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ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND FLAVONOID CONTENT OF *MORINDA CITRIFOLIA* FRUIT EXTRACTS FROM VARIOUS EXTRACTION PROCESSES

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Abstract

Soxhlet, Ultrasonic extract of *Morinda* citrifolia L. fruit and four extracts from high pressure extraction at 10 MPa using ethanol, ethyl acetate as solvent and dried by vacuum oven and spray dryer were analyzed for their antioxidant activity by peroxide value method and diphenylpicrylhydrazyl radical scavenging method. The five extracts along with the reference samples, butylated hydroxyl toluene and tannic acid were further analyzed to determine their total phenolic content by Folin-Ciocalteau method and total flavonoid content by Dowd method. The *M. citrifolia* extract by high pressure extraction with ethyl acetate as solvent and spray dried was found to exhibit highest antioxidant activity and total flavonoid content. High total phenolic content was determined in the high pressure extract using ethyl acetate as solvent and vacuum dried. It was interesting to note that ultrasonic extract exhibited significant antioxidant activity, total phenolic and flavonoid content. High pressure extracted *M. citrifolia* in ethanol was found to express lesser values comparatively. The significant difference in activity among the high pressure extracts was found to be due to the polarity of the solvents used for extraction as *M. citrifolia* fruit contains relatively larger quantity of non-polar antioxidant compounds. It was also found that the drying methods had significant impact on the antioxidant activity, total phenolic and flavonoid content of the extracts.

Keywords: Phytochemical Antioxidant, Reactive Oxygen Species, Radical Scavenging Activity, Peroxide Value, High Pressure Extraction, Ultrasonic Extraction

1. Introduction

Plants such as herbs have been used in folk medicine for centuries in most of the cultures throughout the world. *Morinda citrifolia* L. native to Polynesia is one of the traditional folk medicinal plants that have been used for over 2000 years by polynesians for treating diabetes, high blood pressure, cancer, eye problems and many other illnesses [1].

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress [2]. Oxidative stress plays a role in heart diseases, neurodegenerative diseases, cancer and in the aging process [3]. This concept is supported by increasing evidence that oxidative damage plays a role in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this and lower risk of disease [4]. Antioxidants are the substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance [5].

Apart from their role of health benefactors, antioxidants are added in foods to prevent or delay oxidation of food, initiated by free radicals formed during their exposure to environmental factors such as air, light and temperature [6]. At present most of the antioxidants are manufactured synthetically. They belong to the class of synthetic antioxidants. The main disadvantage with the synthetic antioxidants is the side effects when taken in vivo [7]. Strict governmental rules regarding the safety of the food has necessitated the search for alternatives as food preservatives [8].

Plants are the potential source of natural antioxidants. Natural antioxidants or phytochemical antioxidants are the secondary metabolites of plants [9]. Carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols, tocotrienols etc., are some of the antioxidants produced by the plant for their sustenance. Beta-carotene, ascorbic acid and alpha tocopherol are the widely used antioxidants [10]. *Morinda citrifolia* L. fruit contains a number of antioxidants such as beta-carotene, ascorbic acid, terpenoids, alkaloids, beta-sitosterol, carotene, polyphenols such as flavonoids, flavone glycosides, rutin etc. [1]. Easily cultivable *Morinda citrifolia* L. with its wide range of antioxidants can be a major source of natural or phytochemical antioxidants.

Research has revealed the potential of *Morinda citrifolia* L. as source of various antioxidants in roots, fruits and leaves [11]. Yet, there is no data on the antioxidant activity, total phenolic content and total flavonoid content of the extracts from *Morinda citrifolia* L. fruit obtained by various processes of extraction using various solvents and drying methods.

The objectives of the present study were to determine the antioxidant activity, total phenolic content and total flavonoid content of the extracts from *Morinda citrifolia* L. fruit by ultrasonic extraction and high pressure extraction (HPE) with ethanol and ethyl acetate as solvents followed by drying in vacuum oven and spray dryer. Two methods namely peroxide value method and DPPH radical scavenging method were used to find and correlate the antioxidant activity of the extracts. In the peroxide value method the extracts were added to the peanut oil stored in an oven at

60 ± 0.5 ºC and oxidative deterioration (formation of peroxides) of the oil was measured for 16 days. Butylated Hydroxy Toluene (BHT) was used as reference. In DPPH radical scavenging method the free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used to find the antioxidant (scavenging) activity of various concentration of the extracts. Tannic acid as natural antioxidant and BHT as synthetic antioxidant were used for comparison. The total phenolic content of the extracts was estimated by Folin-Ciocalteau test and total flavonoid content by Dowd method respectively.

