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PHYTOCHEMICAL SCREENING AND *IN VITRO* ANTIOXIDANT STUDIES ON ACETONE EXTRACT OF *MANILKARA ZAPOTA* L. SEEDS

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ABSTRACT: The present study was aimed to investigate the phytochemical compounds and antioxidant activity of acetone extract of *Manilkara zapota* L. seeds. Preliminary phytochemical screening was carried out with the solvents of different polarities and among them acetone extract was selected for further studies. The antioxidant activity of the acetone extract was evaluated by seven different *in vitro* methods namely: DPPH, FTC, TBA, Metal chelation assay, hydroxyl radical scavenging activity, phosphomolybdenum assay, FRAP. Among them, the acetone extract showed good DPPH radical scavenging activity; IC_{50} value 380 µg /ml and was compared to the standard α-tocopherol. The amount of lipid peroxidation was high in initial stages when compared to the later stages when assessed using FTC and TBA assay. The seed extract did not show good hydroxyl radical scavenging activity when compared to the other antioxidant evaluation techniques. In conclusion, the results presented here imply that *Manilkara zapota* seeds could be considered as nutraceutical/functional foods.

INTRODUCTION: Antioxidants are substances or nutrients in our foods which can prevent or slow the oxidative damage to our body. When our body cells use oxygen, they naturally produce free radicals (by-products) which can cause damage. Antioxidants act as "free radical scavengers" and hence prevent and repair damage done by these free radicals. Health problems such as heart disease, macular degeneration, diabetes, cancer etc., are contributed by oxidative damage [1].

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Antioxidants have been detected in a number of agricultural and food products including cereals, fruits, vegetables and oil seeds [2, 3].

Manilkara zapota L. belongs to the family *Sapotaceae.* It is an evergreen, glabrous tree, 8-15 m in height. It is cultivated throughout India, though it is native to Mexico and Central America. The seeds are aperients, diuretic tonic and febrifuge. Bark is antibiotic, astringent and febrifuge. Chicle from bark is used in dental surgery. Fruits are edible, sweet with rich fine flavour.

The present study aims to determine the following four things from the acetone extracts of *Manilkara zapota* L. seeds:

(1) Qualitative analysis of phytochemicals and

(2) Determination of antioxidant activity using various *in vitro* assays such as DPPH radical scavenging activity, metal chelation assay, phosphomolybdenum assay, hydroxyl radical scavenging activity, FTC, TBA and FRAP assays.

MATERIALS AND METHODS:

Collection of seed material: Fresh seeds of *Manilkara zapota* L. were collected in the month of February from the fields located in Chennai, Tamil Nadu.

Preparation of *M. zapota* seed extract: The seeds were carefully washed with tap water, rinsed with distilled water, and air-dried for 1 hour. Then it was cut into small pieces & dried in room temperature for one week. Then they were ground into powder and stored in room temperature. Direct extraction with chloroform, acetone and methanol was used as an extraction method[4] .In this method, finely ground material (1 gm) was extracted with 10 ml of chloroform, acetone and methanol in conical flask in shaking condition. The extract was decanted in to pre-weighed glass vials. The process was repeated 3 times and the same material but using fresh solvent. The solvent was removed by condensation. The extracted residues were weighed and re-dissolved in different solvents to yield 10mg/ml solutions for further analysis.

Chemicals: Analytical grade chemicals supplied by Hi-Media (Mumbai), Merck, India and Sigma Chemicals (USA) were used. 2, 2-diphenyl-1picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide reduced (NADH), gallic acid, ascorbic acid, quercetin, aluminium chloride, thiobarbituric acid (TBA), trichloroacetic acid, tris-HCl, acetone, methanol, chloroform, ferric chloride, Mayer's reagent, sulphuric acid, hydrochloric acid, benzene, ammonia, ethanol, linoleic acid, ammonium thiocyanate, ferrous chloride, α-tocopherol, ethylene diamine tetra acetic acid (EDTA), dimethyl sulphoxide (DMSO) ,glacial acetic acid, acetyl acetone, ferrozine, sodium phosphate, ammonium molybdate, Folin-Ciocalteu's reagent, sodium carbonate, potassium acetate, hexane, sodium chloride.

