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Full Length Research Paper

Insight into the functional and medicinal properties of *Medicago sativa* (Alfalfa) leaves extract

Ehsan Karimi[1], Ehsan Oskoueian[2]*, Armin Oskoueian[3], Vahid Omidvar[4,5], Rudi Hendra[6] and Hani Nazeran[3]

[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
[2]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
[3]Department of Agronomy, Faculty of Agriculture, Islamic Azad University, Mashhad Branch, Iran.
[4]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
[5]Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
[6]Department of Chemistry, Faculty of Mathematic and Natural Sciences, University of Riau, Pekanbaru, Riau, Indonesia.

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This research was carried out to evaluate the phenolics and flavonoids content of *Medicago sativa* leaves extract and to characterize the phenolics and flavonoids profiles using reversed phase-high performance liquid chromatography (RP-HPLC). The antioxidants, anti inflammatory and xanthine oxidase (XO) inhibitory activities of the extract were also determined. The obtained result showed that the total phenolics value was 37.0 ± 0.02 mg gallic acid equivalent (GAE)/g dry matter (DM), and for total flavonoids was 12.6 ± 0.17 mg rutin equivalent/g DM. In addition, the RP-HPLC analyses indicated the presence of gallic acid, pyrogallol, salicylic acid, caffeic acid as phenolics while the detected flavonoids and isoflavonoid were naringenin, apigenin, quercitin, myrcitin and daidezin. The antioxidant activity of Alfalfa by diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) method showed 54.42 and 56.71% inhibition of free radicals at the concentration of 250 µg/ml of leaves crude extract, respectively, but the activities were lower than those of antioxidant standards such as butylated hydroxytoluene (BHT) and α-tocopherol. Nitric oxide (NO) inhibition assay as one of antioxidant potential indicator showed 50.99% inhibitory activity of NO at the concentration of 250 µg/ml, while this value for the vitamins E and C were 91.52 and 94.13%, respectively. Alfalfa exhibited moderate anti-inflammatory activity where the 50% of the NO production by the induced RAW 264.7 cells was inhibited at the concentration of 147.24 µg/ml of Alfalfa crude extract. The cell viability test [3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay] showed that Alfalfa extract was not toxic to macrophage RAW 264.7 cells. Meanwhile, leaves crude extract also inhibited the XO 51.63% at the concentration of 250 µg/ml.

Key words: *Medicago sativa*, Alfalfa, functional properties, antioxidant, anti-inflammatory, xanthine oxidase.

INTRODUCTION

Alfalfa is a perennial flowering herb plant in the pea family Fabaceae, and botanically known as *Medicago sativa*. The Alfalfa is one of the most reputed medicinal plants that grows up to three feet in height with sprightly green leaves and flowers which are bluish-violet in colour (Bagavathiannan and Van Acker, 2009). The Alfalfa plantis beneficial to both humans and animals. Humans benefit from Alfalfa sprouts, tender stems, dehydrated Alfalfa leaf (available as a dietary supplement in forms

*Corresponding author. E-mail: ehs424@yahoo.com. Tel: +60129795283.

such as tablets, powders and tea), while animals enjoy its benefits in the form of forage, harvested hay and feed. *M. sativa* has a long tradition of use as ayurvedic and homoeopathic medicine in central nervous system disorders. The plant has been reported to have antioxidant, anti-inflammatory and antidiabetic effects (Kundan and Anupam, 2011). Phytochemicals are chemicals derived from plant sources such as phenolics, flavonoids, alkaloids, saponins, tannins and lignin which possess biological activities (Oskoueian et al., 2011a; Okwu, 2005).

These bioactive compounds have received considerable attention due to their therapeutic potential as antimicrobial, anti-inflammatory, anticancer and antioxidant activities (Rathee et al., 2009). These biological activities including anti-inflammatory and antioxidant activities could be attributed to the presence of both phenolic and flavonoids compounds which act as free radical scavengers and/or metal chelators (Oskoueian et al., 2011b).

