

Full Length Research Paper

Bioactive compounds, antioxidant, tyrosinase inhibition, xanthine oxidase inhibition, anticholinesterase and anti inflammatory activities of *Prunus mahaleb* L. seed

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Prunus mahaleb L. plant is cultivated for its seeds which are known as a spice. There is lack of information about its biological activities although it has long history of ethnopharmacological application. Therefore, this research was conducted to evaluate the bioactive compounds, antioxidant, tyrosinase inhibition, xanthine oxidase (XO) inhibition, anticholinesterase and anti inflammatory activities of the *P. mahaleb* L. seeds. Results showed that the total phenolic and flavonoid compounds concentration in the methanolic extract were 75.7 ± 0.18 and 28.5 ± 0.37 mg/g dry weight (DW) whereas in the hexane extract were 34.6 ± 0.24 and 16.2 ± 0.31 mg/g dry weight, respectively. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, ferric reducing antioxidant power (FRAP) activity and nitric oxide (NO) scavenging activity of the methanolic extract at the concentration of 100 µg/ml of crude extract were 44.3, 51.9 and 36.9% respectively, while for the hexane extract were 26.8, 32.8 and 21.8%, respectively. Both extracts showed antioxidant activity although they were lower than reference antioxidants (vitamin C, E and butylated hydroxytoluene (BHT)). Ferric thiocyanate (FTC) and thiobarbituric acid (TBA) tests results showed the antioxidant activity of extracts. Methanolic extract in XO, tyrosinase, acetylthiocholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition assays showed the significant ($p < 0.05$) lower IC_{50} with values of 59.8, 21.0, 52.1 and 86.2 µg/ml as compared to the hexane extract with the values of 94.1, 54.2, 85.2 and 110.7 µg/ml. Anti-inflammatory assay using LPS/IFN-γ stimulated RAW 264.7 cells indicated that methanolic extract is a potent source of anti-inflammatory agent. Consequently, the methanolic extracts of *P. mahaleb* seeds possess strong tyrosinase inhibitory activity and moderate antioxidant potential, XO inhibitory, anticholinesterase and anti inflammatory activities.

Key words: *Prunus mahaleb*, phenolics, flavonoids, antioxidant, xanthine oxidase, tyrosinase, anticholinesterase, anti inflammatory.

INTRODUCTION

In the human body, oxidant-antioxidant imbalance impairs cell functions and immunity, and promotes cell death and DNA damage, which can cause mutations and

ultimately contribute towards the development of chronic diseases such as cancer. Food composition and food additives play major role in providing the required antioxidants for the body. Although traditionally spices have been used in food preparations to improve the flavor and taste, today they are also frequently used as antioxidant-food supplements (Karimi et al., 2010). Spices are reported to contain bioactive compounds

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imparting antioxidant, preservative and antimicrobial properties to the food. Several researches have shown that spices containing phenolic and flavonoid compounds indicated antioxidant activities (Hinneburg et al., 2006; Oskoueian et al., 2011c). A positive linear correlation among phenolic and flavonoid compounds with biological activities such as antioxidant, xanthine oxidase (XO), tyrosinase, anticholinesterase and anti inflammatory activities capacity of spices has also been previously reported (Orhan et al., 2007; Cherdshewasart and Sutjit, 2008; Umamaheswari et al., 2009; Oskoueian et al., 2011d). Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been widely used for many years to retard lipid oxidation. However, the safety of using these synthetic antioxidants in food industry has become a concern among scientists and leading to current interest in uncovering natural antioxidants. The seeds of *Mahaleb* which scientifically identified as *Prunus mahaleb* L. have also been identified as a spice with beneficial traits. *P. mahaleb* grows abundantly in the Mediterranean, Southeast Europe and West Asia. Its culinary use is restricted to the South eastern part of Europe (Greece, Armenia) and West Asia (Iran, Turkey, Lebanon, Syria). Studies have shown that cherry plant used in herbal folk medicine to treat diseases such as, blood pressure, diarrhea, anti-bilious material, nausea and anti-kidney stone and also the cream complexion useful for opening and kidney swelling and inflammation and swelling of the stomach and intestines (Mariod et al., 2010). Despite the extensive use of *P. mahaleb* L. seeds as a spice by people living in Mediterranean, Southeast Europe and West Asia, there have been only limited attempts to explore its bioactive compounds, health promoting properties and biological activities in relation to its application as functional food, medicinal purposes and as source of natural bioactive compounds. Therefore, in this investigation into biological activities of this food additive, the total phenolic and flavonoid compounds, antioxidant, XO, tyrosinase, anticholinesterase and anti inflammatory activities of the extracts from this spice was evaluated.