Qualitative Analysis of Phytochemicals: The following tests were performed on the extracts to detect various phytoconstituents present in them [5, 6];

(1) **Alkaloids (Mayer's Test):** 0.5 g of the extract was stirred with few ml of dilute hydrochloric acid and filtered. To a few ml of filtrate, one or two drops of Mayer's reagent were added to the sides of the test tube. A white creamy precipitate demonstrated the test as positive.

(2) **Steroids (Salkwoski's Test):** 0.5 g of the extract was dissolved in 2ml of chloroform. Sulphuric acid was then carefully added to form a lower layer. A reddish-brown colour at the interface indicated the presence of steroids.

(3) **Triterpenoids (Hishorn's Test):** 0.5g of the extract was dissolved in 2ml of chloroform. The mixture was heated for 10min, after the addition of 2ml trichloro acetic acid. The change of yellow colour to red indicates the presence of triterpenoids.

(4) **Flavonoids (Ferric Chloride Test):** 0.5 g of the extract was boiled with distilled water and then filtered. To 2 ml of the filtrate, few drops of 10% ferric chloride solution were then added. A green-blue or violet colouration indicated the presence of flavonoids.

(5) **Phenols (Lead acetate Test):** 0.5 g of the extract was treated with lead acetate solution. Formation of precipitate indicated the presence of phenols.

(6) **Glycosides (Salkwoski's Test):** 0.5 g of the extract was dissolved in 2ml of chloroform. Sulphuric acid was then carefully added to form a lower layer. A reddish-brown colour at the interface demonstrated the presence of glycosides.

(7) **Saponin (Frothing Test):** To 1g of the extract about 3 ml of distilled water was added and shaken vigorously for about 5 min. frothing which persisted on warming was taken as an evidence for the presence of saponins.

(8) **Phlobotannins (Aqueous HCl Test):** Deposition of a red precipitate when an aqueous extract was boiled with 1% aqueous hydrochloric acid indicated the presence of phlobotannins.

(9) Anthraquinones (Borntrager's Test): About 0.2 g of each portion to be tested was shaken with 10 ml of benzene and then filtered.5 ml of the 10% ammonia solution was then added to the filtrate. Appearance of a pink, red or violet colour in the ammonical (lower) phase was taken as the evidence for the presence of free anthraquinones.

***In Vitro* Antioxidant Activity Assays:** The following assays were performed to detect the antioxidant activity of the acetone extract of *M.zapota* seeds.

Radical Scavenging Activity (RSA) using DPPH Assay: The RSA activity of the extract was determined using DPPH assay[7]. The decrease in the absorption at 517 nm of the DPPH solution after addition of the seed extract was measured in a cuvette containing 2960 µl of 0.1 mM ethanolic DPPH solution with 40 µl of the plant extract at various concentrations. Blank containing 0.1 mM ethanolic DPPH solution without plant extract, serves as control. The setup was incubated in dark condition at room temperature. The absorption was monitored after 20 min. α-tocopherol was used as standard. The ability to scavenge DPPH radical was calculated by the following equation;

% of DPPH radical scavenging activity (% RSA)

$$= \frac{Acontrol - Asample}{Acontrol} \times 100$$

Where, $A_{control}$ is the absorbance of DPPH radical + ethanol, A_{sample} is the absorbance of DPPH radical + seed extract.

Measurements were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions.

Ferric thiocyanate (FTC) assay: The seed extract of about 4 mg in 99.5% ethanol were mixed with 2.51% linoleic acid in 99.5% ethanol (4.1 ml), 0.05 M phosphate buffer, pH 7 (8 ml) and distilled water (3.9 ml). The above mentioned chemicals were kept in screw cap containers under dark conditions at 40^{o}C. To 0.1 ml of this solution, 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate were added. After 3 min, 0.1 ml of 2 M ferrous chloride in 3.5% Hcl was added to the reaction mixture and the absorbance of the red color was measured at 500 nm each 24 h until one day after absorbance of the control reached maximum. The control and the standard were subjected to the same procedure as the sample except for the control, where there was no addition of sample, and for the standard 4 mg of sample was replaced with 4 mg of α-tocopherol [8].

Thiobarbituric acid (TBA) assay: The same samples as prepared for the FTC method was used in TBA test. To 1 ml of sample solution, 2 ml of 20% aqueous thiobarbituric acid was added. This mixture was then incubated in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and the absorbance of supernatant was measured at 532 nm. Antioxidant activity was recorded based on absorbance on the final day [9].

