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Antioxidant activity of *M. zapota* and *C. limon* seeds

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Abstract

Seed extracts of *Citrus limon* and *Manilkara zapota* were analyzed for their antioxidant activity, free radical scavenging activity, and lipid peroxidation inhibition capacity. Total phenolic and total flavonoid contents were also estimated, antioxidant activity was found to be correlated to the former. Chloroform-methanol extract of *M. zapota* exhibited the highest total antioxidant capacity among all samples tested. The chloroform-methanol solvent mixture proved most efficient in extracting phenolic constituents.

Key words: Antioxidant; Free radical; *Manilkara zapota*; *Citrus limon*; Total phenolics; Total flavonoids

1. Introduction

Free radical species and antioxidants both play role in maintaining redox equilibrium in the body. Apart from their role in the diseased conditions in the body, reactive oxygen species (ROS) are also known to have a role in the spoilage of food by autoxidation of lipids, enzymatic oxidation, during storage and processing in fats, oils, and fat-containing foods [12]. Human body does not synthesize overwhelming amount of antioxidants to compensate with the damaging effects of ROS. Synthetic antioxidants have been criticized due to possible toxic effects, low solubility along with moderate antioxidant activity. Hence there arises a need to discover new potential natural sources of antioxidants. Present study was made on the seeds of *Citrus limon* (lemon) and *Manilkara zapota* (chikoo), which belongs to family Rutaceae and Sapotaceae respectively. *C. limon* is known to have antioxidant and anticancer activity [18]. Though antioxidant activity of methanol extract of *C. limon* seeds has been reported earlier [17], as all components of a seed can not be extracted with a single solvent, we have evaluated extracts of *C. limon* seeds prepared in different solvents. As any assay alone cannot provide the complete picture of the antioxidant activity of a given sample [7], this study subjected the analytes to multiple methods for assaying antioxidant activity, besides determining their total phenol and flavonoid content. Several studies have correlated the antioxidant activity of plant samples with their total phenolic contents [3, 5, 6, 8].

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2. Materials and Methods

2.1. Extract preparation

Seed extracts were prepared by microwave assisted extraction (MAE) technique. One gram of seed powder was added to 50 mL of respective solvent and pre-leached for 1 min. Five different solvents used were hexane, acetone, chloroform:methanol (2:1), ethanol (50%) and water. The extracts obtained after microwave heating (at 720 W, for 300, 120, 50, 70, 180 second in hexane, acetone, chloroform:methanol, ethanol, and water respectively, with intermittent cooling for avoiding overheating) were cooled, centrifuged at 10,000 rpm for 15 min and filtered through Whatman #1 filter paper (Whatman International Ltd., England). The supernatant were evaporated at the respective boiling points of the solvents. Dried extracts were then reconstituted in respective solvents.

2.2. Antioxidant Assays

2.2.1. Total antioxidant activity by molybdate assay

Method followed was that described by Pilar *et al* (1999) [15]. Standard curve was prepared using concentrations of gallic acid in the range 0.5-13 mM. O.D. was measured at 695 nm.

2.2.2 Ferric thiocyanate (FTC) method

Method as described by Kikuzaki and Nakatani (1993) was followed [11]. Butylated hydroxy toluene (BHT) was used as positive control, while a mixture without sample was used as negative control. The blank contained ethanol instead of linoleic acid. O.D. was measured at 500 nm.

2.2.3 Thiobarbituric acid (TBA) method

The method as described by Ottolenghi (1959) was followed [14]. The assay was performed on the day when the absorbance of the negative control used for the FTC method reached maximum. O.D. was measured at 532 nm. Both FTC and TBA method were used to determine the total antioxidant activity according to the following formula.

Total antioxidant activity = (Absorbance of control on day maximum / Absorbance of sample on the same day) × 100

2.2.4. DPPH radical-scavenging activity

The antioxidant activity of the extracts was measured on the basis of the scavenging activity of the stable 2,2'-diphenyl-2-picrylhydrazyl (DPPH) free radical, as described by Duan *et al* (2007) [1, 4]. The radical scavenging activity was calculated in terms of Ascorbic Acid Equivalent Antioxidant Capacity (AEAC) by using the following formula

$AEAC = A_{control} - A_{sample} / A_{control} - A_{ascorbic\ acid} \times$ concentration of ascorbic acid × (mg/mL) × volume × 100/g of sample

Where, A is Absorbance (at 517 nm).