A positive correlation was observed between the phytochemicals content and the strong biological activities such as antioxidant activity, anti inflammatory (Barros et al., 2007). This investigation for natural antioxidants with the plant origin is also being explored as an alternative to the synthetic antioxidants, such as butylated hydroxyanisole (BHA), propyl gallate (PG) and butylated hydroxytoluene (BHT), used in food and pharmaceutical industries (Brunet et al., 2009) as the synthetic antioxidants may possess some side effects and toxic properties such as carcinogenic to human health (Manian et al., 2008). Searching for a natural bioactive extract with the wide spectrum of functional properties to be applied in various industries beside available information about ethnopha-rmacological applications of *M. sativa* prompted us, to perform this research.

Thus, initially the qualitative and quantitative analyses of phenolics and flavonoids present in *M. sativa* extract were conducted. Moreover, the functional properties of the extract including antioxidant, anti-inflammatory and xanthine oxidase (XO) inhibitory activities were evaluated.

MATERIALS AND METHODS

Plant material

M. sativa used in this study was freshly harvested from a farm (Taghavi farm) located in the city of shirvan, North Khorasan, Iran with the GPS location of latitude 37°25'2.67"N and longitude57°53'28.00"E in June, 2011. The leaves of *M. sativa* were cleaned, separated, and freeze dried for further analysis.

Preparation of extracts

Samples ware extracted using methanol as a solvent based on Crozier et al. (1997), 2 g freeze-dried of leaves were weighed and placed into a 100 ml conical flask, and added with 40 ml of 80% (v/v) methanol. It was followed by an addition of 10 ml of 6 M HCl. The mixture was refluxed for 2 h at 90°C and filtered by using Whatman No. 1 filter paper (Whatman, England) continued by evaporation of filtrate using a vacuumed Rotary Evaporator (Buchii, Switzerland). The dried crude extract was weighed and dissolved in methanol and stored at -20°C for further analyses.

Determination of total phenolic compound

Total phenolic content of the extract was determined colorimetrically using the Folin-Ciocalteu method as illustrated by Ismail et al. (2010). The extract was measured at absorbance 765 nm and the result expressed as milligrams of gallic acid equivalents (GAE) per gram of dry matter (DM). The assay was repeated three times.

Determination of total flavonoid compound

Total flavonoid content was determined using standard flavonoid rutin as described by Ismail et al. (2010). The extract was measured using absorbance at 510 nm and the result was expressed as milligrams of rutin equivalents per gram of dry matter. The assay was repeated three times.

Reversed phase-high performance liquid chromatography (RP-HPLC) analyses of phenolic and flavonoid compounds

The phenolic and flavonoid compounds of saffron were quantitatively measured by a RP-HPLC technique based on the method described by Crozier et al. (1997) with some modifications. The phenolic compound standards used were gallic acid, syringic acid, pyrogallol, salicylic acid, and caffeic acid, and the flavonoid compound standards were quercetin, rutin, myricetin, kaempferol, naringin and apigenin while the isoflavonoid were genistein and daidzein.

An aliquot of sample extract was loaded on an Agilent-1200 series HPLC instrument equipped with an ultraviolet (UV)-Vis photodiode array (DAD) detector, binary pump, vacuum degasser, auto sampler and an analytical column (Intersil ODS-3 5 µm 4.6 × 150 mm GI Science Inc). The solvents used were deionized water and acetonitrile, whilst the pH of water was adjusted with trifluoroacetic acid to 2.5.

The phenolic and iso-flavonoid compounds were detected at 280 nm, while flavonoid compounds were detected at 350 nm. The column was equilibrated by 85% solvent A (which is it) and 15% solvent B then the ratio of solvent B was increased to 85% in 50 min followed by reducing solvent B to 15% in 55 min. This ratio continued to the 60th min for the next analysis at a flow rate at 0.6 ml/min. The analyses were conducted in triplicates.

Free radical scavenging activity

The free radical scavenging activities of the extracts were determined as reported by Gulcin et al. (2004). All determinations were conducted in three replications. Lower absorbance values of the reaction mixture indicated higher free radical scavenging activity. The free radical scavenging activities of the tested samples were expressed as percentage of inhibition and were calculated according to the following equation (Yen and Duh, 1994):

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

Table 1. Total phenolic and flavonoid compounds in *M. sativa* leaves extract.

