IN VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING STUDIES OF ALCOHOLIC EXTRACT OF *MEDICAGO SATIVA* L.

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Free radicals are implicated for many diseases including diabetes mellitus, arthritis, cancer, ageing. *etc*. In the treatment of these diseases, antioxidant therapy has gained utmost importance. Alcoholic extract of *Medicago sativa* was studied for its *in vitro* antioxidant activity using different models *viz.* DPPH radical scavenging, ABTS radical scavenging, iron chelating activity, lipid peroxidation assay, nitric oxide scavenging assay, and alkaline DMSO assay. The results were analyzed statistically by the regression method. Its antioxidant activity was estimated by IC_{50} value and the values are 100.38 µg/ml (DPPH radical scavenging), 12.33 µg/ml (ABTS radical scavenging), 115.79 µg/ml (Iron chelating activity) and 49.06 µg/ml (lipid peroxidation), 21.77 µg/ml (nitric oxide scavenging) and 15.91 µg/ml (alkaline DMSO). In all the testing, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals, metal chelation or inhibition of lipid peroxidation. The antioxidant property may be related to the polyphenols and flavonoids present in the extract. These results clearly indicate that *Medicago sativa* is effective against free radical mediated disease.

Key words: DPPH, lipid peroxidation, nitric oxide, *Medicago sativa*, superoxide.

INTRODUCTION

The plant *Medicago sativa L*. (Alfalfa) is locally known as "buffalo herb of Lucern". Alfalfa (*Medicago sativa L*.) sprouts are often consumed as vegetable salad and their leaves or seeds are also sold as bulk powdered herb, capsules, and tablets for nutritional supplement in health food stores (Nadkarni, 1976). The extracts from alfalfa sprouts, leaves, and roots have been indicated to be helpful in lowering cholesterol levels in animal and human studies (Story, 1984, Molgaard, 1987, Colodny, 2001). In addition, traditional medicinal use of alfalfa sprouts or leaves includes treatment of arthritis, kidney problems, Anodyne, Anti-Cancer, Anti-rheumatic, Cardiotonic, Depurative, Lactagogue, Fever, Emmenagogue, Antiscorbutic and boils (Duke, 1990, Barnes, 2002). However, these treatments still need to be scientifically examined. Our previous study showed that the ethyl acetate extract of alfalfa sprouts ameliorates the autoimmune-prone disease of

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lupus mice, probably by attenuating cytokine and inflammatory responses (Hong, 2009). It is also used in the treatment of neurovegetative menopausal symptoms, as pancreatic and extra-pancreatic effects of the traditional anti-diabetic plant (Ranger, 2004) and the anti-inflammatory effect of the ethyl acetate extract of alfalfa sprouts with *in vitro* cell culture and through *in vivo* LPS-induced inflammatory mice have been reported (Zhao, 1993, Hong, 2009, De Leo, 1998, Gray, 1997).

The purpose of this study was to evaluate *Medicago sativa* as a new potential source of natural antioxidants.

MATERIAL AND METHODS

All chemicals and solvents were of analytical grade and were obtained from Ranbaxy Fine Chemicals, Mumbai, India.

Plant material. The fresh roots of *M. sativa* were collected from the local region of Rajkot, Gujarat, India in November 2007 and were authenticated by Dr. Vishal Muliya, Professor, Department of Botany,Christ College, Udupi, Karnataka, India. A voucher specimen (DPS/Herbs/20) has been deposited at the Museum of the Department of Pharmacognosy, Department of Pharmaceutical Sciences, Rajkot, Guajrat, India.

Plant extract. About 500 g of the root powder was taken and extracted in a soxhelet extractor with methanol (2 Lit.). The crude extract was concentrated to dryness in a rotary flash evaporator under reduced pressure and controlled temperature (40–50 ^{o}C). The extract was preserved in vacuum desiccators for subsequent use in study.

DPPH radical scavenging assay. To the methanolic solution of DPPH (1 mM) an equal volume of the extract dissolved in alcohol was added at various concentrations from 2 to 1000 µg/ml in a final volume of 1.0 ml. An equal amount of alcohol was added to the control. After 20 min, absorbance was recorded at 517 nm. Experiment was performed in triplicate (Sreejayan, 1996, John, 1984).

ABTS radical scavenging assay. To the reaction mixture containing 0.3 ml of ABTS radical, 1.7 ml phosphate buffer and 0.5 ml extract was added at various concentrations from 2 to 500 µg/ml. Blank was carried out without drug. Absorbance was recorded at 734 nm. Experiment was performed in triplicate (Sreejayan, 1996, John, 1984).

Iron chelating activity assay. The reaction mixture containing 1 ml O-Phenanthroline, 2 ml Ferric chloride, and 2 ml extract at various concentrations ranging from 2 to 1000 µg/ml in a final volume of 5 ml was incubated for 10 minutes at ambient temperature. The absorbance at 510 nm was recorded. Ascorbic acid was added instead of extract and absorbance obtained was taken as equivalent to 100% reduction of all ferric ions. Blank was carried out without drug.