2.2.5. Trolox® Equivalent Antioxidant Capacity (TEAC) assay

The $ABTS^+$ stock solution (7 mM) was prepared [13] using potassium persulphate as the oxidizing agent. The working solution of ABTS radical was prepared by diluting the stock solution in ethanol to give absorption of 0.70 ± 0.02 at 734 nm. Each sample was tested for two different concentrations. Trolox® was used as a standard. The curves of % change in absorbance vs. conc. of ABTS were plotted for each sample as well as for Trolox®, for each min.% change in absorbance was calculated as,

% change in absorbance = (Initial absorbance of $ABTS^+$ radical (734 nm)–New mean absorbance of ABTS radical (734 nm)/ Initial absorbance of ABTS+ radical (734 nm) x 100

Table 1. Results of different assays for *C. limon* and *M. zapota* seed extracts

Seed	Extract prepared in	Total antioxidant activity (g GAE/g of dry extract)	Lipid peroxidation inhibition*		DPPH radical scavenging activity (g AEAC / 100 g of dry extract)	ABTS scavenging assay (TEAC)	Total phenolic content (gGAE/g of dry extract)	Total flavonoid content (mg QE / g of dry extract)
			FTC assay	TBA assay				
	Hexane	413.90 ± 35.23	120.77 ± 2.9	45.31 ± 1.9	106.75 ± 24.9	0.008 ± 0.002	70.23 ± 2.92	144.72 ± 10.3
	Acetone	533.06 ± 38.7	136.16 ± 9.8	78.9 ± 8.72	215.34 ± 23.7	0.0007 ± 0.0002	313.06 ± 13	4.32 ± 1.3
C. limon	Chloroform-Methanol	766.27 ± 54	296.17 ± 0.8	268.88 ± 0.68	371.23 ± 30.1	11.92 ± 0.02	219.62 ± 62.9	15.65 ± 5.8
	Ethanol	977.85 ± 185.3	978.94 ± 184	1273.68 ± 194.6	148.14 ± 2.8	0.148 ± 0.03	137.83 ± 9.7	24.42 ± 0.81
	Water	1820.96 ± 40.20	349.62 ± 15.62	474.50 ± 36.32	4663.20 ± 187.67	0.010 ± 0.003	165.86 ± 8.32	12.38 ± 0.47
	Hexane	18.81 ± 0.52	1271.42 ± 180	120.01 ± 3.65	104.59 ± 5.98	0.016 ± 0.012	48.9 ± 0.47	19.84 ± 0.32
	Acetone	5.87 ± 0.8	164.81 ± 43	114.89 ± 52	377.67 ± 87	0.013 ± 0.007	174.5 ± 5.56	25.8 ± 3.9
M. zapota	Chloroform-Methanol	4171.25 ± 192.68	251.59 ± 67.40	91.86 ± 1.29	150.55 ± 53	0.009 ± 0.003	378.7 ± 31.20	10.53 ± 3.30
	Ethanol	194.01 ± 47	1056.81 ± 188.83	691.42 ± 62.12	672.37 ± 60.23	0.014 ± 0.009	115.89 ± 4.52	7.2 ± 1.40
	Water	424.99 ± 29.65	161.73 ± 38.56	20.19 ± 6.30	256.86 ± 66.80	0.002 ± 0.001	1.005 ± 0.007	7.26 ± 1.86

*For FTC and TBA assays the limit (range) of absorbance applied was 0.01-0.93, which were the values for positive control and negative control, respectively. GAE: Gallic acid equivalent, AEAC: Ascorbic acid equivalent antioxidant capacity, QE: Quercetin equivalent, TEAC: Trolox® equivalent antioxidant capacity, DPPH·: 2,2'-diphenyl-2-picrylhydrazyl, TBA: Thiobarbituric acid, ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), FTC: Ferric thiocyanate.

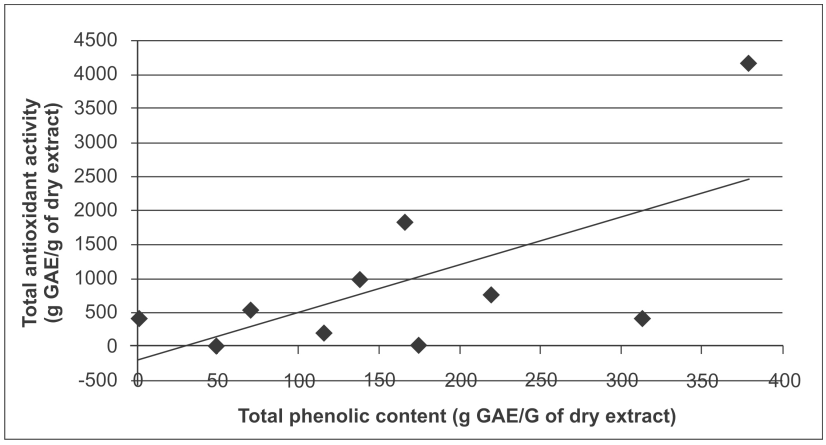

Fig. 1. Correlation of total phenolic content with antioxidant activity

The antioxidant activity was calculated in terms of TEAC by dividing slope of the curve of the sample by that of Trolox® for the same time.

2.2.6. Estimation of total phenolic content

Folin-Ciocalteu method was used to determine total phenolic content of the sample [16]. The calibration curve was prepared by employing gallic acid at concentrations of 0.4 to 1.6 mM. O.D. was measured at 765 nm.