Sample	Total flavonoid (mg rutin eq./g DM)	Total phenolic (mg GAE/g DM)
M. sativa	12.6 ± 0.17	37.0 ± 0.02

Table 2. Concentration of different phenolic compounds in *M. sativa* leaves extract.

Phenolic contents (µg g/g DM)				
Gallic acid	Pyrogallol	Caffeic acid	salicylic acid	Syringic acid
452.5 ± 0.11	715.2 ± 0.07	348.9 ± 0.02	116.1 ± 0.09	ND

ND, Not detected.

Table 3. Concentration of different flavonoids and isoflavonoids compounds in *M. sativa* leaves extract.

Flavonoid and isoflavonoid contents (µg/g DM)							
Apigenin	Kaempferol	Myricetin	Naringin	Quercetin	Rutin	Daidzein	Genistein
213.9 ± 0.05	ND	456.6 ± 0.02	738.2 ± 0.12	574.21 ± 0.06	ND	335.1 ± 0.04	ND

ND, Not detected.

Where A_0 is the absorbance value of the blank sample or control reaction and A_1 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 value for each of the test sample was presented as inhibition curve at 50% or IC_{50}.

Ferric reducing antioxidant power (FRAP)

The ferric reducing property of the extracts was determined using an assay described by Yen and Chen (1995). The assay was carried out in triplicate. BHT, α-tocoferol and Vitamin C were used were used as standard antioxidant.

Nitric oxide (NO) scavenging activity

The NO-scavenging activity of extracts was determined according to Tsai et al. (2007). The activity was expressed as percentage of inhibition and IC50 (extract concentration to quench 50% of the NO radicals released by sodium nitroprusside. The experiment was performed in triplicate.

XO inhibitory activity

The XO inhibitory activity was performed based on Orhan et al. (2007). Twenty microliters (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 dimethyl sulfoxide (DMSO) in a 96-well plate and incubated for 10 min at room temperature. 20 µl of 0.1 mM xanthine was added to the mixture. The uric acid formation was measured by a spectrophotometer (Molecular Devices Inc., USA) at 295 nm. Allopurinol was used as a positive control. The experiment was performed in triplicate.

Anti-inflammatory assay

The presence of nitrite was determined in cell culture media by Griess reagent and absorbance was read at 550 nm using a micro plate reader (Spectra Max Plus 384, Molecular Devices Inc., USA). Nitrite concentration in the supernatants was determined by comparison with a sodium nitrite standard curve. The amount of cell viability was detected by 3-4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay. N-Nitro-L-Arginine Methyl Ester (L-NAME) was used as iNOS inhibitor (control) at concentration 250 µM (Ahmad et al., 2005). The experiment was performed in triplicate.

RESULT

Total Phenolics and flavonoids contents

Phenolic and flavonoid compounds belong to large secondary metabolites which are synthesized by plants and indicated several biological activities in human (Marinova et al., 2005). The obtained result showed that the total flavonoids was 12.62 ± 0.17 mg rutin equivalent/g dry matter and total phenolics value was37.0 ± 0.02 mg GAE/g dry matter (Table 1).

RP-HPLC analysis of phenolic and flavonoid compounds

In this study, the RP chromatography was used to determine the phenolic and flavonoid compounds in leaves of *M. sativa* leaves extracts. The RP-HPLC analyses indicated the presence of gallic acid, pyrogallol, caffeic acid and salicylic acid as phenolics, while the main flavonoids and isoflavonoid were apigenin myrcitin, naringenin, quercitin and daidezin (Tables 2 and 3). The HPLC chromatograms shows the various type of phenolic and flavonoid compounds in the leaves of *M. sativa*