Experiment was performed in triplicate (Sreejayan, 1996, John, 1984, Benzie, 1996).

Lipid peroxidation assay. The mixture (Egg phosphatidylcholine in 5 ml saline) was sonicated to get a homogeneous suspension of liposome. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1 ml). The pink chromogen was extracted with a constant volume of n-butanol and absorbance of the upper organic layer was measured at 532 nm. The experiment was performed in triplicate (Sudheerkumar, 2003).

Nitric oxide radical scavenging. Sodium nitroprusside 5 mM was prepared in phosphate buffer pH 7.4. To 1 ml of various concentrations of test compound, sodium nitroprusside 0.3 ml was added. The test tubes were incubated at 25 ºC for 5 hr after which, 0.5 ml of Griess reagent was added. The absorbance of the chromophore was read at 546 nm. The experiment was performed in triplicate (Sreejayan, 1996).

Superoxide scavenging. Alkaline DMSO was used as a super oxide generating system. To 0.5 ml of different concentrations of the test compound, 1 ml of alkaline DMSO and 0.2 ml of NBT 20 mM in phosphate buffer pH 7.4 was added. The experiment was performed in triplicate (Govindarajan, 2003).

Statistical analysis. All results are expressed as mean ± S.E.M. Linear regression analysis (Origin 6.0 version) was used to calculate the IC_{50} values.

RESULTS AND DISCUSSION

Several concentrations ranging from 2–1,000 μg/ml of the alcoholic extract of *M. sativa* were tested for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models. The percentage scavenging and IC_{50} values were calculated for all models given in Table 1.

Table 1

Comparison of IC_{50} values of extract with standard

Crt. No.	Model	IC_{50} value of Methanolic extract (μg/ml)	IC_{50} value of standard (μg/ml)
1.	DPPH radical scavenging activity	100.381	9.9982
2.	ABTS radical scavenging activity	12.33	36.42
3.	Iron chelating method	115.79	2.017
4.	Lipid peroxidation method	49.060	30.67

Table 1
(continued)

Crt. No.	Model	IC_{50} value of Methanolic extract (μg/ml)	IC_{50} value of standard (μg/ml)
5.	Nitric oxide scavenging method	21.77	38.68
6.	Super oxide scavenging method	15.91	16.33
7.	Total antioxidant capacity	10 mg/ml Ethanolic extract of *M. sativa* is equivalent to 91.0 μg/ml of ascorbic acid	

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing (Joyce, 1987, Velioglu, 1998). The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm (Ganapaty, 2007) (Fig. 1).

The antioxidant activity of the extract by this assay implies that action may be by either inhibiting or scavenging the ABTS radicals since both inhibition and scavenging properties of antioxidants towards this radical have been reported in earlier studies (Rice-Evans, 1997) (Fig. 2).

O-phenanthroline quantitatively forms complexes with Fe^{+2} which get disrupted in the presence of chelating agents (Mahakunakorn, 2004). The alcoholic extract interfered with the formation of a ferrous-o-phenanthroline complex, thereby suggesting that the extract has metal chelating activity (Fig. 3).

Initiation of the lipid peroxidation by ferrous sulphate takes place either through the ferryl-perferryl complex or through $^{\cdot}OH$ radical by Fenton's reaction. Thus the decrease in the MDA level in egg phosphatidylcholine with the increase in the concentration of the extract indicates the role of the extract as an antioxidant (Fig. 4).

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity (Hagerman, 1998). *M. sativa* inhibit nitric oxide in a dose dependent manner (Fig. 5).

Superoxide can cause oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyse the breakdown of superoxide radical (Shirwaiar, 2007). In our study, alkaline DMSO used for superoxide generation indicates that *M. sativa* is a potent superoxide scavenger (Fig. 6).

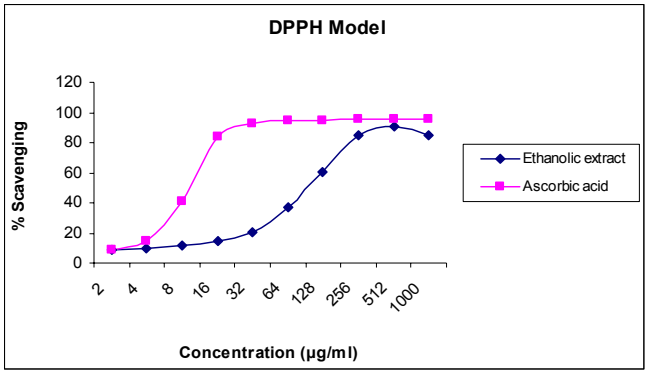

Fig. 1. Antioxidant activity of different concentrations of alcoholic extract and ascorbic acid in DPPH radical scavenging method. Each value represents mean ± SEM.

