DPPH radical scavenging assay IC_{50} values of *Manilkara zapota* leaves (MZL) and ascorbic acid (standard) were found to be 68.27 and 16.17 µg/ml, respectively. *In vivo*, the extract was evaluated by carbon tetrachloride (CCl_4) induced liver damage rats in hepatoprotective model. CCl_4 produced significant alteration of serum marker enzymes, total bilirubin, total protein and liver weight. Restoration of these values towards normal, which is comparable to control group, indicated hepatoprotective activity, which reflects the antioxidant potential of the extract. Results presented here indicate that MZL possess strong antioxidant activity and they can therefore be used as a good natural source of antioxidant.

Keywords: MZL; DPPH; Scavenging activity; Serum marker enzymes.

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1. Introduction

A common theme, which underlines etiology of several degenerative disorders, is free radical stress. The production of free radicals is inextricably linked to the inflammatory process. Free radicals prime the immune response, recruit inflammatory cells and are innately bactericidal [1, 2]. Some of these free radicals play a positive role *in vivo* such as energy production, phagocytosis, regulation of cell growth and intercellular signaling and synthesis of biologically important compounds [3]. However, free radicals are very detrimental in attacking lipids in cell membranes and also DNA, inducing oxidations that cause membrane damage such as membrane lipid peroxidation and a decrease in

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membrane fluidity and also cause DNA mutation leading to cancer [4]. A potent scavenger of these free radical species may serve as a possible preventive intervention for free radical mediated diseases [5]. Recent studies showed that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plants or crude extracts exert potent antioxidant actions [6-10]. Antioxidant action has also been reported to play a crucial role in the hepatoprotective capacity of many plants, such as *Curcuma longa, Phoneix dactylifera, Punica granatum, Phyllanthus niruri, and Solanum nigrum* (11-15). Ayurveda, an indigenous system of medicine in India, has a long tradition of treating liver disorders, with plant drugs. Thus search for crude drugs of plant origin with antioxidant activity has become a central focus of study of hepatoprotection.

The *Manilkara zapota* is a plant of Sapotaceae family, which is abundantly found in Bangladesh. MZL has not been studied much for significant chemical as well as biological studies; the fruits of this plant were reported to contain polyphenolic compounds that showed antioxidant activity [16]. The plant has been used in the indigenous system of medicine for the treatment of various ailments. Decoction of the bark used for diarrhea and fever. An infusion of the young fruits and the flowers is drunk to relieve pulmonary complaints and fever. Leaf decoction used for fever, hemorrhage, wounds and ulcers. The crushed seeds have a diuretic action and are claimed to expel bladder and kidney stones and effective in rheumatism. For neuralgia, leaves with tallow or oil, applied as compress to the temples [17]. The present work has been designed to evaluate the antioxidant potential of extracts from the leaves of *M. zapota* and to explore the basis for its use as a preventive intervention for free radical mediated diseases.

2. Materials and Methods

2.1. *Chemicals*

DPPH (1, 1-diphenyl, 2-picrylhydrazyl), TCA (trichloroacetic acid), ferric chloride, Gallic acid and Quercetin were obtained from Sigma Chemical Co. USA. Ascorbic acid and Aluminium chloride were obtained from SD Fine Chem. Ltd., Biosar, India. Ammonium molybdate, Methanol, Sodium Phosphate, Concentrated H_2SO_4, Folin-ciocalteu reagent, Sodium carbonate, Potassium Acetate, Mono-Sodium phosphate, Bi-sodium phosphate, Potassium ferricyanide and Trichloro acetic acid were purchased from Merck, Germany.

2.2. *Preparation of plant extract*

Ethanol extract of leaves was used in the present study. The matured leaves were collected in the month of July 2009 from Rajshahi, Bangladesh. The leaves were dried under shade and pulverized in a mechanical grinder. The powder was extracted with ethanol. The mixture was filtered and the filtrate was concentrated in Rotary vacuum evaporator to yield semisolid (7.12 % w/w). The extract was preserved in a refrigerator till further use.

3. Experimental Procedure

All the following experiments were repeated three times and the results averaged.

3.1. *In vitro antioxidant activity*

3.1.1. *DPPH radical scavenging activity*

The free radical scavenging capacity of the extract was determined using stable free radical 1, 1-Diphenyl-2-picrylhydrazyl, (DPPH) [18]. The extract was mixed with 95 % ethanol to prepare the stock solution (5 mg/ml). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and the extract was added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer). Ascorbic acid was used as a reference standard at the same concentration of the extract. The % scavenging of the DPPH free radical was measured by using the following equation:

$$\%\,\text{Scavenging activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100$$

IC_{50} value was determined from the plotted graph of scavenging activity against the different concentrations of the extracts, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. The measurements were triplicates and their scavenging effect was calculated based on the percentage of DPPH scavenged.