Hydroxyl radical scavenging activity assay: Various concentrations (50, 100, 150 and 200 µg) of extracts were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%) and 1.0 ml of dimethyl sulphoxide (DMSO) (0.85% v/v in 0.1M phosphate buffer, pH 7.4).The reaction was initiated by adding 0.5ml of ascorbic acid (0.22%) and incubated at 80–90ºC for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1.0ml of ice-cold TCA (17.5% w/v). 3ml of nash reagent (75.0g of ammonium acetate, 3.0ml of glacial acetic acid, and 2ml of acetyl acetone were mixed and raised to 1l with distilled water) was added and left at room temperature for 15min.

The reaction mixture without sample was used as control. Ascorbic acid was used as control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank [10]. The % hydroxyl radical scavenging activity was calculated by the following formula:

$$\% \text{ HRSA} = [(A_0 - A_1)/A_o] \text{ X100},$$

Where, A_0 - absorbance of the control and A_1- absorbance of the extract/standard.

Metal chelation assay: Briefly the extract samples (250µl) were added to a solution of 2mmol/l $FeCl_2$ (0.05ml). The reaction was initiated by the addition of 5mmol/l ferrozine (0.2ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. The above mixture without the seed extract serves as control. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent/g extract. The assay was carried out in triplicate and the mean values with ± SEM are presented [11].

Phosphomolybdenum assay: An aliquot of 100µl of sample solution was combined with 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) in a 4ml vial. The vials were capped and incubated in a water bath at 95ºC for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank [12].

The above mixture without the seed extract serves as control. The formation of green phosphomolybdenum complex of the extracts was evaluated using ascorbic acid as standard. The results were expressed as mg ascorbic acid equivalent/g extract. The assay was carried out in triplicate and the mean values with ± SEM are presented.

Ferric Reducing antioxidant power (FRAP) assay: FRAP reagent (1.8ml) was mixed with 0.2ml of test sample, then incubated at 37ºC for 10min in a water bath. The FRAP reagent contains 20mm TPTZ solution 20mm $Fecl_3.6H_2O$ and 0.3M acetate buffer with pH 3.6.

After incubation the absorbance were measured immediately at 593nm. The above mixture without the seed extract serves as control [22].

Methanolic solutions of known Fe (II) concentration were used as standards. The values were expressed as mmol Fe (II)/g extract. The assay was carried out in triplicate and the mean values with ± SEM are presented.

Statistical analysis: The experimental results were given as mean±SD of three parallel measurements. The experimental values were evaluated by using one-way analyses of variance (ANOVA). P values < 0.05 were regarded as ‘‘significant” .The SPSS 16.0 (Statistical Program for Social Sciences) was used for statistical analysis.

RESULTS AND DISCUSSION: The results of present study are encouraging as the tested seed extract revealed the presence of phytoconstituents with good antioxidant potential.

Qualitative analysis of phytochemicals: Table 1 demonstrates the results for phytochemical screening of three different seed extracts of *M. zapota* namely acetone, chloroform and methanol extracts. The low polar chloroform extract indicated the presence of steroids, glycosides and saponins.

The high polar methanol extract revealed the presence of steroids, phenols, glycosides and saponins. The medium polar acetone extract showed maximum number of phytochemicals such as tannins, flavonoids, alkaloids, phenols, steroids, glycosides and saponins.

Presence of tested secondary metabolites in the acetone extract of *M. zapota* seeds are in line with earlier reports [14]. The phytoconstituents detected in the seeds could be responsible for their antioxidant activity.

Taking this into consideration we choose only the acetone extract for further studies since this indicates maximum number of phytoconstituents.

TABLE 1: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS IN VARIOUS EXTRACTS OF *M. ZAPOTA* SEEDS

Phytochemicals/Extracts	Methanol extract	Chloroform extract	Acetone extract
Alkaloids	-	-	+
Steroids	+	+	+
Triterpenoids	-	-	-
Flavonoids	-	-	+
Tannins	-	-	+
Phenols	+	-	+
Glycosides	+	+	+
Saponins	+	+	+
Phlobotannins	-	-	-
Anthraquinones	-	-	-

***In Vitro* Antioxidant Activity Assays:**

Radical Scavenging Activity (RSA) using DPPH Assay: The measurement of radical scavenging activity of any antioxidant is commonly associated with the usage of DPPH method because it is quick, reliable and reproducible method. It is widely used to test the ability of compounds as free radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts [15].