MATERIALS AND METHODS

Plant material

Seed of *P. mahaleb* L. were extracted using methanol and hexane as a solvent and for extraction, the method of Crozier et al. (1977) was followed. An air dried sample (4 g) was weighed and placed in a 100 ml conical flask, and 80% (v/v) methanol (40 ml) were added, followed by an addition of 6 M HCl (10 ml) for the preparation of methanolic extract while another extraction was done by addition of hexane.

The mixture was refluxed for 2 h at 90°C and filtered using Whatman No. 1 filter paper (Whatman, U.K.), followed by evaporation of the filtrate under vacuum using a rotary evaporator (Buchi, Switzerland). The dried crude extract was weighed and dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C for further analysis.

Chemicals

Methanol, hydrochloric acid, Folin-Ciocalteu reagent, sodium carbonate, aluminium chloride, sodium hydroxide, ascorbic acid, alpha-tocopherol, BHT, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and DMSO were purchased from Fisher Scientifics, USA. Dulbecco's Modified Eagle Medium (DMEM), 3-(4,5-dimethylthiazol-2,5-diphenyl)tetrazolium bromide thiazol blue (MTT), foetal bovine serum, phosphoric acid, sulfanilamide, naphtyl ethylene diaminedihydrochloride, N-nitro-L-arginine-methyl ester (L-NAME) N ω-nitro-L-arginine methyl ester, lipopolysaccharide (LPS), tyrosinase, XO and all phenolics and flavonoids standard were purchased from Sigma Aldrich and Interferon gamma (IFN-γ) was purchased from eBioscience, Inc. The other chemicals used in this study were bought from Merck.

Total phenolic content

Total phenolic compounds of *P. mahaleb* extract were determined according to Halici et al. (2005). 0.5 ml of each extract, 2.5 ml Folin-Ciocalteu reagent, 2 ml of 7.5% (w/v) Na₂CO₃ were mixed. The mixture was vortex and incubated at room temperature for 90 min. The absorbance was read using visible spectrophotometer (Novaspec II Visiblespectro) at 765 nm. The results were expressed as mg gallic acid equivalents/g dry weight (DW).

Total flavonoid content

The total flavonoid compounds in the extract was determined according to Gulcin et al. (2004). An aliquot (0.1 ml) of extract was added to 0.3 ml 5% (w/v) NaNO₂ and incubated for 5 min. 0.3 ml 10% (w/v) AlCl₃ and 2 ml 1 N NaOH was added and the total volume was made up to 5 ml with distilled water. The absorbance was measured at 510 nm by using visible spectrophotometer (Novaspec II Visiblespectro) at 510 nm. The results were expressed as mg rutin equivalents/g DW.

Antioxidant activity (1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity)

The free radical scavenging activity of extract was evaluated by DPPH according to Gulcin et al. (2004). Briefly, 0.1 mM solution of DPPH in methanol was prepared and 3 ml of this solution was added to 1 ml of each extracts with different concentrations (100, 150, 200, 250 and 300 µg/ml). The mixture was shaken vigorously for a few second and incubated at room temperature for 30 min. Then the absorbance was measured at 517 nm using a visible spectrophotometer (Novaspec II Visiblespectro). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity which calculated by using the following equation:

$$\text{Percent (\%)} \text{ inhibition of DPPH activity} = \left[\frac{(A_0 - A_1)}{(A_0)} \right] \times 100\%$$

Where A₀ is the absorbance value of the blank sample or control reaction and A₁ is the absorbance value of the test sample. A curve of percent inhibition or percent scavenging effect against samples concentrations was plotted and the concentration of the sample required for 50% inhibition was determined. The assay was carried out in triplicate. BHT, Vitamin C and Vitamin E were used as standard antioxidants.

Ferric reducing antioxidant power (FRAP)

The ferric reducing property of the extracts was determined by

using assay described by Yen and Chen (1995). 1 ml (concentration of 100, 150, 200, 250, and 300 µg/ml) of extract were mixed with 2.5 ml of potassium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1 g/100 ml). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%) was added to the mixture to stop the reaction. Equal volume of distilled water was added followed by 0.5 ml ferric chloride (0.1 g/100 ml) (FeCl₃). The procedure was carried out in triplicate and allowed to stand for 30 min before measuring the absorbance at 700 nm. BHT, Vitamin C and Vitamin E were used as standard antioxidants. The percentage of antioxidant activity in FRAP assay of the samples was calculated according to the formula:

$$\text{Antioxidant activity (\%)} = \left(\frac{A_1 - A_0}{A_1} \right) \times 100\%$$

A₀, Absorbance of the control (potassium phosphate buffer + FRAP reagent); A₁, absorbance of sample.

