PHYTOCHEMICAL ANALYSIS AND IN VITRO FREE RADICAL SCAVENGING ACTIVITIES OF MEDICAGO SATIVA SEEDS

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ABSTRACT

Phytochemical analysis and in vitro free radical scavenging activities were analyzed in the various extracts of Medicago sativa seeds. The phytochemical analysis showed the presence of alkaloids, carbohydrates, flavonoids, glycosides, saponins, phytosterols, tannins, terpenoids and phenols. Among the various extracts, phytochemicals were extracted best in ethanol. Free radical scavenging activities such as 2,2'-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, superoxide, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ferrous ion chleting activity and non radicals such as hydrogen peroxide and nitric oxide were analyzed in the various extracts of Medicago sativa seeds and were compared with standard antioxidant ascorbic acid. All the extracts of Medicago sativa seeds scavenged the free radicals in a concentration dependent manner. The antioxidative activity of all the extracts was found to be more pronounced than that of the standard antioxidant ascorbic acid. Among the various extracts, the antioxidant activity was found to be more pronounced in ethanolic extract of Medicago sativa seeds.

Keywords: Medicago sativa, Alfalfa, phytochemicals, DPPH, ABTS, free radicals and antioxidant.

1. INTRODUCTION

Plant materials remain an important resource to combat serious diseases in the world. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries. The medicinal value of these plants lies in some chemical active substances that produce a definite physiological action on the human body. The most important bioactive constituents of plants are alkaloids, tannin, flavonoid and phenolic compounds (Edeoga et al., 2005).

Reactive oxygen species such as superoxide anions (O2•−), hydroxyl, nitric oxide radicals and hydrogen peroxide (H2O2) play an important role in oxidative stress related to the pathogenesis of various important diseases (Halliwell and Gutteride, 1999; Finkel and Holbrook, 2000). Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals. Antioxidant based drugs/formulations for the prevention and treatment of complex diseases, like atherosclerosis, stroke, diabetes, Alzheimer’s disease and cancer, have appeared in the last three decades (Devasagayam et al., 2004).

Medicago sativa L., or Alfalfa is a member of Leguminosae or Fabaceae family, which has a long history of dietary and medicinal uses in traditional herbal medicine in China, America, India and many Middle Eastern countries for the treatment of a variety of ailments and is also used as a fodder for animals (Al-Dosari, 2012). 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. The extracts from alfalfa sprouts, leaves and roots have been indicated to be helpful in lowering cholesterol levels in animal and human studies (Hong et al., 2009). In this regard, the present investigation was aimed to analyze the phytochemical constituents and free radical scavenging activity of M. sativa raw seeds and germinated seeds. The present study was aimed to analyze the phytochemical constituents and in vitro free radical scavenging activities of M. sativa seeds.

2. MATERIALS AND METHODS

2.1. Plant material

The M.sativa seeds were collected from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India and authenticated (BSI/SRC/5/23/2014-15/Tech/394) by the authority of the botanical survey of India (BSI), Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

2.2. Preparation of the plant sample

After that, the seeds were washed with water to remove contamination and shade dried at room
temperature. The dried samples were ground into coarse powder with the help of mechanical grinder and stored in airtight containers for further studies. 20 grams of powdered samples were weighed and wrapped separately with whatmann No.1 filter paper and extracted using various solvents such as petroleum ether, benzene, chloroform, ethyl acetate, ethanol, methanol and aqueous with the help of soxhlet extractor. Then, the various plant extracts were concentrated using rotary evaporator and then preserved for future analysis.

2.3. Qualitative analysis of phytochemicals

The phytochemical constituents of various extracts of Medicago sativa seeds were qualitatively analyzed using standard procedure (Iyengar, 1995; Siddiqui and Ali, 1997; Raaman, 2006).

3.4. Determination of free radical scavenging activity

Free radical scavenging activities such as DPPH, hydroxyl, hydrogen peroxide, nitric oxide, superoxide, ABTS and ferrous ion radical scavenging activities were analyzed using standard procedures (Carter, 1971; Misra and Fridovich, 1972; Green et al., 1984; Ruch et al., 1989; Elizabeth and Rao, 1990; Mensor et al., 2001; Shirwaiker et al., 2006).

3. RESULTS AND DISCUSSION

Table 1 indicates the phytochemical analysis of Medicago sativa seeds. The alkaloids and flavonoids were found to be present in all the extracts including petroleum ether, benzene, ethanol, methanol and aqueous extracts. The anthraquinones were found to be present in all the extracts except petroleum ether and aqueous extract. Saponins were present in chloroform, ethanol and methanol extracts. Phenols were present in all the extracts except ethyl acetate. Steroids were present in petroleum ether, benzene, ethyl acetate, and aqueous extracts. Tannins were found in chloroform, ethyl acetate, ethanol and methanol extracts. Glycosides were found to be present in all the extracts except benzene and ethyl acetate extracts.

| Phytochemical constituents | PE | B   | C   | EA  | E   | M   | A   |
|----------------------------|----|-----|-----|-----|-----|-----|-----|
| Amino acids                | -  | -   | -   | +   | +   | +   | -   |
| Anthraquinones             | -  | +   | +   | +   | +   | +   | -   |
| Alkaloids                  | +  | +   | -   | -   | +   | +   | +   |
| Carbohydrates              | +  | +   | -   | +   | +   | -   | -   |
| Flavonoids                 | +  | -   | -   | +   | +   | +   | -   |
| Glycosides                 | -  | -   | -   | +   | +   | -   | -   |
| Phenols                    | +  | +   | -   | -   | -   | -   | -   |
| Saponins                   | -  | -   | +   | -   | +   | -   | +   |
| Steroids                   | +  | +   | -   | +   | +   | -   | -   |
| Tannins                    | +  | -   | -   | +   | +   | +   | -   |
| Terpenoids                 | +  | -   | -   | +   | +   | +   | +   |

Among the various extracts, the ethanolic extract of Medicago sativa seeds showed maximum phytochemical constituents. So, the ethanolic extract of Medicago sativa seeds were taken for further study.