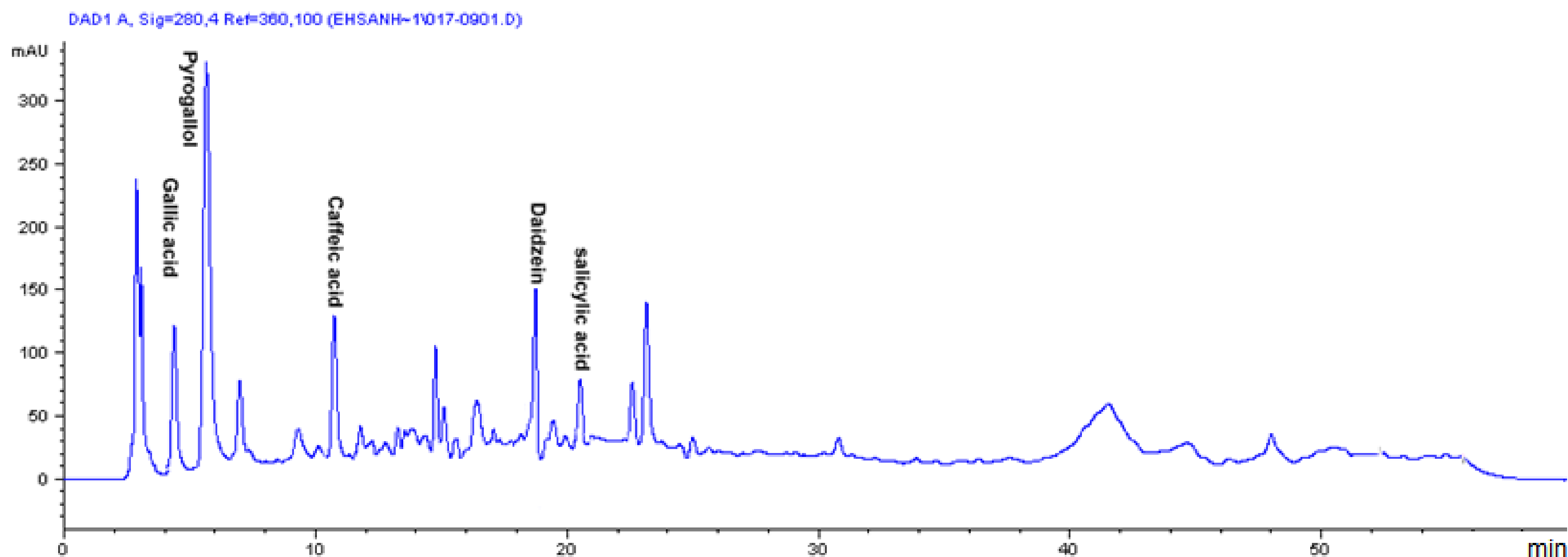

Figure 1. The RP-HPLC chromatogram of phenolic and isoflavonoid compounds in the *M. sativa* leaves extract.

extracts (Figures 1 and 2).

Antioxidant activity

Free radical scavenging activity

The diphenyl-1-picrylhydrazyl (DPPH) method was applied since this method relatively required shorter time compared to other methods to evaluate the free radical scavenging activity of the plants extract. The result of the DPPH scavenging activity indicated a steady increase in the scavenging activity of free radicals in the extract and the standards in the range of 0 to 250 μg/ml (Figure 3). The antioxidant activity of Alfalfa by DPPH method showed 54.42% inhibition of free radicals at the concentration of 250 μg/ml of Alfalfa crude extract, while this value for the Vitamin E and C were 90.25 and 91.06%, respectively.

FRAP

Transition metals like iron are potentially pro-oxidative and the capability of an antioxidant to maintain the transition metal in its reduced state is an effective means of preventing lipid oxidation (Halliwell, 2007). The FRAP assay is a commonly used assay to evaluate the antioxidant potent of fruits and vegetables. FRAP assay similar to the DPPH results, indicated that the reductive potential of leaf extracts and standards increased with increasing samples concentration (Figure 4). The obtained results showed 56.71% inhibition of free radicals at the concentration of 250 μg/ml of Alfalfa crude extract, while this value for the BHT and Vitamin C were 89.02 and 92.21%, respectively.