3.1.2. *Reducing power capacity*

Reducing power of the extract was evaluated by Oyaizu method [19]. Different concentrations of MZL extract (125, 250, 500, 1000 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1% w/v). The mixture was incubated at 50°C for 20 min. After incubation, 2.5ml of 10% trichlorocacetic acid solution was added to each tube and the mixture was centrifuged at 3000 rpm for 10 minutes. 5 ml of the upper layer solution was mixed with 5 ml of distilled water and 1 ml of ferric chloride solution (0.1% w/v) and the absorbance was measured at 700 nm. The reducing power of the extract was linearly proportional to the concentration of the sample. Ascorbic acid was taken as reference standard. Phosphate buffer (P^H 6.6) was used as blank solution.

3.1.3. *Estimation of total phenolic compounds*

Total phenol content in the extract was determined with Folin-Ciocalteu reagent [20]. Extract (200 µg/ml) was mixed with 400 µl of the Folin-Ciocalteu reagent and 1.5 ml of

20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 hrs. Then the absorbance at 765 nm was determined. The concentration of total phenol content in MZL was then determined as mg of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph.

3.1.4. *Estimation of total flavonoid content*

The total flavonoid content was determined using a method previously described by Kumaran and Karunakaran [21]. 1 ml of plant extract in ethanol (200 μg/ml) was mixed with 1 ml aluminium trichloride in ethanol (20 mg/ml) and a drop of acetic acid, and then diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 1 ml of plant extract and a drop of acetic acid, and then diluted to 25 ml with ethanol. The total flavonoid content was determined using a standard curve of quercetin (12.5-100 μg/ml) and expressed as mg of quercetin equivalent (QE/gm of extract).

3.2. ***In vivo antioxidant study***

3.2.1. *Preparation of test sample*

Three different doses (150, 300 and 600 mg/kg body weight) were selected for *in vivo* antioxidant activity. The stock solution was prepared by dissolving 0.36 gm of the extract of MZL in 4.8 ml of distilled water. Then 0.2, 0.4 and 0.6 ml of the stock solution per 100 gm rat were given orally so that the dose would be 150, 300 and 600 mg/kg body weight, respectively.

3.2.2. *Animals*

Male Wister albino rats (150 gm) were used for the present study. They were purchased from ICDDR, B and placed in plastic cages with mesh bottoms in a room temperature. Prior to the commencement of the experiment, all the rats were acclimatized to the new environmental condition for a period of one week. They were maintained with 12h light and dark cycles and fed on a standard pellet diet supplied from ICDDR, B and fresh drinking water *ad libitum*.

3.2.3. *Experimental design*

The hepatoprotective activity of MZL extract was determined by using carbon tetrachloride induced hepatotoxic rat model. After seven days of acclimatization, the rats were divided into four groups each comprising of three rats and treatment was done for 8 days.

Group I : Normal control (0.9% normal saline; 1 ml/kg i.p.)
Group II : CCl_4 control (CCl_4: liquid paraffin (1:2); 1ml/kg i.p.)
Group III : CCl_4 + MZL (300 mg/kg/day; p.o)
Group IV : CCl_4 + standard drug Silymarin (25 mg/kg/day; p.o)
Group II-IV: Received CCl_4 in liquid paraffin (1:2) (1.0 ml/kg i.p.) once in every 72 h.

After 24 hrs of the last dose, blood was withdrawn from retro-orbital plexus under sodium phenobarbital anesthesia and the rats were dissected to isolate liver. Before collecting of the blood, the syringe was ringed with heparin to prevent haemolysis/clotting. The blood samples were then centrifuged at 2500 rpm at 37°C to separate serum and were used for estimation of the biochemical markers of liver damage viz. SGOT, SGPT [22], ALP [23], Bilirubin [24] and total protein levels [25].

3.3. *Statistical analysis*

Linear regression analysis was used to calculate the values wherever needed. All the results are shown as average ± S.E.M. Data was statistically evaluated by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test using instat software. $P < 0.05$ was considered as statistically significant.

4. Results

4.1. *In Vitro Antioxidant Activity*

4.1.1. *DPPH radical scavenging activity*

The free radical scavenging activity of MZL has been evaluated by using the DPPH free radical. The antioxidant quality of an extract is determined by the IC_{50} value. The result of the DPPH scavenging activity of *M. zapota* leaves extract is shown in Fig. 1. The extract exhibited DPPH radical scavenging activity with IC_{50} values of 68.27 µg/ml compared to ascorbic acid (IC_{50} 16.17 µg/ml).