Nitric oxide (NO) scavenging activity

The nitric oxide (NO) scavenging activity of each plant extract was determined by the method of Tsai et al. (2007). 60 µl of two-fold diluted sample were mixed with 60 µl of 10 mM sodium nitroprusside in phosphate buffered saline (PBS) in a 96-well flat-bottomed plate and incubated under light at room temperature for 150 min. Finally, an equal volume of Griess reagent was added into each well in order to measure the NO content. Ascorbic acid and α-tocopherol were used as controls. The NO scavenging activity was calculated according to the formula: $\left(\frac{A_0 - A_1}{A_0} \right) \times 100\%$; where A₀ was the absorbance of the control reaction and A₁ was the absorbance in the presence of the sample.

Total antioxidant activity assay

Ferric thiocyanate (FTC) test

This test was carried out according to the method described by Ismail et al. (2010). In this study 4 mg of extract was dissolved in 4 ml of methanol followed by mixing with linoleic acid (4.1 ml), 0.05 M phosphate buffer pH 7.0 (8 ml) and distilled water (3.9 ml). This mixture was then kept in screw-cap containers at 40°C in dark. For the FTC test, 0.1 ml of this mixture was respectively added into 9.7 ml of 75% ethanol and 0.1 ml of this mixture was added into 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. After 3 min, the addition of 0.1 ml of ferrous chloride solution (in 3.5% HCl) to the reaction mixture, the absorbance of the samples was read at 500 nm by using a spectrophotometer (Molecular Devices Inc., USA). This procedure was repeated every 24 h until the control sample reached its maximum absorbance value. BHT was used as standard antioxidants in this test.

Thiobarbituric acid (TBA) test

This test was carried out according to the method by Ismail et al. (2010). In brief, 1.0 ml of 20% aqueous trichloroacetic acid and 2.0 ml of 0.67% aqueous TBA were added to 2 ml of sample solutions acquired from FTC test. The mixtures were then placed in boiling water bath for 10 min. After cooling under the running tap water, the mixtures were centrifuged at 3000 g for 30 min. The absorbance of the reaction was measured at 532 nm using a spectrophotometer (Molecular Devices Inc., USA).

Tyrosinase inhibitory activity

The tyrosinase inhibition activity of the extracts was determined

based on Lee et al. (2009). Briefly, the extracts were serially diluted with phosphate buffer (50 mM) in 96-well microtiter plate. Equal volume of tyrosinase (SIGMA) (333 units ml⁻¹) was added into wells. After 5 min incubation at room temperature, L-DOPA (6 mM) was added. The absorbance was measured at 492 nm using spectrophotometer (Molecular Devices Inc., USA).

Xanthine oxidase (XO) inhibitory activity

The XO inhibition was performed based on Orhan et al. (2007). 20 µl XO (0.003 unit/well) dissolved in phosphate buffer (0.1 M, pH = 7.5) were mixed with various concentrations of each sample in 10 µl of DMSO in a 96-well plate and incubated for 10 min at room temperature. The mixture was added to 20 µl of 0.1 mM xanthine. The uric acid formation was measured by a spectrophotometer (Molecular Devices Inc., USA) at 295 nm. Allopurinol was used as a positive control.

Anticholinesterase inhibitory activities

Determination of acetylthiocholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities were measured according to Orhan et al. (2007). Briefly, 140 µl of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µl of DTNB, 20 µl of test solution and 20 µl of AChE/BChE solution were added in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 µl of acetylthiocholine iodide/butyrylthiocholine chloride. The hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate-reader (Spectramax Plus-384, Molecular Devices, CA, USA). Percentage of inhibition of AChE/BChE was determined by comparison of rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula $(E-S)/E \times 100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galanthamine (Sigma St. Louis, MO, USA) was used as reference.

Anti-inflammatory activity

The murine monocytic macrophage RAW 264.7 cell line (European Cell Culture Collection, CAMR, UK) was cultured in DMEM (2 mM L-glutamine, 45 g/L glucose, 1 mM sodium pyruvate) with 10% fetal bovine serum (FBS). The cells were cultured at 37°C with 5% CO₂ and were subcultured twice a week. The cells were seeded in 96-well tissue culture plates (1×10⁶ cells/ml) and incubated for 24 h at 37°C with 5% CO₂. Then, 100 µl of test extract in DMSO was then added and serially diluted to give a final concentration of 200 µg/ml in 0.1% DMSO. Cells were then stimulated with 200 U/ml of recombinant mouse IFN-γ and 10 µg/ml *Escherichia coli* LPS and incubated at 37°C for another 17 h.

