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Seasonal variation of phenolics, flavonoids, antioxidant and lipid peroxidation inhibitory activity of methanolic extract of Melilotus indicus and its sub-fractions in different solvents

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Abstract

In the present work, seasonal variations of total phenolic and flavonoid contents, antioxidant activity and lipid peroxidation inhibitory activities of methanolic extract of Melilotus indicus and its sub-fractions in different solvents have been evaluated. The study was conducted on plant samples collected in February and April which are its flowering and fruiting seasons respectively. Both phenolic and flavonoid contents were higher in methanolic extract of the plant collected in April than in February. Free radical scavenging activity measured through DPPH method was low in methanolic extracts of both the samples, although it decreased as we go from February to April. Ethyl acetate fractions showed highest % inhibition. Total antioxidant activity of methanolic extracts measured through phosphomolybdate assay remained almost same. Chloroform fraction showed the highest value in both the seasons with value in April higher than in February, 57.24 and 46.44 µg of ascorbic acid equivalents per mg of the dried mass, respectively. The methanolic extract of April sample showed higher FRAP (ferric reducing antioxidant power) value than that of February. In February, ethyl acetate fraction showed highest FRAP value, while in April, chloroform exhibited the highest value. Methanolic extracts and fractions in all solvents showed remarkable ability to inhibit peroxidation in linoleic acid. After 72 hours, all samples were much more effective than the standard BHA. Seasonal variations affect biosynthesis of different types of antioxidant and free radical scavenging compounds in Melilotus indicus. Thus, the medicinal and nutritional value of the plant will depend on harvesting season.

Keywords: Melilotus indicus, phenolics, antioxidant, peroxidation, seasonal variations

Introduction

Melilotus indicus (Linn.) All. is a small herb (family Fabeaceae). The genus Melilotus consists of about 25 species, four of which are found in Pakistan, namely, *M. indicus, M. officinalis, M. alba,* and *M. messangensis* [1]. *M. indicus* (syn. *M. indica*) is distributed in many parts of the world including Indo-Pakistan Subcontinent, Mediterranean region, central Asia, Europe and Africa [2-5]. *Melilotus indicus*, is commonly consumed as a vegetable and is known for its ethnomedicinal activities such as antibacterial, anticoagulant, astringent, emollient, laxative and narcotic [6]. The plant is also used in infantile diarrhea, and is externally applied as poultice or plaster on swellings [4,7]. Phytochemical investigations have shown the plant to contain flavonoid glycosides, coumarins, terpenoids, and steroids [6,8-11]. The plant contains methoxyflavone, meliternatin which experimentally inhibited cell growth, induced granularity, retraction and then lysis of cells [12]. Flowering period of the herb is March-August [2].

Free radicals are the chemical species with odd electrons, which are produced in human body during metabolism. Natural defense system operative in the body normally keeps these reactive species in control. However, when an imbalance occurs and free radicals are produced in excessive amounts they then cause numerous harmful effects [13,14]. To inhibit and scavenge the surplus free radicals antioxidants are needed. Although a number of synthetic antioxidants are available, but they have toxic side effects and, therefore, there is a growing need to discover newer, natural and safer antioxidants [15,16]. Plant products including extracts and pure compounds furnish a promising alternative. Growing number of scientists have, thus, directed their efforts to investigate plants for medicines including antioxidants [17-19]. As per the current understanding, plants produce natural products, or secondary metabolites, as part of their defense mechanism. Therefore, climatic and ecological variations, such as the duration and intensity of sunlight, have significant effect on the nature and quantity of natural products produced in the plant body [20,21].

The purpose of the present study was to evaluate phenolic and flavonoid contents, antioxidant and free radical scavenging potential and lipid peroxidation inhibitory activity of the methanolic extract of the medicinal herb *Melilotus indicus* and its sub-fractions in different solvents and to analyze the seasonal effects on these properties.