2. Material and Methods

2.1 Chemicals

Chloroform, methanol, ethanol and ethyl acetate (HPLC grade) were obtained from J.T. Baker (USA). Butylated Hydroxy Toluene, Folin and Ciocalteau reagent, Tannic acid and catechin were obtained from sigma Chemicals (St. Louis, USA). 2, 2-Diphenyl picryl hydrazyl (DPPH), Na_2CO_3, $Alcl_3$ were from Fluka Chemie, Germany. Acetic acid from May & Baker Ltd. (Dagenham, England), Sodium thiosulphate from Ajax chemicals (Auburn, Australia) and Potassium Iodide from Hannschweile lab chemicals (GmbH, Germany) were obtained respectively.

The fresh and double refined peanut oil was bought from Tirupathi oil mills, India. It contained no synthetic antioxidants (peroxide value (PV) 0.47 meq / kg). 10 kgs of *Morinda citrifolia* L. fruit before ripening stage were obtained from kota kinabalu market, Sabah, Malaysia.

2.2 Preparation of plant extracts

The *Morinda citrifolia* L. fruit was tap washed followed by washing with distilled water. The fruit was cut and dried at 29 ºC. The dried fruit was then finely powdered (100 – 500 µm). To about 60 grams of the sample, 0.5 litre of ethanol was added and ultrasonic extraction at a frequency of 24 KHz was performed for time duration of 4 hours. The supernatant was then separated from the residue by filtration using whatman no.4 filter paper and dried in Jeiotech OV-02 vacuum oven maintained at 130 mm Hg and 40 ºC.

To the second portion of 60 grams, 2 litres of ethanol were added and subjected to high pressure extraction in a parr 4842 series high pressure reactor with nitrogen gas at 10 MPa and 60 ºC for 48 hours with continuous stirring at 60 rpm. The supernatant was then filtered and divided into two parts. One part was dried in Jeiotech OV-02 vacuum oven maintained at 130 mm Hg and 40 ºC. The other part was spray dried in labplant SD-05 spray drier at an air pressure of 1.4 bar and operating temperature of 149 ºC.

To another portion of 60 grams of the dried *Morinda citrifolia* L., 2 litres of ethyl acetate were added and the above procedure of high pressure extraction was repeated. One part of the filtered supernatant was dried in vacuum oven at 120 mm Hg and 40

ºC. The other part dried by spray dryer at an air pressure of 1.4 bar and operating temperature of 145 ºC.

The five extracts were preserved in Stable Temp vacuum oven, Model 282A at 0 mm Hg and 22 ºC.

2.3 Peroxide value determination of antioxidant activity

Extracts of about 0.2 % of the oil weight were added to the 50 g peanut oil. The samples were kept in an incubator maintained at 60 ºC with constant stirring. A blank sample was prepared under the same conditions, without adding any additives. Synthetic antioxidant, BHT was used as reference substance for comparative purposes. The rate of autoxidation of peanut oil was estimated according to the increase of its peroxide value by the peroxide value method described by American oil chemist's society [12].

The changes in induction period (IP) of oil after the addition of each extract were determined. The IP was considered as the number of days needed for the peroxide value (PV) of the sample to reach the value of 20 meq of O_2 / kg of fat. This is in agreement with a general consideration that oils become rancid at peroxide values higher than 20 [6].

Protection factor (PF) values of peanut oil and antioxidant activities (AA) of the extracts were calculated by the following formulas:

$$PF = \frac{IP_A}{IP_B} \tag{1}$$

$$AA = \frac{IP_A - IP_B}{IP_{BHT} - IP_B} \tag{2}$$

where, IP_A - induction period of sample with additive, days; IP_B - induction period of sample without additive, days; IP_{BHT} - induction period of sample with added synthetic antioxidant BHT, days. The following scale is proposed for the PF values: 1.0 - 1.5 (very low), 1.5 - 2.0 (low), 2.0 - 2.5 (medium), 2.5 - 3.0 (high) and > 3.0 (very high). PF is defined as a stability value with additive divided by that of the blank sample [8].

2.4 DPPH radical scavenging activity method

The DPPH free radical method is based on the determination of the concentration of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) at steady state in a methanol solution, after adding the mixture of antioxidants. DPPH absorbs at 515 nm, and as its concentration is reduced by the existence of an antioxidant, the absorption gradually disappears with time. A PerkinElmer UV–VIS lambda 25 spectrophotometer was used and the quantity of the mixture of antioxidants needed to reduce by 50 % the initial DPPH concentration was evaluated [13]. This characteristic parameter is called efficient

concentration (EC_{50}) or oxidation index. The lower the EC_{50}, higher is the antioxidant activity of the examined compound.