The phytochemical screening of C. lanatus seeds showed the presence of phytochemical constituents such as alkaloids, flavonoids, tannins, aminoacids, carbohydrates, cardioglycosides, terpenoids, oils and fats in the ethanolic extract of plant material when compared with other solvents (Varghese et al., 2013).

3.1. Free radical scavenging activity

The scavenging of stable DPPH radical model is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH radical is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997).

Figure 1 indicates the DPPH radical scavenging activity of various extracts of Medicago sativa seeds. Ethanolic extract of Medicago sativa seeds showed the highest DPPH scavenging activity which was followed by standard antioxidant ascorbic acid, methanol and chloroform extracts. This may due to the extraction of maximum phytochemicals in ethanolic extract. The least activity was noted in aqueous extract.
3.2. Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology capable of damaging the biomolecules of living cells (Zhang et al., 2009).

Figure 2 illustrates the hydroxyl radical scavenging activity of various extracts of Medicago sativa seeds. Ethanolic extract of Medicago sativa seeds showed the highest radical scavenging activity which was followed by standard antioxidant ascorbic acid, methanol and ethyl acetate extracts. The least activity was noted in aqueous extract.

3.3. Scavenging of hydrogen peroxides (H₂O₂)

\[ \text{H}_2\text{O}_2 \] is highly important because of its ability to penetrate biological membranes. \( \text{H}_2\text{O}_2 \) itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. Thus, removing \( \text{H}_2\text{O}_2 \) is very important for the protection of living systems.

Figure 3 illustrates the hydrogen peroxide radical scavenging activity of various extracts of Medicago sativa seeds. Ethanolic extract of Medicago sativa seeds showed the highest radical scavenging activity which was followed by standard antioxidant ascorbic acid and methanol. The least activity was noted in aqueous extract.

3.4. Nitric oxide scavenging activity

Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases. Nitric oxide is a very unstable species under aerobic condition. It reacts with \( \text{O}_2 \) to produce stable product nitrate and nitrite through intermediates \( \text{NO}_2 \), \( \text{N}_2\text{O}_4 \) and \( \text{N}_3\text{O}_4 \) (Jayakumari et al., 2012).

Figure 4 illustrates the nitric oxide radical scavenging activity of various extracts of Medicago sativa seeds. Ethanolic extract of Medicago sativa seeds showed the highest radical scavenging activity which was followed by standard antioxidant ascorbic acid, methanol and ethyl acetate extracts. The least activity was noted in aqueous extract.

3.5. Superoxide anion radical scavenging activity

Superoxide anion radicals are produced endogenously by flavoenzymes like xanthine oxidase, which converts hypoxanthine to xanthine and subsequently to uric acid in ischemia-reperfusion (Bora and Sharma, 2010). Superoxide is generated \textit{in vivo} by several oxidative enzymes, including xanthine oxidase. In PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with
antioxidants indicates the consumption of superoxide anion in the reaction mixture.

Fig. 5. Superoxide anion radical scavenging activity of various extracts of *Medicago sativa* seeds

Figure 5 illustrates the inhibition of superoxide radical generation of various extracts of *Medicago sativa* seeds. Ethanolic extract of *Medicago sativa* seeds showed the highest radical scavenging activity which was followed by standard antioxidant ascorbic acid, methanol and ethyl acetate extracts. The least activity was noted in aqueous extract.

3.6. ABTS radical scavenging activity

ABTS is frequently used by the food industry and agricultural researcher to measure the antioxidant capacities of foods (Peiyuan *et al.*, 2010). Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals. ABTS is a blue green chromogen and this assay is based on the ability of the antioxidants to scavenge long – life radical cation ABTS.

Fig. 6. ABTS radical scavenging activity of various extracts of *Medicago sativa* seeds

Figure 6 illustrates the ABTS radical scavenging activity of various extracts of *Medicago sativa* seeds. Ethanolic extract of *Medicago sativa* seeds showed the highest radical scavenging activity which was followed by standard antioxidant ascorbic acid, methanol and ethyl acetate extracts. The least activity was noted in aqueous extract.

3.7. Ferrous metal ion chelating activity

The chelating of ferrous ions by various extracts of Medicago sativa seeds were estimated by the method of Carter. Ferrozine can quantitatively form complexes with Fe$^{2+}$. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of colour reduction, therefore, allows estimation of the chelating activity of the coexisting chelator. In this assay MMS and standard antioxidant compounds interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine. Iron can stimulate lipid peroxidation by Fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell, 1991; Gulcin *et al.*, 2003).

Fig. 7. Ferrous ion chelating activity of various extracts of *Medicago sativa* seeds.

Figure 7 illustrates the ferrous ion radical scavenging activity of various extracts of *Medicago sativa* seeds. Ethanolic extract of *Medicago sativa* seeds showed the highest radical scavenging activity which was followed by standard antioxidant ascorbic acid and methanol. The least activity was noted in aqueous extract.

5. CONCLUSION

From the results, it clearly indicates that *M. sativa* seeds possess powerful *in vitro* antioxidant activity. The encouraging results of various extracts *M. sativa* seeds with the various *in vitro* antioxidant tests proved the plant as a metal chelator, its
hydrogen donating ability, and effectiveness as scavengers of free radicals, superoxide anions, hydroxyl and hydrogen peroxide. Further work will be carried out to isolate and elucidate the bioactive principle(s) responsible for the antioxidant activity.

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