Materials and methods

Plant collection and extract preparation

The aerial parts of *Melilotus indicus* were collected from Hazara, Pakistan, in February (the flowering season) and April (fruiting season) 2010 and were identified by the authors. In each case, the plant sample, after drying under shade for 15 days, was ground to obtain a powder material. The dried powder (100 g) was extracted in 100 % methanol at room temperature (300 mL x 15 days x 3). The three extracts were filtered and the filtrates were combined and concentrated on rotary evaporator under reduced pressure at 30 °C. This crude methanolic (MeOH) extract (8.068 g) was suspended in water (30 mL) and extracted with hexane, ethyl acetate (EtOAc), chloroform ($CHCl_3$) and n-butanol (n-BuOH) respectively, thus affording the crude MeOH extract and its sub-fractions: hexane, EtOAc, $CHCl_3$, n-BuOH and residual H_2O. Each sample was dried under reduced pressure and weighed.

Chemicals and Apparatus

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical and ascorbic acid were purchased from MP Biomedicals, (France). Linoleic acid was obtained from Sigma-Aldrich, (Germany). Sodium hydroxide, sodium nitrite, rutin, Folin-Ciocalteu reagent, sodium carbonate, sulfuric acid, ammonium molybdate, ferric chloride, potassium thiosulfate, sodium chloride, iron(II) sulfate, sodium acetate trihydrate, hydrochloric acid, iron(II) chloride, Tween 20, dipotassium phosphate, butylated hydroxyanisole (BHA) and all solvents used were of analytical grade and were purchased from Merck (Germany). Glacial acetic acid was purchased from PRS Pancreac, (France), Aluminium chloride was obtained from BDH Labs., (England). Gallic acid was purchased from Scharlau, (Switzerland). UV-Visible Spectrophotometer UVD-3200 Labomed, Inc. was used to measure absorbance and Rotavapor R-210/A (Buchi) was used to evaporate solvents under reduced pressure.

Total Flavonoid Content (TFC)

The total flavonoid contents in samples of the plant collected in February and April were determined by a known protocol [22]. In each case, the working sample was obtained by dissolving 30 mg of a fraction in 10 mL of methanol. In a glass vial, 3.4 mL of 30% aqueous methanol was taken and 300 µL of plant sample or standard was added and mixed followed by the addition of 150 µL of $NaNO_2$ (0.5 M). After an interval of 5 min, 150 µL of $AlCl_3$ (0.3 M) solution, and after another 5 min, 1 mL of NaOH (1 M) solution was added and thoroughly mixed. The absorbance of the mixture was then measured at 506 nm. Rutin was used as a standard.

Total Phenolic Content (TPC)

The total phenolic contents of the MeOH extracts and fractions in different solvents were determined by the method reported by Slinkard [23]. To prepare each of the sample solutions, 30 mg of an extract or fraction was dissolved in 10 mL of methanol. In a glass cuvette, 3.16 mL of distilled water, 40 µL of a sample or standard solution and 200 µL of Folin–Ciocalteu reagent was added and the solutions were mixed thoroughly. To this, 600 µL of sodium carbonate (7%) solution was mixed after incubation of eight minutes. The glass cuvettes containing the samples were incubated at 40 °C for 30 min, and absorbance of the mixture was observed against a blank at 765 nm. Gallic acid was used as a standard.

DPPH Radical Scavenging

The radical scavenging activity of all the plant samples using DPPH assay was determined by the method reported by Brand-Williams *et al* [24]. In each case, 10 mg of a plant extract/fraction was dissolved in 10 mL of methanol to be used in the experiment. To prepare the stock solution of DPPH radical, 24 mg of DPPH was dissolved in 100 mL of methanol and kept in a refrigerator until used. The stock solution was diluted with MeOH to obtain the working solution having about 0.98 (±0.02) absorbance at 517 nm. Then, to 3 mL of the working solution of DPPH taken in a vial, 100 µL of the plant extract/fraction or the standard was added and mixed to obtain a clear solution. The absorbance of this mixture was noted at 517 nm for a 30 minutes. The percent scavenging activity, or inhibition, was calculated using the following formula:

$$\% \text{ Inhibition} = [A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of DPPH after reacting with antioxidant sample after 30 min. Ascorbic acid was used as a standard.