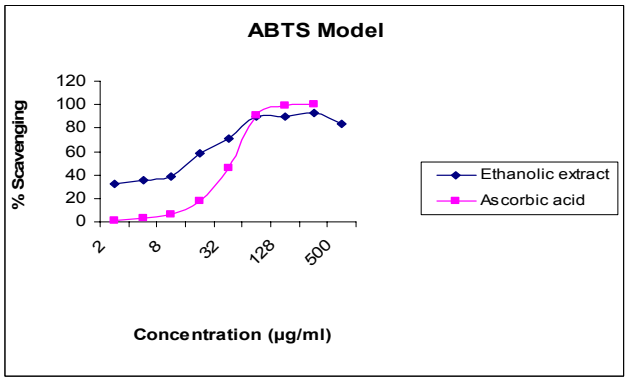

Fig. 2. Antioxidant activity of different concentrations of alcoholic extract and ascorbic acid in ABTS radical scavenging method. Each value represents mean ± SEM.

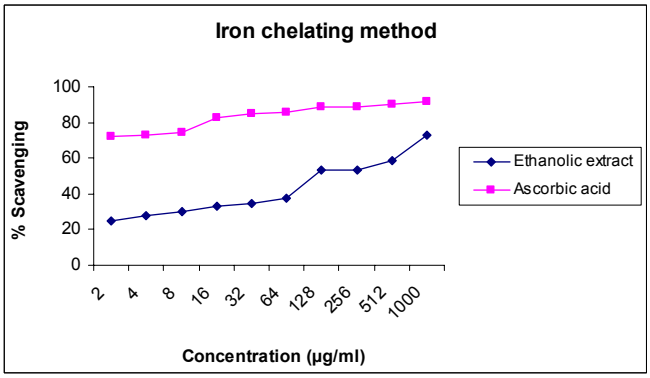

Fig. 3. Antioxidant activity of different concentrations of alcoholic extract and ascorbic acid in iron chelating method. Each value represents mean ± SEM.

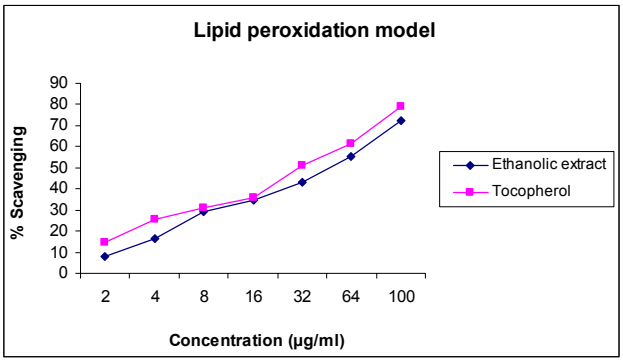

Fig. 4. Antioxidant activity of different concentrations of alcoholic extract and ascorbic acid in lipid peroxidation method. Each value represents mean ± SEM.

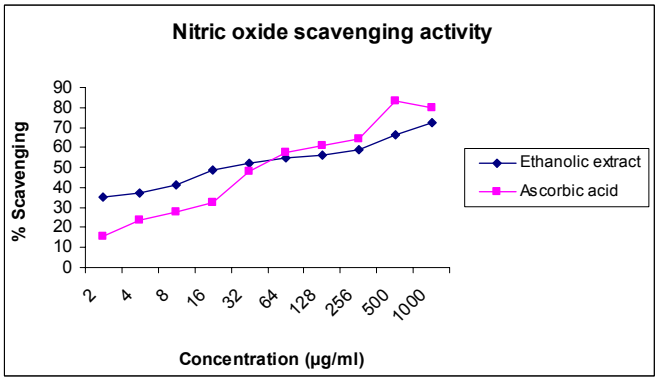

Fig. 5. Antioxidant activity of different concentrations of alcoholic extract and ascorbic acid in nitric oxide scavenging method. Each value represents mean ± SEM.

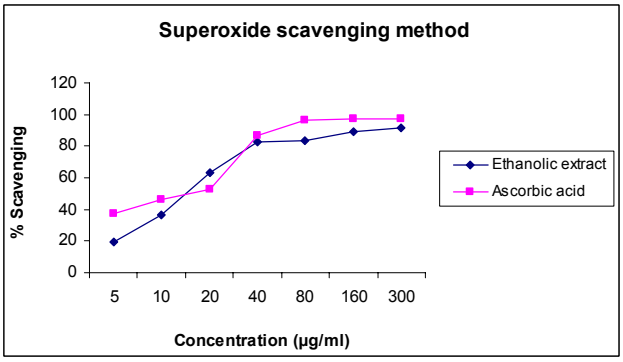

Fig. 6. Antioxidant activity of different concentrations of alcoholic extract and ascorbic acid in superoxide scavenging method. Each value represents mean ± SEM.

CONCLUSION

The results of the present study show that the extract of *M. sativa* contains the highest amount of polyphenol compounds and exhibits the greatest antioxidant activity through the scavenging of free radicals which participate in various pathophysiology of diseases including ageing.

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