The presence of nitrite was determined in cell culture medium by Griess reagent and cell viability was detected by using MTT cytotoxicity assay as described by Ahmad et al. (2005). L-NAME was used as inducible NO synthase (iNOS) inhibitor (control) at a concentration of 250 µM.

Statistical analysis

The antioxidant activity values were analyzed using analysis of variance (ANOVA) with Statistical Analysis System (SAS) Version 9 (SAS Institute, Cary, NC). Significant differences among means

Table 1. Total phenolic and flavonoid content of *P. mahaleb* seed.

<i>P. mahaleb</i> seed	Phenolics ¹	Flavonoids ²
Methanolic extract	75.7±0.18 ^a	28.5±0.37 ^a
Hexane extract	34.6±0.24 ^b	16.2±0.31 ^b
S.E.M	0.26	0.19

¹mg gallic acid equivalent/g DW; ²mg rutin equivalent/g DW. Means in the same column with the different superscripts are significantly different at P < 0.05.

from triplicate analyses (p < 0.05) were determined by Duncan's multiple range test.

RESULTS AND DISCUSSION

Plant materials and crude extracts preparation

The *P. mahaleb* L. fresh whole plant (Rosaceae) was collected from the torghabeh's mountains, a place located nearby Mashhad, Iran and identified by Mr. Joharchi from the Department of Botanic, Faculty of Agriculture, Ferdowsi University of Mashhad. A voucher specimen (GF2142/2010) was deposited in the Phytomedicinal Herbarium, Department of Botanic, Faculty of Agriculture, Ferdowsi University of Mashhad, Khorasan Razavi, Iran.

Total phenolic and flavonoid content

Phenolic and flavonoid compounds, which are widely found as secondary metabolites in plants, are important due to their ability to serve as antioxidants. Many phenolic compounds have been reported to possess potent antioxidant activity and anti-cancer, anti-carcinogenic, anti-bacterial, antiviral or anti-inflammatory activities in a greater or lesser extent (Soooratttee et al., 2005; Hendra et al., 2011; Namuli et al., 2011). Flavonoids, which are found commonly in the leaves, seed, flowering tissues and pollens, are an important part of the diet because of their effects on human nutrition. The most important function of flavonoids is their antioxidant activity, as they have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Pietta, 2000). The phenolic and flavonoid content of different extracts of *P. mahaleb* seeds are presented in Table 1. Results showed that the total phenolic and flavonoid compounds concentration in the methanolic extract were 75.7±0.18 and 28.5±0.37 mg/g DW whereas in the hexane extract were 34.6±0.24 and 16.2±0.31 mg/g DW, respectively. The significant (p<0.05) lower content of phenolics and flavonoids in hexane extract indicated the effects of the nature of solvent on extraction of phenolics and flavonoids.

Free radical scavenging activity (DPPH)

The DPPH method has been recommended as an easy and accurate technique to measure the antioxidant activity of fruit and vegetable juices or extracts. The results are highly reproducible and comparable to other free radical scavenging method such as ABTS (Sanchez-Moreno, 2002). The extracts and reference antioxidants at the 100 µg/ml concentration inhibited the DPPH (Table 2) with the significant (p<0.05) differences in their percentage inhibition. The percentage inhibition values of methanolic extract, hexane extract, vitamin C, BHT and vitamin E were 44.3, 26.8, 92.2, 95.1, 92.5%, respectively. Extracts showed lower activity as compared to the reference antioxidant and methanolic extract was found to be more active in inhibition of free radicals as compared to hexane extract.

Ferric reducing antioxidant power (FRAP)

FRAP of extracts and reference antioxidants (vitamin E, C and BHT) are presented in Table 2. Reference antioxidants showed higher reducing power followed by methanolic and hexane extract. At the concentration of 100 µg/ml the reduction of Fe³⁺ to Fe²⁺ (as an index for antioxidant potential in FRAP) for vitamin C, E and BHT were 91.4, 90.8 and 91.6 %, respectively (Table 2), while for the methanolic and hexane extracts were 51.9 and 32.8%, respectively. In FRAP assay, the general ability of the extracts to donate electrons is tested whereas, in the DPPH assay, reduction by hydrogen atoms are also involved. Therefore, both extracts showed antioxidant activity and the results indicated that the extracts were more active in reduction and electron donation as compared to scavenging free radicals. In the complex systems, such as food, various different mechanisms may contribute to oxidative processes. Therefore, it is important to characterize the extracts by a variety of antioxidant assays (Ismail et al., 2010; Oskoueian et al., 2011a; Oskoueian et al., 2011b).