The DPPH radical- scavenging activity in terms of percentage was calculated according to the following equation [14].

$$\text{DPPH scavenging activity (\%)} = \{1 - (\text{Abs}_{515}\ \text{sample} / \text{Abs}_{515}\ \text{DPPH solution})\} \times 100\ \% \qquad (3)$$

2.5 Total phenolic content

The total phenolic content of the extracts were determined by the Folin-Ciocalteau method with some modifications [15]. 5 grams per 50 mL of sample was filtered with whatman no.1 paper. 0.5 mL of the sample was added to 2.5 mL of 0.2 N Folin-Ciocalteau reagent and placed for 5 minutes. 2 mL of 75 g/L of Na_2CO_3 were then added and the total volume made upto 25 mL using distilled water. The above solution was then kept for incubation at room temperature for 2 hours. Absorbance was measured at 760 nm using 1 cm cuvette in a perkin-elmer UV-VIS lambda 25 spectrophotometer. Tannic acid (0 - 800 mg/L) was used to produce standard calibration curve. The total phenolic content was expressed in mg of Tannic acid equivalents (TAE) / g of extract.

2.6 Total flavonoid content

The total flavonoid content was determined using the Dowd method [16]. 5 mL of 2 % aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (0.4 mg/mL). Absorption readings at 415 nm using PerkinElmer UV-VIS lambda 25 spectrophotometer were taken after 10 minutes against a blank sample consisting of a 5 mL extract solution with 5 mL methanol without $AlCl_3$. The total flavonoid content was determined using a standard curve with catechin (0 - 100 mg/L) as the standard. Total flavonoid content is expressed as mg of catechin equivalents (CE) / g of extract.

2.7 Statistical analysis

Data are reported as mean of three determinations. The results obtained were statistically analyzed with the Student's t-test using a significance level of $P < 0.05$. Microcal origin (version 6.0) was used for graph plotting.

3. Results and Discussion

The peroxide values of peanut oil with and without antioxidants at 60 ºC are presented in Fig. 1. High pressure extracted *M. citrifolia* in ethyl acetate and spray dried was found to retard the hydroperoxide formation substantially. The addition of this extract to peanut oil lowered the final peroxide value after 16 days from 152, as by blank sample, to 25 meq/kg. This was significantly followed by ultrasonically derived extract, BHT and high pressure extracted *M. citrifolia* in ethyl acetate and vacuum dried. Peroxide value of BHT rose significantly after 11 days. Least effect on the

retardation of hydroperoxide formation was exhibited by the high pressure extracted m.citrifolia fruit extracts with ethanol as solvent.

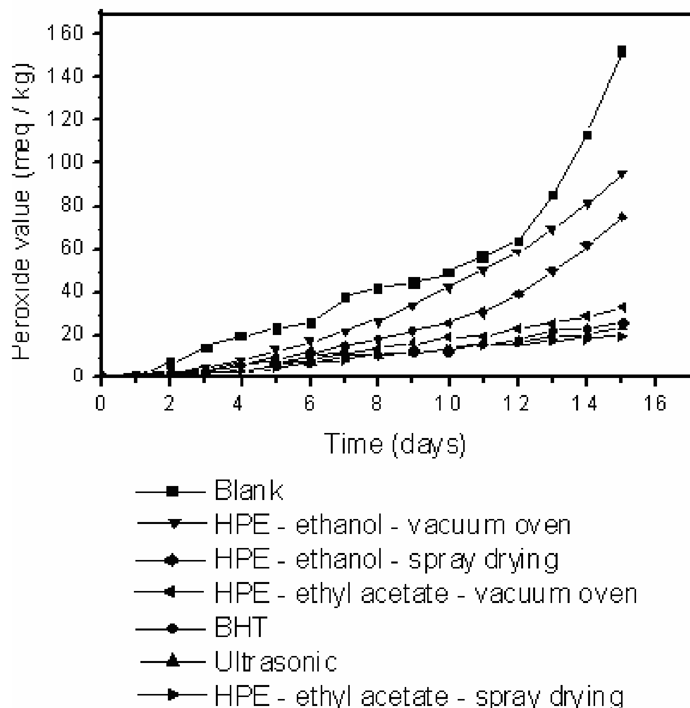

Fig. 1. Peroxide Value Determination of Peanut Oil with and without Extracts.