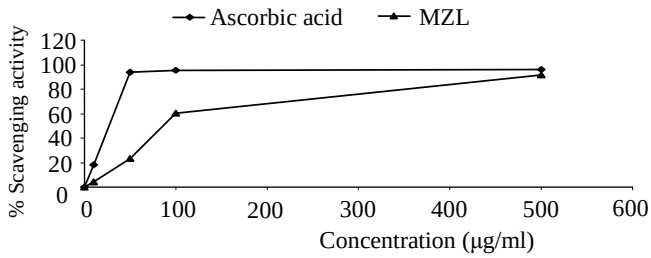

Fig. 1. DPPH radical scavenging activity of the ethanolic extract of *Manilkara zapota* leaves.

4.1.2. *Reducing power capacity*

The extract showed significant reducing power activities as compared to ascorbic acid and proportionally increased with the increasing concentration of the extract, which is shown in Fig. 2. Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample.

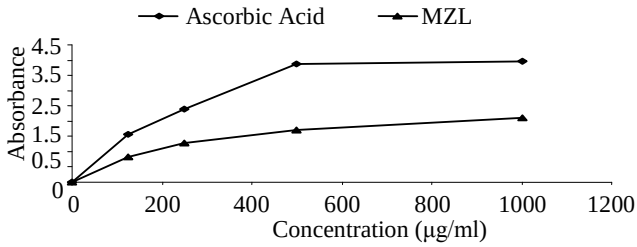

Fig. 2. Reducing power of *M. zapota* leaves and ascorbic acid.

4.1.3. *Estimation of total phenolic compounds*

The Folin-Ciocalteu reagent was used to estimate total phenols present in the extract and the value was expressed as Gallic Acid Equivalents (GAE). As shown in Fig. 3, it was found that total phenolic content of the sample, calculated on the basis of the standard curve for gallic acid, was 89.67 ± 3.074 mg gallic acid equivalent /gm of MZL extract.

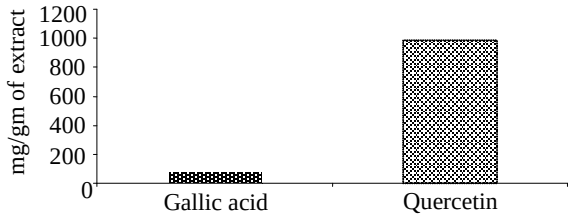

Fig. 3. Total phenol and flavonoid content of *M. zapota* leaves represented as equivalent of gallic acid and quercetin, respectively.

4.1.4. *Estimation of total flavonoid content*

The amount of total flavonoids determined by spectrophotometer is shown in Fig. 3. The total flavonoid contents of MZL was 984.13 ± 31.39 mg of quercetin per gm of extract.

4.2. *In vivo antioxidant study*

Biochemical parameters (SGOT, SGPT, ALP, total protein, total bilirubin) and liver weight are shown in Table 1. The level of SGPT, SGOT, ALP, total bilirubin, total protein and liver weight were restored towards the normal value in MZL at dose of 300 mg/kg body weight and silymarin treated carbon tetrachloride intoxicated rats was the index of their hepatoprotective effects. At higher dose the rats were died and lower dose the values of the biochemical parameters were same like CCl_4 treated rats and the data are not shown here.

Table 1. Effect of ethanolic extract of *M. zapota* leaves and Silymarin on serum biochemical parameters in CCl_4 induced liver damage in rats[a].

Treated Group	Serum biochemical parameters					Liver weight (g)
	SGPT (U/L)	SGOT (U/L)	ALP (KA)	Bilirubin (mg/dL)	Total protein (mg/dL)	
Normal control	22.1±0.33	38.8±0.39	19.26±0.0 4	1.06±0.016	12.14±0.181	5.96± 0.18
CCl_4	74.3±0.51$^{\#}$	85.4±0.39$^{\#}$	68.12±0.122$^{\#}$	5.68±0.027$^{\#}$	7.67±0.064$^{\#}$	9.57±0.31$^{\#}$
MZL	39.77±0.6*	63.44±0.34*	45.38±0.036*	4.32±0.04*	8.88±0.06*	7.29±0.36*
Silymarin	26.8±0.33*	46±0.34*	24.47±0.125*	2.33±0.036*	11.08±0.082*	6.98±0.42*

[a]Values are mean±S.E.M; $n = 3$ in each group; Drug treatment was done for 8 days; $^{\#}P < 0.001$ CCl_4 treated group compared with normal group; $^{*}P < 0.05$ Experimental groups compared with control group, where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test.