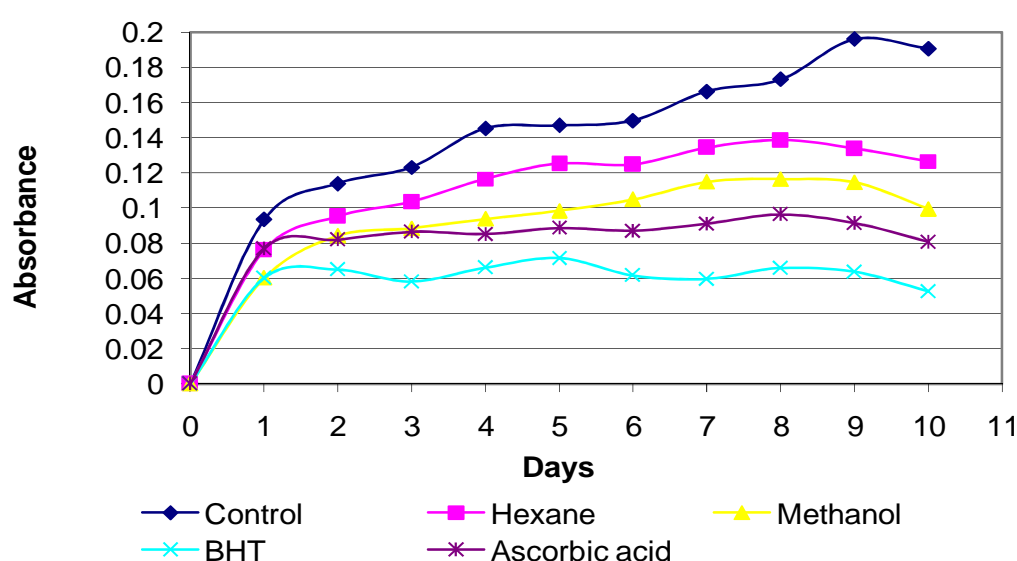
Nitric oxide (NO) scavenging activity

Nitric oxide radicals radical generated from sodium nitroprusside was found to be inhibited by *P. mahaleb* seeds extracts (Table 2). Both extracts exhibited NO scavenging activity but in different levels. Methanolic extract showed 36.9% inhibition at 100 µg/ml, indicating good NO scavenging activity, whereas, hexane extract with 21.8% inhibition at 100 µg/ml indicated moderate activity. The NO scavenging activities of the extracts were lower than vitamin C (53.5%) and vitamin E (71.8%). However, NO scavenging activity of the BHT was similar to methanolic extract. All the NO-scavenging values were categorised according to Tsai et al. (2007). The results of NO scavenging activity demonstrated that

Table 2. Free radical scavenging and ferric reducing power activity of *P. mahaleb* seeds extracts at 100 µg/ml and reference antioxidant.

Antioxidant activity	Methanolic extract	Hexane extract	Vitamin C (%)	Vitamin E (%)	BHT (%)	SEM
Free radical scavenging activity (DPPH)	44.3 ^b	26.8 ^c	92.2 ^a	95.1 ^a	92.5 ^a	5.83
Ferric reducing antioxidant power (FRAP)	51.9 ^b	32.8 ^c	91.4 ^a	90.8 ^a	91.6 ^a	4.95
Nitric oxide scavenging activity	36.9 ^c	21.8 ^d	53.5 ^b	71.8 ^a	35.6 ^c	6.12

Means in the same raw with the different superscripts are significantly different at $P < 0.05$.

**Figure 1.** Absorbance values of samples at 200 µg/ml concentration using FTC method.

methanolic extract was a good scavenger of nitric oxides showing its antioxidant potential. Probably the phenolic and flavonoid compounds present in *P. mahaleb* seeds extracts contributed in scavenging of nitric oxide. Similarly, Srinivasa et al. (2010) also reported the high positive correlation of phenolic and flavonoid content with NO scavenging activity of extract obtained from *Chromolaena odorata* leaves. NOs as free radicals involves in various types of inflammation in the body therefore, with regard to the NO scavenging activity of the extracts they could be applied as anti inflammatory agent.

Total antioxidant activity (ferric thiocyanate (FTC) and thiobarbituric acid (TBA) tests)

Hydroperoxides inhibition activity of extracts in FTC test is presented in Figure 1. Almost both extracts reduced

the hydroperoxides formation in the linoleic acid emulsion throughout the incubation period when compared to the negative control. The percentage inhibitions of the methanolic and hexane extracts in the last day of the assay were 47.79 and 33.63%, respectively while for the vitamin C and BHT were 57.69 and 72.45% (Figure 2). The extracts showed lower percentage inhibition value to that of vitamin C and BHT at the end of the incubation period. TBA test determined the content of thiobarbituric acid reactive substances at the end of lipid oxidation. Thiobarbituric acid reactive substances inhibition activities for the methanolic extract, hexane extract, BHT and vitamin C were 48.32, 35.87, 84.07 and 74.70%, respectively (Figure 2). The results showed that methanolic extract exhibited the stronger significant activity ($P < 0.05$) as compared to hexane extract. The result suggests that the methanolic extract contains antioxidative compounds as shown in Table 1 react