In Table. 1 the induction period (IP), protection factor (PF) and antioxidant activity (AA) of the extracts (0.20%) are presented. High pressure extracted *M.citrifolia* in ethyl acetate and spray dried exhibited the highest antioxidant activity, protection factor and induction period. Ultrasonically extracted *M. citrifolia* was also found to exhibit values higher than that of BHT. As BHT is a pure compound, while the extracts are complex mixtures containing ineffective substances in terms of their antioxidative activity it could be suggested that *M. citrifolia* fruit contains very strong constituents retarding lipid peroxidation.

The least antioxidant activity was exhibited by the high pressure extracted *M. citrifolia* in ethanol and vacuum dried. The effectiveness of antioxidants under the conditions used is ranged in the following descending order: HPE - $CH_3COOC_2H_5$ - Spray Dried > Ultrasonic > BHT > HPE - $CH_3COOC_2H_5$ - Vacuum Oven > HPE - C_2H_5OH - Spray Dried > HPE - C_2H_5OH - Vacuum Oven. This is in agreement with findings of Mohd. Zin *et al.* (2002) that the *M. citrifolia* fruit contains comparatively higher amounts of non-polar antioxidant compounds. Ethyl acetate used as solvent during high pressure extraction was able to extract these non-polar antioxidants which are mostly of alkaloid nature.

Table 1. IP, PF & AA of Extracts from various Process & Drying Methods.

Additive	Blank	BHT	Ultrasonic-Vacuum oven	HPE-C_2H_5OH Vacuum oven [a]	HPE-C_2H_5OH Spray drying	HPE-$CH_3COOC_2H_5$ Vacuum oven	HPE-$CH_3COOC_2H_5$ Spray drying
Induction Period	4	13	14	7	9	11	15
PF	1	3.25	3.5	1.75	2.25	2.75	3.75
AA	-	1	1.11	0.33	0.55	0.77	1.22

Figure 2 illustrates the antioxidant activity by DPPH radical scavenging method at various concentration of extracts. Steady state absorbance value for pure tannic acid (reference sample) was the least 0.0800, exhibiting the highest antioxidant activity followed by high pressure extracted *M. citrifolia* in ethyl acetate and spray dried. There was no significant difference in antioxidant activity between high pressure extracted *M. citrifolia* in ethyl acetate dried in vacuum oven, BHT and ultrasonically extracted *M. citrifolia*. Higher absorbance and least antioxidant activity were observed in the high pressure extracted sample in ethanol and vacuum dried. The order of antioxidant activity for the extracts was found to be similar to that of peroxide value method.

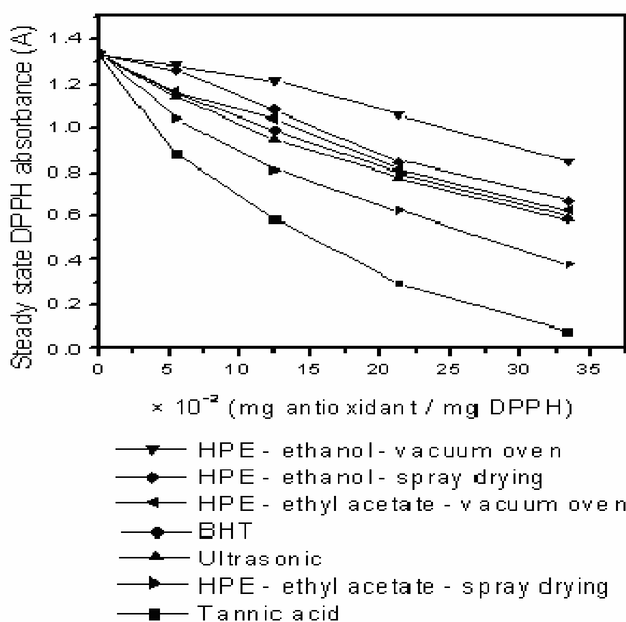

Fig. 2. Absorbance of DPPH after Addition of *M. Citrifolia* Extracts.

Percentage of radical scavenging activity is depicted in Fig. 3. Of the extracts, high pressure extracted *M. citrifolia* in ethyl acetate and spray dried at a concentration of 0.333 mg / mg DPPH, exhibited the highest radical scavenging activity of 58.3 % and the least of 36.6 % by the ethanol extract dried in vacuum oven. In Table 2, EC_{50}, the effective concentration of the extracts (mg antioxidant / mg DPPH) required to scavenge 50 % of DPPH radical are presented. Significant difference in EC_{50} between the vacuum dried and spray dried HPE extracts in ethanol may be due to the different drying methods.