NO scavenging activity

NO inhibition assay as one of antioxidant potential

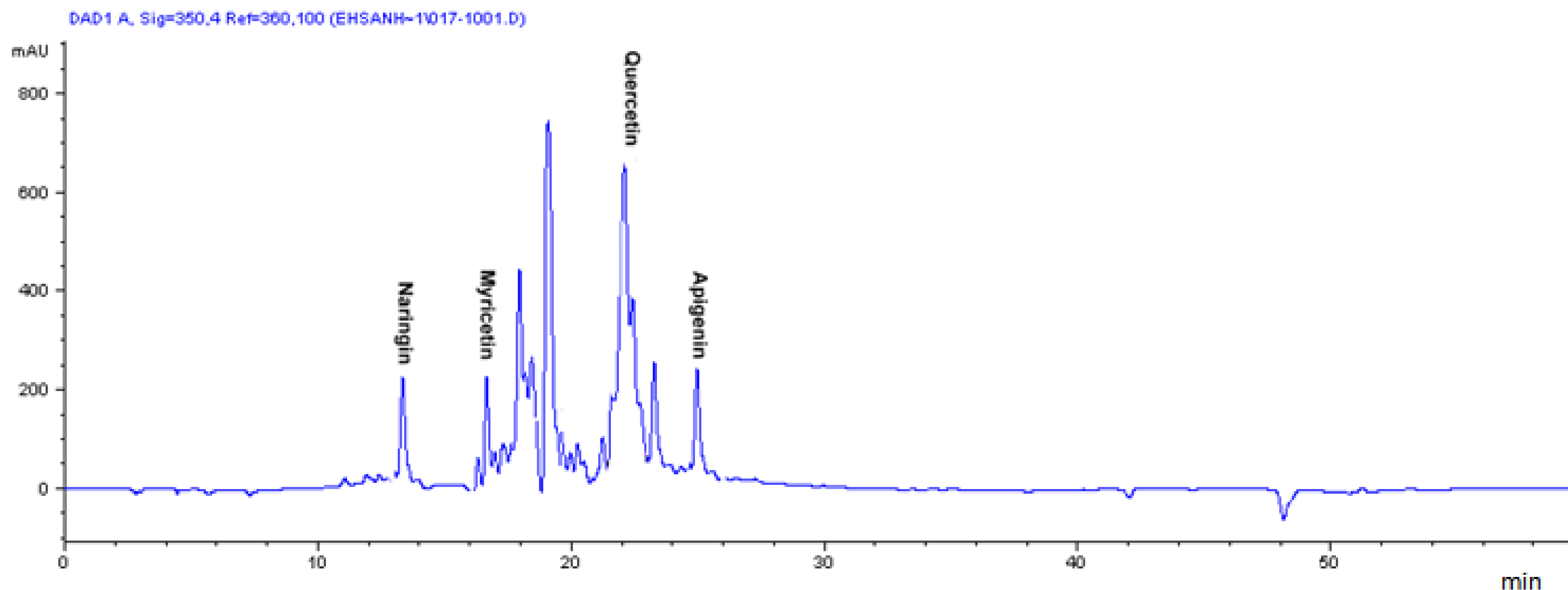

Figure 2. The RP-HPLC chromatogram of flavonoid compounds in the leaves extract of *M. sativa* leaves extract.

indicator showed 50.99% inhibitory activity of NO at the concentration of 250 µg/ml, while this value for Vitamin C and E were 94.13 and 91.52%, respectively (Figure 5).

Anti-inflammatory activity

Anti-inflammatory activity of leaves extract of *M. sativa* were carried out based on colorimetric NO assay. In this study, extracts were examined for their inhibitory ability on NO released in RAW 264.7 cells, stimulated with bacterial lipopolysaccharides (LPS) and interferon-gamma (IFN-γ). All extracts were assayed at the concentration of two-fold dilution range (7.8 to 500 µg/ml) dissolved in 0.2% DMSO and compared with L-NAME. The viability of RAW 264.7 cells after treatment was assessed by MTT method. The activity profiles of the extracts in terms of percentage of NO inhibition were categorized according to Kim et al. (1998). Inhibition rate of NO production in LPS/IFN-γ stimulated RAW 264.7 cell line and can be classified into four ranks which are: strongly active (70% and above), moderately active (50 to 69%), weakly active (30 to 49%) and very weak active (29% or less). Alfalfa exhibited moderate anti-inflammatory activity where the 50% of the NO production by the induced RAW 264.7 cells was inhibited at the concentration of 147.2 µg/ml of Alfalfa crude extract. The cell viability test (MTT assay) showed that Alfalfa extract was not toxic to macrophage RAW 264.7 cells (Figure 6).