Total antioxidant activity by Phosphomolybdate Antioxidant Assay

Total antioxidant activity of the methanolic extracts of M. Indicus collected in flowering and fruiting seasons and their fractions in different solvents was determined through the phosphomolybdate antioxidant assay which was carried out according to a known procedure [25]. In each case, 25 mg of an extract/fraction was dissolved in 10 mL of methanol to prepare test samples. Phosphomolybdate reagent was prepared by mixing 0.6 M sulfuric acid (100 mL), 4 mM ammonium molybdate (100 mL) and 28 mM sodium phosphate (100 mL) solution. In a test tube, 3 mL of phosphomolybdate reagent, 300 µL of the test sample or standard mixed. After capping with a silver foil, the test tube was heated at 95 °C in a water bath for 90 min. Then the content of the test tube was allowed to cool down, and the absorbance was noted at 765

nm against a blank. For this evaluation, ascorbic acid was used as a standard.

Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing antioxidant power (FRAP) assay involves reduction of ferric into ferrous. It was carried out in accordance with a reported method [26]. The FRAP reagent was prepared by mixing 25 mL of acetate buffer (300 mM, pH 3.6), 2.5 mL of ferric chloride (20 mM) and 2.5 mL of TPTZ solution (10 mM) prepared in HCl (40 mM). The mixture was warmed at 37 ℃ for 15 min. The FRAP reagent (2.85 mL) was mixed with 150 µL of a plant extract/fraction or standard. After incubating the mixture for 30 min in dark, absorbance was recorded at 593 nm.

Lipid Peroxidation Inhibition Assay

The lipid peroxidation inhibitory activities of extracts and fractions of the plant were determined according to the method described by Mitsuda et al [27]. To prepare an emulsion of linoleic acid, 155 µL of the acid were mixed with 175 µg of Tween 20 and the volume was made 50 mL by adding 0.05 M of potassium phosphate buffer (pH 7). In a test tube, a plant extract or fraction (100 µL) was dissolved in potassium phosphate buffer (2.4 mL, pH 7). To this, 2.5 mL of linoleic acid emulsion was added. After incubating the mixture at 37 ˚C for 25 min, an aliquot of 100 µL was regularly taken at 24 h intervals and dissolved in 3.7 mL of ethanol. To this, 100 µL of 20 mM ferrous chloride solution and 100 µL of 30% potassium thiocyanate solution were added and absorbance of the clear mixture was noted at 500 nm. A 5 mL solution consisting of linoleic acid emulsion (2.5 mL) and potassium phosphate buffer (2.5 mL) was used as blank. BHA (butylated hydroxyanisole) was used as a standard antioxidant.

Statistical Analysis

To ensure reproducibility, each experiment was carried out three times and a mean was calculated. One way analysis of variance (ANOVA) was applied and the results were correlated.

Results and Discussion

Total Flavonoid Content (TFC)

Total flavonoid contents of the methanolic extracts and their sub-fractions in hexane, chloroform ethyl acetate, n-butanol and water of Melilotus indicus collected in February and April were determined in terms of µg of rutin equivalents per mg of the dried mass (µg RE/mg) of the extract or fraction, and the results are shown in Table 1. The plant contains good quantity of flavonoids which are known to possess biochemical and antioxidant properties (Dajas et al., 2005) [28]. As the results showed, there is a notable difference in TFC in various solvents from one season to the other. MeOH extract that had a low value (150 µg RE/mg) in February attained a value as high as 550 µg RE/mg in April. This is a manifold increase and can be attributed to the change in ecological parameters such as the sunlight and day length. As we move from February to April, weather becomes gradually hotter with increase in the day length. The plants thus are exposed to stronger sunlight for a longer duration inducing enhanced biosynthesis of flavonoids which being antioxidant are an important part of the plants' defence system [29,30]. The fractionation of the MeOH extract into different solvents resulted into an interesting distribution of flavonoids. The TFC in the fractions of the sample collected in February decreased in the order $CHCl_3$> EtOAc > n-BuOH > H_2O > Hexane, while in the fractions of the sample collected in April it decreased in the order n-BuOH > EtOAc > H_2O > Hexane > $CHCl_3$. Thus, the $CHCl_3$ fraction that contained the highest TFC in February (575 µgRE/mg), had the lowest in April (60 µgRE/mg). The EtOAc fractions also showed a notable decline from 410 to 170 µgRE/mg. On the other hand, there is an increase in TFC in Hexane, n-BuOH and H_2O fractions. This variation of flavonoids in solvents of different polarity indicates a variation in the structures and types of flavonoids, hence a variation in biosynthesis of these compounds from season to season.