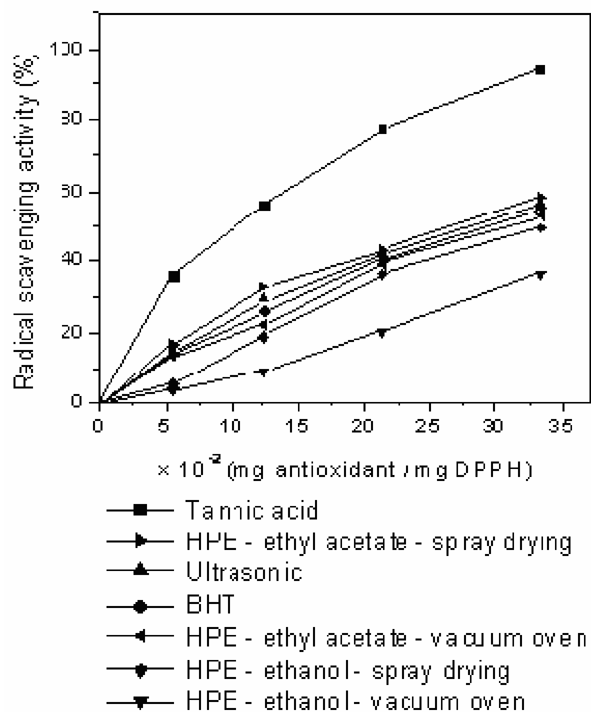

Fig.3. Percentage of Radical Scavenging Activity by *M. Citrifolia* Extracts.

Table 2. EC_{50} of Extracts by DPPH Free Radical Scavenging Method.

Antioxidants	$\times 10^{-2}$ EC_{50} (mg antioxidant /mg DPPH)
Tannic acid	12.5
10Mpa - $CH_3COOC_2H_5$ - Spray drying	26
Ultrasonic	30.75
BHT	31.75
10Mpa - $CH_3COOC_2H_5$ – Vacuum oven	33.5
10Mpa - C_2H_5OH - Spray drying	36.25
10Mpa - C_2H_5OH – Vacuum oven	43

The total phenolic content of the *M. citrifolia* extracts in tannic acid equivalents are presented in Table 3. The highest value was obtained for high pressure extracted *M. citrifolia* in ethyl acetate and vacuum dried and the lowest by the extract with ethanol as solvent and spray dried. It was observed that drying method affected the total phenolic content of the extracts. This may be due to the loss of phenolics through exhaust in spray drier during the drying process.

Table 3. Total Phenolic Content of Ultrasonic & High Pressure Extracts.

Extracts	TAE[a]
HPE- C_2H_5OH-spray drying	13.3
HPE- C_2H_5OH-vacuum oven	16.1
Ultrasonic extract	25
HPE- $CH_3COOC_2H_5$-spray drying	28
HPE- $CH_3COOC_2H_5$-vacuum oven	30

[a] Total phenolic content is expressed as tannic acid equivalents (TAE; mg tannic acid /g of extract).

High pressure extracted *M. citrifolia* with ethyl acetate and spray dried expressed the highest total flavonoid content in catechin equivalents, as shown in Table. 4. This was significantly followed by the high pressure extract in ethyl acetate and vacuum dried. Ultrasonic extract exhibited significant total flavonoid content.

Table 4. Total Flavonoid Content of Ultrasonic & High Pressure Extracts.

Extracts	CE[a]
HPE- C_2H_5OH-spray drying	5
HPE- C_2H_5OH-vacuum oven	6
Ultrasonic extract	10.75
HPE- $CH_3COOC_2H_5$-vacuum oven	11.5
HPE- $CH_3COOC_2H_5$-spray drying	12.3

[a] Total flavonoid content is expressed as catechin equivalents (CE; mg catechin/g of extract.

4. Conclusions

The results of this study showed that the highest antioxidant activity, total phenolic content and total flavonoid content were exhibited by the extracts obtained by high pressure extraction with ethyl acetate as solvent. It has been reported that *M. citrifolia* fruit contains relatively larger quantity of non-polar antioxidant compounds. However, among the ethylacetate extracts spray dried extract exhibited highest antioxidant activity and total flavonoid content and vacuum dried, the highest total

phenolic content. This may be due to the different drying methods used. It is interesting to note that the ultrasonic extracts at 24 KHz in ethanol and vacuum dried exhibited significant antioxidant activity, total phenolic content and flavonoid content comparable to ethyl acetate extracts. Further work is possible on finding the optimum frequency for the separation of phytochemicals from the fruit matrices of *Morinda citrifolia* L. Comparatively, least activity were expressed by the ethanol extracts. It can be said that the polarity of the solvent had significant impact on the extraction of phytochemicals such as antioxidants, phenolics and flavonoids from the fruit matrices of *Morinda citrifolia* L.

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