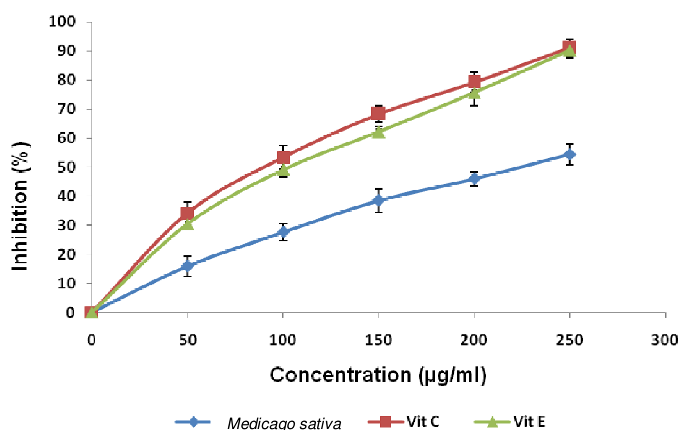

Figure 3. Free radical scvenging activity of *M. sativa* leaves extract, Vitamin C and E. n = 3.

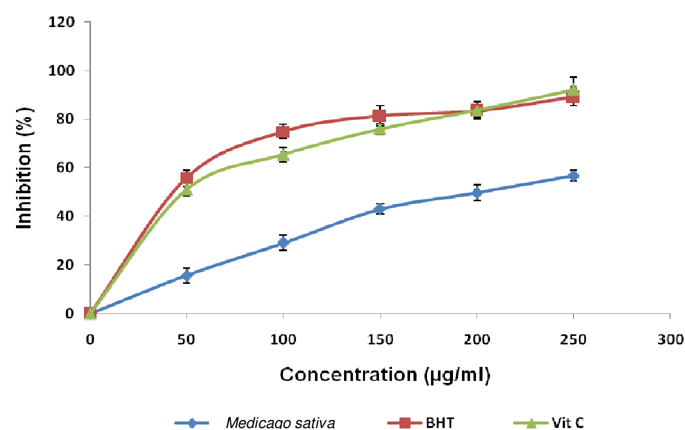

Figure 4. Ferric reducing activity power of *M. sativa* leaves extract, BHT and vitamin C. n = 3.

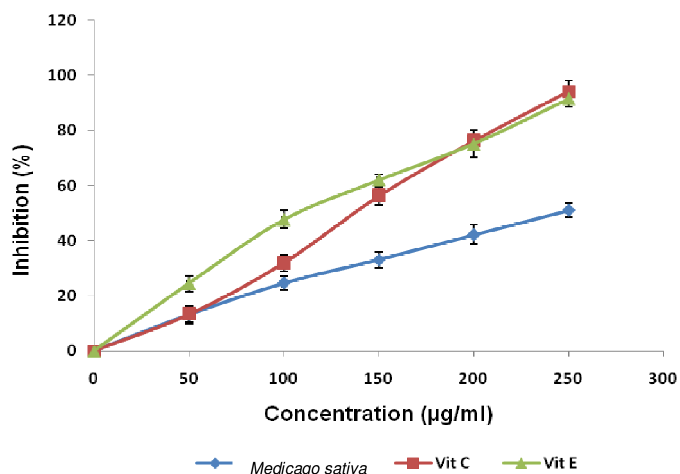

Figure 5. NO scavenging activity of leaves extract of *M. sativa* leaves extract, Vitamins C and E. n = 3.

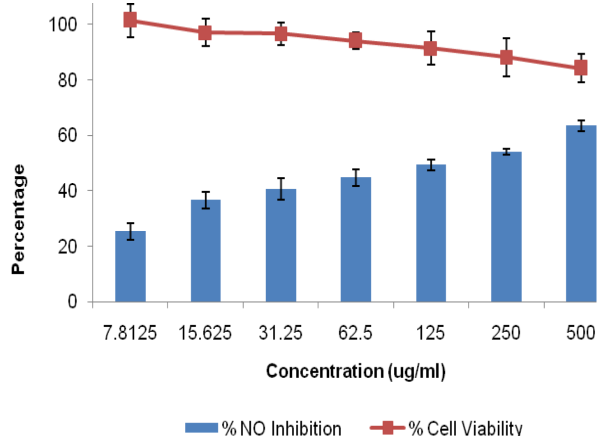

Figure 6. Percentage values of NO inhibition and cell viability of *M. sativa* leaves extract. All values represent mean ± standard deviation.