Total Phenolic Content (TPC)

Total phenolic contents of different samples of *M. indicus* collected in February and April were determined in term of µg of gallic acid equivalents per mg of the dried mass of the extract or fraction (µg GAE/mg) and the results are displayed in Table 1. The plant has much lower TPC than TFC in methanolic extracts. However, there is almost a two times increase in TPC, from 25 to 45 µg GAE/mg, in MeOH extracts as we go from colder February to hotter April. Stronger sunlight for a longer duration induced enhanced biosynthesis of phenolic compounds [20]. Distribution of phenolic compounds also varied in sub-fractions of MeOH extract in different solvents across the season. EtOAc fraction had the highest TPC content in both the seasons, although in February the value (150µg GAE/mg) was much higher than in April (75 µg GAE/mg). In the two seasons, February and April, the TPC decreased in the order of EtOAc > $CHCl_3$ > n-BuOH > H_2O > Hexane and EtOAc > n-BuOH > $CHCl_3$ = H_2O > Hexane. Hexane has the lowest content in both the cases, but there is a threefold increase (10-28 µg GAE/mg) in going from February to April. The n-BuOH and H_2O fractions in both the seasons have almost equal values.

Free Radical Scavenging by DPPH Assay

Free radical scavenging activity of MeOH extracts of *M. indicus*, collected in February and April, and their sub-fractions in different solvents were determined using DPPH assay and the results are shown in Table 2. The DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical. It can accept a hydrogen fee radical or an electron to form diamagnetic species (Soares et al., 1997) and in doing this it changes its colour from purple to pale yellow. The reaction can be monitored spectrophotometrically by observing the

Table 1 – Total Flavonoid and Phenolic Contents of MeOH extract of *Melilotus indicus* and its sub-fractions in different solvents in flowering and fruiting seasons

Extract /Fraction	Total flavonoid content (µg) expressed as Rutin equivalents per mL of plant extract		Total phenolic content (µg) expressed as Gallic acid equivalents per mL of plant extract	
	Flowering Season	Fruiting Season	Flowering Season	Fruiting Season
Methanolic	150	550	25	45
Hexane	60	150	10	28
Chloroform	575	60	42	30
Ethyl Acetate	410	170	150	75
n-Butanol	200	300	38	42
Aqueous	110	165	29	30