5. Discussion

Antioxidants fight free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms. The leaves extract of *M. zapota* showed potent *in vitro* antioxidant activity, in comparison to the known antioxidants, such as ascorbic acid.

The DPPH test provided information on the reactivity of test compounds with a stable free radical. Because of its odd electron, 2, 2-Diphenyl-Picryl Hydrazyl radical (DPPH) gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). The efficacies of anti-oxidants are often associated with their ability to scavenge stable free radicals [26]. The ethanol extract showed DPPH radical scavenging activity with IC_{50} values 68.27 μg/ml compared to vitamin C (IC_{50} 16.17 μg/ml). Although the IC_{50} value of MZL was greater than reference antioxidant (vitamin C) it was comparable to other plant with good antioxidant activity (*Centella asitiaca*, 0.2 mg mL^{-1}; *Pisonia alba*, 0.18 mg mL^{-1}; *Orthosiphon stamineus*, 0.21 mg mL^{-1}; *Mentha arvensis*, 0.22 mg mL^{-1};

Ocimum basilicum, 0.19 mg mL^{-1}) [27, 28]. So the result indicates that MZL have bioactive constituents which act as hydrogen donor to stabilize free radical.

The reducing power of the plant extract components might serve as a significant indicator of its potential antioxidant activity [29, 30]. With regards to reducing capacity, higher reducing powers might be attributed to higher amounts of total phenolic and flavonoid and the reducing power of a compound may reflect its antioxidant potential [31]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Zhang *et al.* [32] mentioned that ethanolic extract of areca seed may have the highest amounts of reductones and polyphenolics. The reducing capacity of MZL was investigated by Fe^{3+}-Fe^{2+} transformation. Presence of reductones causes the reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} form. Reducing power indicates compounds that are electron donors, which can act as primary and secondary antioxidants [33].

Flavonoids possess an ideal structure for the scavenging of free radicals, since they present a number of hydroxyls acting as hydrogen-donators, which makes them important antioxidant agents [34, 35]. The key role of phenolic compounds as free radical scavengers is emphasized in two important reports [36, 37]. It has been long recognized that plant flavonoids possess antioxidant activity, with considerable beneficial effects on human nutrition and health; their mechanisms of action are believed to be through scavenging or chelating process [38, 39]. MZL showed significantly inhibition percentage (stronger hydrogen-donating ability) positively correlated with total phenolic content. Therefore, phenolic components of MZL may contribute directly to antioxidant action in this study.

CCl_4 mediated hepatotoxicity was taken here as the experimental model for liver injury. It has been established that CCI_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P-450 dependent mono-oxygenases to form a trichloromethyl free radical ($CCI_3^{\cdot}$). This then readily interacts with molecular oxygen to form trichloromethyl peroxy radical ($CCl_3OO^{\cdot}$). These free radicals bind covalently to cellular proteins or lipids or abstract a hydrogen atom from an unsaturated lipid, thereby initiating lipid peroxidation and consequently leading to liver damage.

The leaves extracts of *M. zapota* offering the hepatoprotection at dose of 300 mg/kg body weight with respect to different liver marker enzymes (SGOT, SGPT and ALP) and total bilirubin. The rat's death at high dose may be due to the toxicity of the extract at this dose. Restoration of the elevated levels of serum enzymes towards the near normal value in case of leaves extracts as well as standard Silymarin treated groups is the indication of stabilization of plasma membrane and repair of hepatic tissue damage that occurred by CCl_4. The reduction of the level of total proteins in CCl_4 challenged animals (Table 1) is attributed to the damage produced localized in the endoplasmic reticulum which results in the loss of P-450 leading to its functional failure with a decrease in protein synthesis. The rise in protein levels in the treated groups suggests the stabilization of endoplasmic reticulum leading to protein synthesis. The CCl_4 induced a significant increase in liver weight, which is due to blocking of secretion of hepatic triglycerides in plasma [40].

6. Conclusion

The MZL extract demonstrated potential in vitro and in vivo antioxidant activities and moderate cytotoxic property. If this study is extended for assessment of antioxidant activities after isolation of pure compounds some useful drugs may develop out of the research. Hence, there is a need to purify and characterize the individual components present in the leaf extract responsible for the scavenging activity of DPPH radical. However, further investigations are necessary to find out their ability to protect various diseases that are usually developed by reactive oxygen species or free radicals.

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