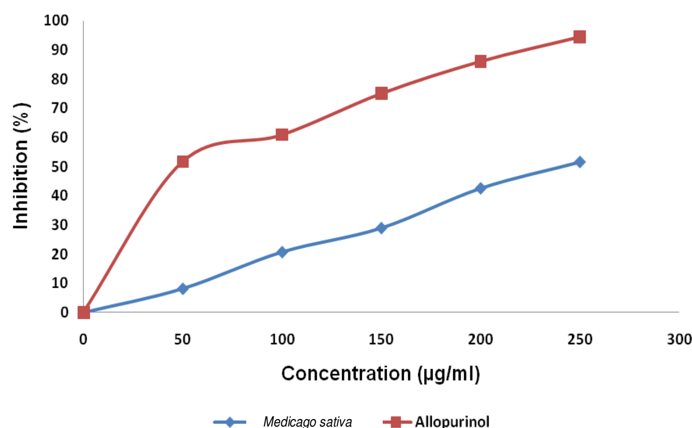

Figure 7. XO inhibitory activity of leaves extract of *M. sativa* leaves extract and allopurinol n = 3.

XO inhibitory activity

The leave extracts XO inhibitory activities are shown in Figure 7. Inhibition of XO led to a decrease in production of uric acid, which was determined spectrophotometrically. The crude extract inhibited the XO in dose-dependent manner. The inhibition of *M. sativa* and allopurinol (positive control) at the concentration of 250 µg/ml were 51.63 and 94.49%, respectively.

DISCUSSION

In the present study, the phenolics and flavonoids content and antioxidant, anti-inflammatory and XO inhibitory acti-

vities of *M. sativa* leaves extract were investigated. The overall results showed gallic acid, pyrogallol, salicylic acid, caffeic acid as phenolics and naringenin, apigenin, quercitin, myrcitin and daidezin are the main flavonoids and isoflavonoid compounds in the leaves extract of *M. sativa.*

The functional properties including antioxidant, anti-inflammatory and XO inhibitory activities observed in this study attributed to the presence of phenolics and flavonoids. These results are in agreement with Manpong et al. (2009) and Nicolis et al. (2008) who reported that some flavonoids and phenolic compounds such as pyrogallol, gallic acid, naringin and quercetin possess antioxidantive properties as well as anti-inflammatory activities. In another research conducted by Tepe and Sokmen (2007), a positive correlation between total phenolic content with antioxidant activity of *Tanacetum subspecies* has been reported. In addition, Firuzi et al. (2005) has also stated the direct relationship among phenolics and flavonoids content with FRAP of plant extract. They reported that flavonols such as quercitin, fisetin and myricetin showed the highest FRAP value compared to others subclass of flavonoid.

As phenolic and flavonoid compounds occur ubiquitously in plants and a variety of biological activities such as antimicrobial, antiviral, anti-ulcerogenic, cytotoxic, anti-neoplastic, mutagenic, antioxidant, anti-hepatotoxic, anti-hypertensive, hypolipidemic, anti-platelet and anti-inflammatory activities were attributed to them (Karimi et al., 2010).

Many of these biological functions corresponded to their free radical scavenging and antioxidant activities (Oskoueian et al., 2011a). Antioxidants are responsible in preventing oxidative damages to the cellular components as a consequence of biochemical reactions. Some phenolics and flavonoids appeared to be more active than vitamins for this purpose and their activities depend on the structure and total number of hydroxyl groups (Prochazkova et al., 2011).

Conclusion

The *M. sativa* leaves extract was found to possess various beneficial phenolics and flavonoids together with isoflavonoids. The antioxidant, anti-inflammatory and XO inhibitory activities observed in *M. sativa* could be due to the presence of various detected phenolic and flavonoid as active compounds.

The functional properties observed in the extract obtained from leaves of *M. sativa* are beneficial for pharmaceutical, food and feed industries. For instance, the crude extract could be applied as a natural source of antioxidant in the human and animal nutrition to maintain and preserve the diet quality. Further experiments on evaluation of different extraction methods in order to have efficient and higher yield extract with enhanced biological activities are in progress.

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