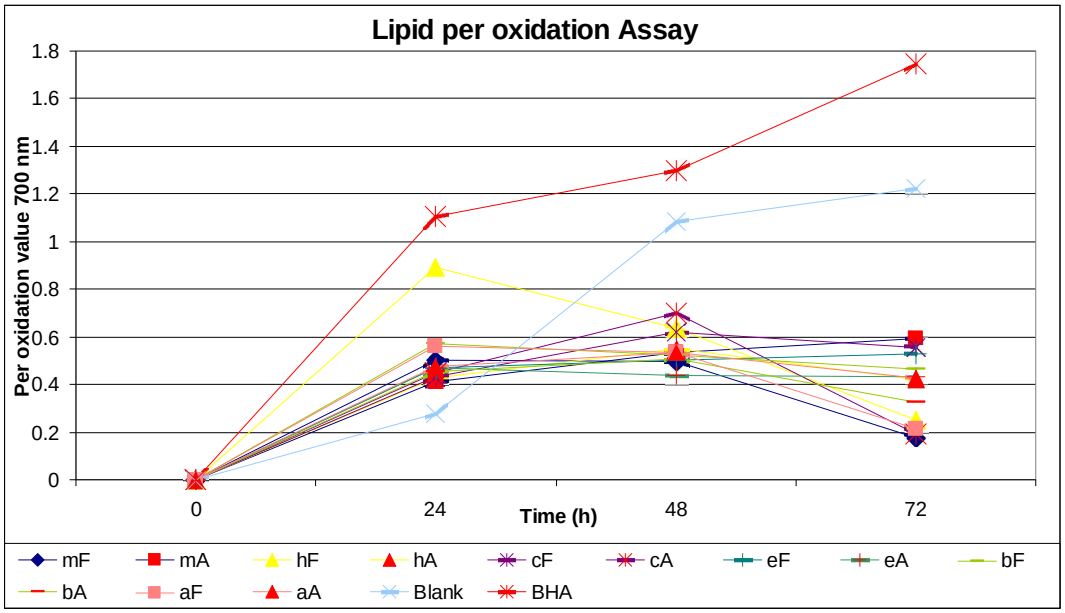
Table 2 – DPPH Radical scavenging activity of MeOH extract of *Melilotus indicus* and its sub-fractions in flowering and fruiting seasons

Extract / Fraction	Radical Scavenging Activity (%)	
	Flowering Season	Fruiting Season
Methanolic	6.55	2.78
Hexane	3.67	1.20
Chloroform	1.48	6.35
Ethyl Acetate	44.50	16.38
n-butanol	19.76	6.65
Aqueous	1.20	1.90

Table 3 – Antioxidant Effect and Ferric Reducing Antioxidant Potential (FRAP) of MeOH extract of *Melilotus indicus* and its sub-fractions in flowering and fruiting seasons in terms of ascorbic acid equivalents (AAE)

Extract/Fraction	Antioxidant Effect (µg/mL) of AAE		Ferric Reducing Antioxidant Potential (µg/mL) of AAE	
	Flowering Season	Fruiting Season	Flowering Season	Fruiting Season
Methanolic	22.84	20.04	14.61	17.30
Hexane	33.64	31.64	101.53	47.69
Chloroform	46.44	57.24	68.84	94.61
Ethyl Acetate	40.44	33.24	139.61	17.69
Butanolic	24.44	24.84	14.61	16.15
Aqueous	20.04	15.24	0	2.30

Figure 1 – Lipid peroxidation inhibition activities of *Melilotus indicus* methanolic extract and fractions in different solvents in flowering and fruiting season.



(m = MeOH, h = hexane, c = chloroform, e = ethyl acetate, b = n-butanol, a = aqueous; F = February, A = April; BHA = butylated hydroxyanisole

decrease in absorbance at 517 nm [31,32]. The plant did not show much appreciable free radical scavenging potential in both the seasons. Interestingly, the value for MeOH extracts is higher in February than April. Out of the fractions, EtOAc fractions showed the highest DPPH scavenging activity in both the seasons and activity n-BuOH fractions was also higher than the other fractions. The trend is supported by other studies as well [33]. The poor free radical scavenging activity of extracts may be due to low phenolic contents in the plant samples. There is a good correlation between the two; EtOAc fractions having highest phenolic contents are the strongest radical scavengers.

Total Antioxidant Activity

Total antioxidant activity through phosphomolybdate assay was determined for the MeOH extracts of *M. indicus* collected in February and April, and their fractions in different solvents. The results expressed in terms of µg of Ascorbic acid equivalents per mg (µgAAE/mg) of the dried extract or fraction are displayed in Table 3. The phosphomolybdate assay involves reduction of molybdenum(VI) to molybdenum(V) in the presence of a antioxidant (reducing agent). The product is a green phosphomolybdate(V) complex whose formation can be stoichiometrically monitored with a spectrophotometer at 765 nm.
The MeOH extracts showed almost same total antioxidant activity in both the months (20.04-22.84 µg AAE/mg). Different fractions of the plant also exhibited almost same total antioxidant activities in both the seasons (Fig 1). Values among solvent to solvent also change in the same order: $CHCl_3$ > EtOAc > Hexane > n-BuOH > H_2O. The $CHCl_3$ fraction showed higher activity in April than February, other fractions were more potent in February than April.