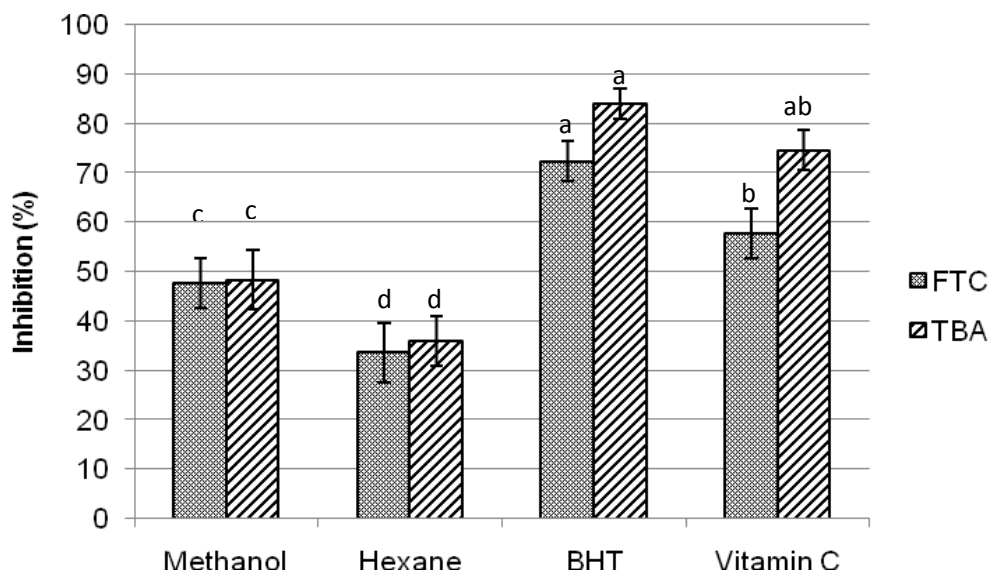


Figure 2. Total antioxidant activity determined by the FTC and TBA methods on day 9. Each bar represents the mean \pm standard error in three independent experiments.

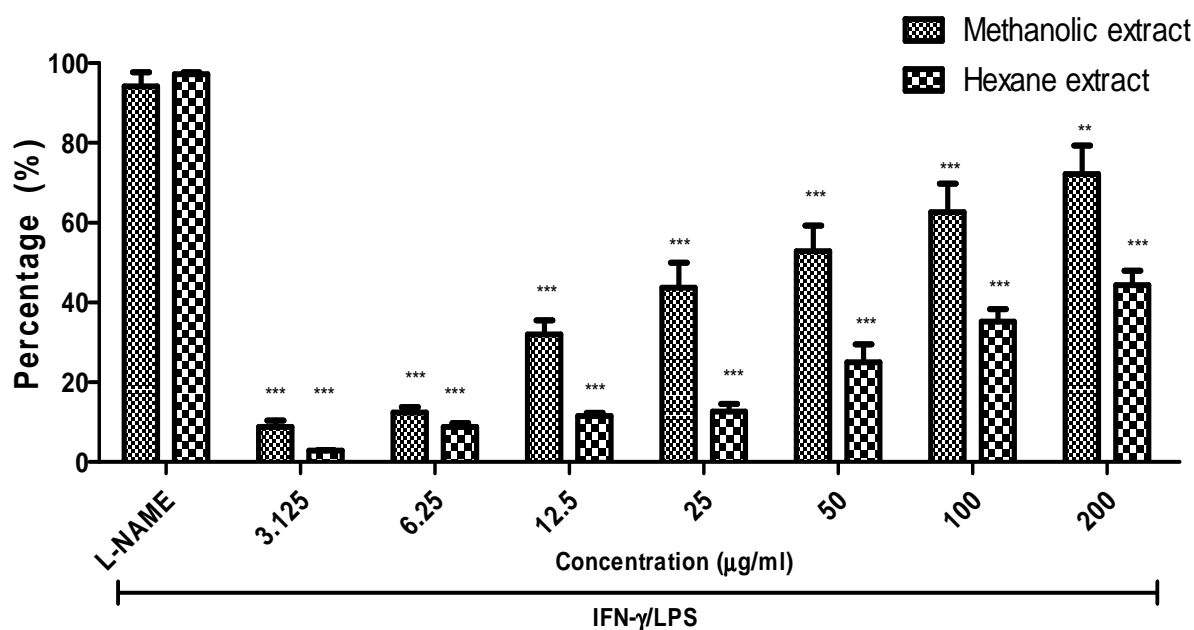


Figure 3. Effects of various concentrations of methanolic and hexane extracts on the NO percentage inhibition of LPS/IFN- γ stimulated RAW 264.7 cells. Each bar represents the mean \pm standard error in three independent experiments. *** $P < 0.0001$; ** $P < 0.001$ indicates significant difference as compared to the control (L-NAME).

aggressively toward hydroxyl radicals and retard the formation of hydroperoxides. Phenolic and flavonoid compounds are important due to the ability to serve as antioxidants, which are widely found as secondary metabolites in plants.