2.2.7. Estimation of total flavonoid content

Aluminium chloride colorimetric method was used for flavonoids determination [2]. The calibration curve was prepared by using quercetin at concentrations of 12.5 to 100 µg/mL in methanol. O.D. was measured at 415 nm. All the chemicals used were of analytical grade.

2.2.8. Statistical analysis

Results are presented as mean value ± standard deviation. Statistical significance between experimental results was evaluated with a Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant. Value of correlation coefficient (r) for all the standard curves used was > 0.95.

3. Results

Results of various assays for all the extracts are summarized in table 1.

4. Discussion

Chloroform-methanol extract of *M. Zapata* seeds followed by water extract of *C. limon* showed maximum antioxidant activity. As evident from Table 1, water extracts exhibit significant total antioxidant activity, indicating notable contribution of polar phytochemicals in it. BHT was found to give higher lipid peroxidation inhibition activity than any of the sample extract. BHT is known to give 100% lipid peroxidation inhibition in both FTC and TBA assays [6]. The highest antioxidant activity in FTC assay was exhibited by acetone extract of *M. zapota* followed by its ethanol extract (Table 1). The highest & lowest antioxidant activity in second stage of lipid peroxidation as measured by TBA method was measured in ethanol and acetone extracts of *C. limon* respectively. Ethanol and water extracts of both the seeds exhibited high antioxidant activity against second stage of lipid peroxidation suggesting contribution of polar phytoconstituents in inhibiting lipid peroxidation. Antioxidant activity in FTC method is higher than that of TBA method in all of the extracts except water and ethanol extracts of *C. limon*.

In case of *C. limon*, water extract, whereas in case of *M. zapota*, ethanol extract showed the highest DPPH radical scavenging activity. Again high DPPH scavenging activity in water extracts and low activity in hexane extracts suggest that polar phytochemicals are largely responsible for scavenging DPPH. Except the chloroform-methanol extract of *C. limon* no other extract was found to possess significant $ABTS^+$ radical scavenging activity. The amount of total phenolics varied widely and ranged from 1.005 to 378.7 g GAE/g dry extract in case of *M. zapota* seeds and 70.238 to 313.08 g GAE/g dry material in *C. limon* seeds. Maximum total phenolic content was found to be present in chloroform-methanol extract and acetone extract of *M. zapota* and *C. limon* seeds respectively. Least presence of phenolics was observed in water extract of *M. zapota* and hexane extract of *C. limon* seeds. This makes it difficult to comment whether polar or non polar solvent extract phenols better as hexane and water lie on two opposite extremes of polarity. Chloroform-methanol mixture seems to be efficient in extracting phenols from both the seeds.

The flavonoid content was obtained in the range of 4.325 to 144.720 mg QE/g of dry extract in

C. limon seeds, and 7.2 to 25.8 mg QE/g of dry extract in *M. zapota* seeds. Highest flavonoid content was recorded in hexane extracts of *C. limon*. Acetone extract of *C. limon* seeds exhibited lowest, whereas acetone extract of *M. zapota* seeds exhibited highest total flavonoid content, which makes it difficult to comment on ability of acetone for extracting flavonoids - which are polar phytochemicals [9] - from the seeds.

Values of total antioxidant activity of the extracts were plotted against values of their total phenolic content (Fig 1). Correlation coefficient (r) was 0.651, which indicates a positive linear correlation between phenolic content and antioxidant activity. This correlation is more positively linear (r = 0.86) in case of *M. zapota* seeds alone. Antioxidant activity of many fruits and vegetables have been shown to be correlated with their total flavonoid content [10]. However in this study no such correlation appeared to exist. In case of *C. limon* seeds, a negative linear correlation was found between total flavonoid and total phenolic contents (r = -0.75), which indicates possibility of the presence of such flavonoids which do not have phenolic ring structure.

From the results discussed above it may be assumed that majority of the phytochemicals in the seed extracts under question which contribute to their total antioxidant activity may belong to the phenol group of secondary plant metabolites, but not to the flavonoid category. Further it becomes clear that flavonoids are not contributing significantly to the total phenolic content of the seeds investigated.

Seed extracts of *M. zapota* and *C. limon* were found to have good antioxidant activity, lipid peroxidation inhibition activity as well as DPPH free radical scavenging capacity, but not so for ABTS radical. This study has identified, chloroform-methanol extract of *M. zapota*, water, chloroform-methanol and ethanol extract of *C. limon* as strong antioxidant, strong DPPH radical scavenger, strong lipid peroxidation inhibitor and strong $ABTS^{+}$ radical scavenger, respectively. Such findings can contribute to the increasing database of the medicinal plants and may be of importance in varietal improvement, food preservatives, nutraceuticals, cosmetics and biopharmaceuticals in a race with the degenerative diseases.

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