Thus, the extracts of flowering season, in general, are slightly more powerful antioxidants than those of fruiting seasons.

Reducing Power with FRAP Assay

Reducing antioxidant power of various samples of *M. indicus* collected in February and April was determined using well-known FRAP (ferrous reducing antioxidant power) assay and the results expressed in terms of µg of gallic acid equivalents per mg of dried mass of extract/fraction (µg GAE/mg) are shown Table 3. In this assay, ferric ions are reduced to ferrous ions in the presence of an antioxidant (or, a reducing agent) which form a coloured ferrous tripyridyltriazine complex at pH 3.6. The change is monitored spectrophotometrically at λ_{max} 593 nm [34].

The MeOH extract showed a higher FRAP value in hotter April (17.31 µg GAE/mg) than colder February 14.62 µgGAE/mg). The increase can be attributed to higher TPC and TFC in April than February. In various sub-fractions, the order of change in the FRAP values in February and April was EtOAc > Hexane > $CHCl_3$ > n-BuOH > H_2O, and $CHCl_3$ > Hexane > EtOAc > n-BuOH > H_2O respectively. The $CHCl_3$, n-BuOH and H_2O fractions have higher values in April while EtOAc and Hexane fractions have higher values in February. The result showed that there is a good correlation between TPC and FRAP values. The EtOAc fraction that had the highest TPC, showed strongest FRAP value (139.62 µgGAE/mg) in February. The aqueous fractions in both the cases showed almost no activity. Hexane fractions presented an interesting case having second highest values in both types of fractions. The samples having higher FRAP values also have comparatively higher total antioxidant activity measured through phosphomolybdate assay.

Lipid Peroxidation Inhibition

To determine the ability of a substance to inhibit peroxidation in lipids, this assay is commonly used. Linoleic acid (a polyunsaturated fatty acid), is allowed to undergo auto-oxidation which produces peroxyl radicals. These radicals are then used to oxidize ferrous ions to ferric ions, which form a coloured complex with SCN^- ions. The formation of the complex is estimated spectrophotometrically at 500 nm. Low absorbance means low peroxidation and thus high peroxidation inhibitory activity of a sample. In our experiment, the process was monitored over a course of time and the results are shown in the form a graph in Figure 1.

As compared to the standard used (BHA), the plant samples of both the seasons exhibited much better and more sustainable ability to inhibit peroxidation in linoleic acid. Lipid peroxidation inhibitory activity of MeOH after 72 h was higher in February than in April. Among the fractions, $CHCl_3$ (Apr), hexane (Feb) and H_2O (Feb) were showing high efficacy. Hexane (Feb) which initially had low inhibitory activity, became more effective with time. More interesting was the behavior of $CHCl_3$ fraction. It was very weak in February but became very strong in April when it also had strong phosphomolybdate and FRAP values, indicating a possible relationship between them.

Conclusions

Seasonal variations effect biosynthesis of antioxidant compounds in *Melilotus indicus*. Total flavonoid and phenolic contents increase as we go from colder to hotter seasons when plants receive stronger sunlight for a longer duration. Types of polyphenolics also vary with change in ecological conditions as we have noticed variations in their contents in solvents of different polarities. Antioxidant activities and lipid peroxidation inhibitory activities of methanolic extracts and their fractions also showed variation with the seasonal change. It is thus advisable that when harvesting the plant for the purpose of isolating antioxidant constituents or using the crude extracts as such seasonal variations should be taken into account.

Authors' contribution

DA conceived of the study, supervised the work, evaluated the results and corrected the manuscript for publication. HB carried out the extraction, and determined the Total Flavonoid Content (TFC), Total Phenolic Content (TPC) and the radical scavenging activity. SZ carried out the total antioxidant activity, Ferric Reducing Antioxidant Power (FRAP), and Lipid Peroxidatin Inhibition assay. All authors read and approved the final manuscript.

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