In the DPPH assay, the antioxidants reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine which has maximum absorption at 517 nm. **Table 2** demonstrates the free radical scavenging activity of the acetone extract. The acetone extract showed DPPH free radical scavenging activity in a concentration range of 220-400 µg/ml and its radical scavenging activity ranged from 22-54%; Whereas the standard α-tocopherol showed DPPH free radical scavenging activity in a concentration range of 20-200 µg/ml and its radical scavenging activity ranged from 18-84%;

The IC_{50} value, a measure of the extract concentration which is required for 50% inhibition of the free radical DPPH, was determined. The IC_{50} value for the acetone extract was 380µg/ml and that of standard α-tocopherol was 80µg/ml. The involvement of free radical, especially their increased production, is a common feature of many dreadful human diseases, including cardiovascular diseases and cancer. Acetone extract of *M. zapota* leaves, showed scavenging activity in a concentration range of 10-35 µg/ml and its inhibition ranged from 23-83% [16].

Table 2: DPPH free radical scavenging activity by acetone extract of *M.zapota* seeds

Standard (α-Tocopherol)		Acetone extract of *M.zapota* seeds	
Concentration (µg/ml)	**% Radical Scavenging Activity**	**Concentration (µg/ml)**	**% Radical Scavenging Activity**
20	18	220	22
40	35	240	25
60	47	260	28
80	50	280	29
100	55	300	36
120	59	320	38
140	66	340	42
160	72	360	46
180	74	380	51
200	84	400	54

Ferric thiocyanate (FTC) assay: Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation [17].

Figure 1 and 2 illustrates that the total antioxidant activity determined by the FTC method was high when compared to the antioxidant activity determined by the TBA method.

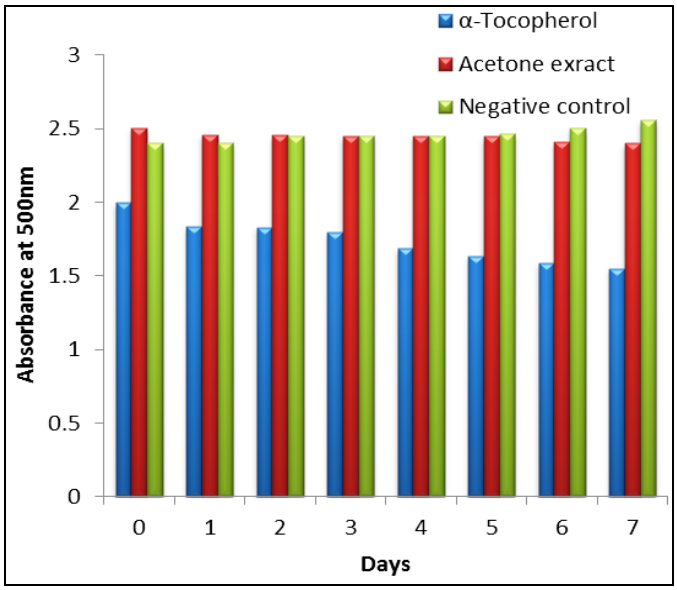

FIGURE 1: ANTIOXIDANT ACTIVITY OF *M. ZAPOTA* SEEDS EXTRACT DETERMINED WITH THE FTC METHOD

TBA Assay: This may indicate that the amount of peroxide in the initial stage of lipid peroxidation is greater than the amount of peroxide in the secondary stage. Furthermore, the secondary product such as malonaldehyde is not stable for a period of time. It will turn into alcohol and acid which cannot be detected by spectrophotometer [18]. The results in **figure 2** indicates that the acetone extract of *M. zapota* seeds exert greater antioxidant activity determined by FTC method.