Many phenolic compounds have been reported to possess potent antioxidant activity. In addition, flavonoids are found commonly in the leaves, flowering tissues and pollens. Plant flavonoids are an important part of the diet because of their effects on human nutrition. The most

Table 3. The IC₅₀ values of xanthine oxidase, tyrosinase and anticholinesterase inhibitory activities of *P. mahaleb* seeds extracts and positive controls.

Sample	IC ₅₀ µg/ml			
	Xanthine oxidase inhibitory activity	Tyrosinase inhibitory activity	Acetyl cholinesterase inhibitory activity	Butyryl cholinesterase inhibitory activity
Methanolic extract	59.8 ^b	21.0 ^b	52.1 ^b	86.2 ^b
Hexane extract	94.1 ^a	54.2 ^a	85.2 ^a	110.7 ^a
Allopurinol	29.7 ^c	-	-	-
Kojic acid	-	11.4 ^b	-	-
Galantamine	-	-	12.8 ^c	10.7 ^c
S.E.M	9.32	7.37	6.45	7.23

Means in the same columns with the different superscripts are significantly different at P <0.05.

important function of flavonoids is the antioxidants properties. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Karimi et al., 2010).

Xanthine oxidase (XO) inhibition activity

The extracts XO inhibition activities are presented in Table 3. Inhibition of XO led to a decrease in production of uric acid, which was determined spectrophotometrically. Both extracts inhibited the XO but in different levels. The IC₅₀ concentrations of allopurinol (positive control) (Table 3) was significantly ($p < 0.05$) lower than that of the methanolic and hexane extracts. The results indicated that both extracts could be a potential source of bioactive compounds with the ability to inhibit the XO enzyme. Umamaheswari et al. (2009) reported the contribution of phenolics and flavonoids toward XO inhibition through interaction in the reactive sites.

Tyrosinase inhibitory activity

Tyrosinase enzyme activity was inhibited by extracts and kojic acid (positive control) (Table 3). The IC₅₀ concentration of extracts (Table 3) showed significant difference ($P < 0.05$) and interestingly methanolic extract showed the stronger inhibitory activity similar to kojic acid. The result of Table 1 indicated the presence of phenolic and flavonoid compounds in the *P. mahaleb* seeds which might contribute in the inhibition of tyrosinase, however Kubo et al. (2003) and Nagendra et al. (2009) also previously reported the tyrosinase inhibitory activity of phenolic and flavonoid compounds. The tyrosinase inhibitory activity might depend on the hydroxyl groups of the phenolic compounds which could form a hydrogen bond to the active site of the enzyme and leading to steric hindrance, conformation changes and finally enzymatic activity suppression. Furthermore,

the antioxidant activity may also affect tyrosinase activity (Kim et al., 2008).

Anticholinesterase activities

The role of oxidative stress in the cancer induction and Alzheimer's disease is clear (Praticò, 2008). Therefore, here, we also aimed to look into anticholinesterase activity together with antioxidant potential of *P. mahaleb* seeds extracted by different polarity solvents. AChE and BChE inhibitory activities of the *P. mahaleb* seeds are presented in Table 3. Both extracts could inhibit the AChE and BChE, where, the methanolic extract could inhibit AChE and BChE significantly ($p < 0.05$) higher than hexane extract. The difference observed among the AChE and BChE inhibitory activity of extracts could be due to their difference in bioactive compounds presence in the extracts. In addition, literature survey highlighted the role of phenolic compounds in anticholinesterase inhibitory activities (Mata et al., 2007; Fawole et al., 2010). In accordance with literature, the result indicated that the phenolic and flavonoid as bioactive compounds (Table 1) presence in the seed may have AChE and BChE inhibitory activities. Preventive and symptomatic treatment of Alzheimer's disease requires multitarget drug strategy. Therefore, it is suitable to explore a crude extract having both anticholinesterase and antioxidant activities.

Anti-inflammatory activity

The extracts were analyzed for their NO production inhibition in RAW 264.7 cells induced by LPS and IFN- γ as well as the effect of extracts on cell viability. Figures 3 and 4 show the NO inhibition and cell viability, respectively for methanolic and hexane extracts. Induced cells produced NO through iNOS as symptoms of inflammation. Both extracts inhibited the NO production in a dose dependent manner and methanolic extract

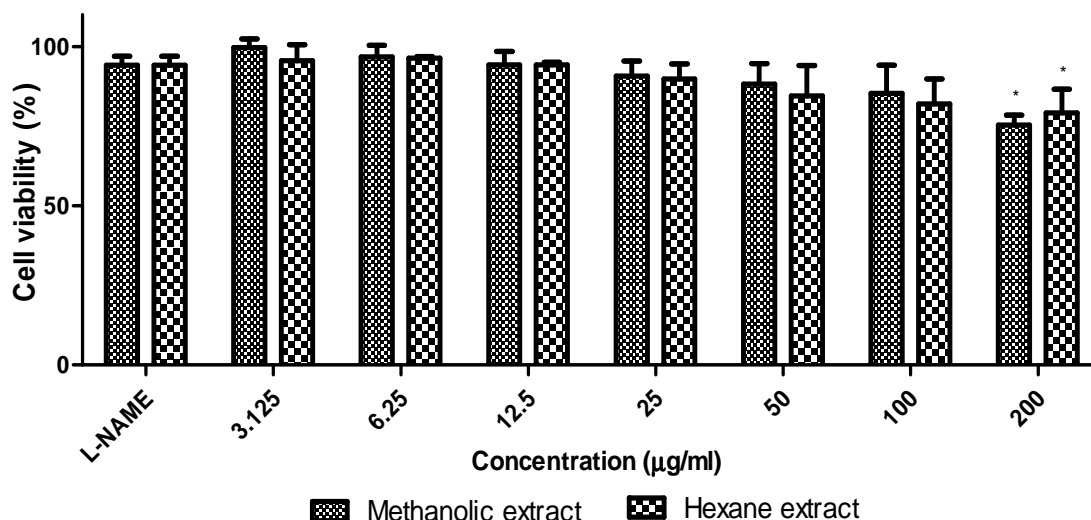


Figure 4. Effects of various concentrations of methanolic and hexane extracts on the cell viability of the LPS/IFN- γ stimulated RAW 264.7 cells. Each bar represents the mean \pm standard error in three independent experiments. * $P < 0.01$ indicates significant difference as compared to the control (L-NAME).

appeared to be more active than hexane extract. Increase in extract concentration up to 200 $\mu\text{g/ml}$ did not inhibit the NO production similar to L-NAME revealing the moderate anti inflammatory activity of methanolic *P. mahaleb* seeds extract. The cell viability result (Figure 4) shows that the *P. mahaleb* seeds extracts just affected the cell viability significantly ($P < 0.01$) at 200 $\mu\text{g/ml}$ indicating its safety and low level of toxicity. The iNOS suppressing effect of the *P. mahaleb* seeds methanolic extract could be due to the blocking of iNOS expression, inactivation of iNOS catalytic function or scavenging of NO radicals (Tsai et al., 2007). Various compounds could be involved in iNOS suppression by additive or synergistic effects although the roles of phenolics and flavonoids as main constituents of plant material responsible in iNOS suppression have been previously demonstrated (Kang et al., 2011; Nwosu et al., 2011; Oskoueian et al., 2011d). Therefore, among extracts used in this study, methanolic extract seemed to be promising as a source of anti inflammatory compounds with the ability to inhibit iNOS and at the same time maintaining the cell viability.

Most probably the phenolics and flavonoids present in the seed were the major contributors of antioxidant, XO inhibition, tyrosinase inhibition, anticholinesterase and anti-inflammatory activities. These results are consistent with those reported by Mariod et al. (2010) and Lee et al. (2011) who demonstrated the role of phenolics and flavonoids in antioxidant and anti inflammatory activities. Owan and Johns (1999) and Wang et al. (2011) also reported the positive correlation of phenolics and flavonoids with anticholinesterase, xanthine and tyrosinase inhibitory activities.

Conclusions

P. mahaleb seeds contain phenolic and flavonoid compounds. The methanolic extracts of *P. mahaleb* seeds possess strong tyrosinase inhibitory activity and moderate antioxidant potential, XO inhibitory, anticholinesterase and anti inflammatory activities therefore, it could be applied as a natural source of bioactive compounds applicable in food, pharmaceutical and cosmetic industries. However, the bioactive compounds responsible for the observed biological activities should be identified and activity of the isolated compounds should also be compared with that of the crude extract to reveal possible synergistic interaction. Hence, the further experiment on isolation, structure elucidation and identification of bioactive compounds are in progress.

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