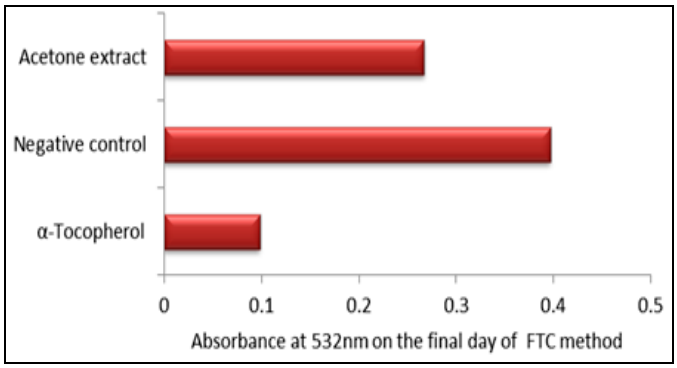

FIGURE 2: ANTIOXIDANT ACTIVITY OF *M. ZAPOTA* SEEDS EXTRACT DETERMINED WITH THE TBA METHOD

The seed extract tested exhibited low absorbance values in the later stages when compared to the initial stages, which indicated a high level of antioxidant activity. The seed extract showed absorbance values greater than the negative controls (without extracts) at the end point, indicating the presence of antioxidant activity.

However, the seed extracts exhibited strong antioxidant activity as determined by both the FTC and TBA methods, surpassing the activity of the standard commercial antioxidant alpha-tocopherol.

Hydroxyl radical scavenging activity assay: Scavenging of hydroxyl radical is an important antioxidant activity because of very high reactivity of the OH radical, enabling it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids, and nucleotides[19]. Thus, removing OH• is very important for the protection of living systems. The acetone extract of *M.zapota* seeds did not show any hydroxyl radical scavenging activity. This is because of the different mechanism of action exhibited by the phytoconstituents present in the acetone extract of *M.zapota* seeds. Similar results were predicted in the leaves of *M.zapota* [16]

Metal chelation assay: Presence of transition metal ions in a biological system could catalyse the Haber-Weiss and Fenton-type reactions, resulting in generation of hydroxyl radicals (OH•). However, these transition metal ions could form chelates with the antioxidants, which result in the suppression of OH• generation, and inhibit ion of peroxidation processes of biological molecules. In this assay, the presence of chelating agents in the extracts of *M. zapota* disrupts the ferrozine–Fe^{2+} complex formation, thus decreasing the red colour. The metal ion scavenging effects of the acetone extract of *M. zapota* seeds was found to be 34.13±0.67 (mg EDTA/g extract) and the data's were presented as the mean ± SD of three measurements. It is reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [20]. The data presented in this study indicated that *M. zapota* seeds have the ability for iron binding and could reduce the generation of hydroxyl radicals.

Phosphomolybdenum assay: Acetone extract of *M. zapota* seeds were used to determine their antioxidant capacities by the formation of green phosphomolybdenum complex. The formation of the complex at 95ºC was measured by the intensity of absorbance (695 nm) in extracts.

The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of green phosphate/Mo (V) complex with the maximal absorption at 695 nm.

In the present study, the phosphomolybdenum reduction potential of acetone extract was found to be 49±0.04 (mg AA/g extract). This may be because of the fact that the transfer of electrons/hydrogen from antioxidants occurs at different redox potential in various assay systems and the transfer also depends on the structure of the antioxidants [21]. Total antioxidant capacity of the *M. zapota* bark was determined by phosphomolybdenum assay using a standard curve of ascorbic acid and value was expressed as ascorbic acid equivalent (AE), which is 462.44 mg AE/gm of extract [22].

Ferric Reducing antioxidant power (FRAP) assay: Antioxidants, explained as reductants and inactivation of oxidants by reductants, are involved in redox reactions in which one reaction species is reduced at the expense of the oxidation of another antioxidant. The antioxidant potential of various extracts of *M. zapota* were estimated from their ability to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex and the results are expressed as concentration of substance having ferric-TPTZ reducing ability equivalent that of 1 mmol concentration of Fe(II). The ferric reducing antioxidant power of the acetone extract of *M.zapota* seeds was found to be 168.36± 13.06 (mmol Fe (II)/ g extract). The presences of total phenolics in the seeds corresponds to the FRAP value. Hence they should be able to donate electrons to free radicals in actual biological or food systems also, making the radicals stable.

CONCLUSION: The results of the present study indicate that acetone extract of M. zapota seeds are high in phenolic and steroidal contents through preliminary screening and these extracts exhibit strong antioxidant activities.

The scavenging activities observed against DPPH, FTC as well as metal chelation assay lead us to propose *M. zapota* seeds as good natural source of antioxidants suitable for application in nutritional/ pharmaceutical fields, in the prevention of free radical- mediated diseases. Therefore, it is suggested that these seeds could be used as an additive in the food industry providing good protection against oxidative damage.

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