

Research Article

Open Access

Nuraniye Eruygur, Burak Dincel, Nazire Gulsah Kutuk Dincel, Esra Ucar

Comparative study of *in vitro* antioxidant, acetylcholinesterase and butyrylcholinesterase activity of alfalfa (*Medicago sativa* L.) collected during different growth stages

<https://doi.org/10.1515/chem-2018-0088>

received January 20, 2018; accepted May 3, 2018.

Abstract: *Medicago* sp. are often consumed as vegetable, salad and herbal tea as a form of fresh leaves and herbs. It is also very important in animal feeding, because it contains high percentage of protein and some important phytochemicals. In addition, traditionally, the leaves or other parts of the plant were used for treatment and preventing of kidney disorders, osteoporosis, anemia, diabetes, ulcer, coronary diseases, some cancers, and menopausal symptoms. In previous reports, it has been demonstrated that this plant has scavenging activity of the free radicals, which are involve in the development of the hypertension, Ischemia, neurodegenerative and rheumatoid diseases. In this study, the antioxidant, enzyme inhibitory activities of *M. sativa* L. collected in two growing period and phenolic substances that may be responsible for these activities were investigated. In this way, the beneficial effects of the plant will be revealed and a scientific work will be provided for the evaluation of the pharmaceutical and food industry.

Keywords: *Medicago sativa*; different growing stages; antioxidant; enzyme inhibitory activities.

1 Introduction

Alfalfa (*Medicago sativa* L.) belongs to the family of Leguminosae and is used intensively all over the world

to feed animals. Alfalfa contains fiber, protein, saponins, flavonoids, minerals, vitamins and phenolic compounds that have potential therapeutic benefits [1-4]. In animal production, rich protein containing foods as well as effective digestible characteristics are important. *Medicago sativa* L. plant has been used as a reference forage in evaluating the digestibility of diets containing native shrubs [5]. The plant is used extensively in our country and all over the world as feeding plant for animals, therefore it is called as the queen of the forages [6]. Traditionally, the leaves or other parts of this plant were used for treatment and preventing of kidney disorders, osteoporosis, anemia, diabetes, ulcer, coronary diseases, some cancers, and menopausal symptoms [7-10]. In previous reports, it has been demonstrated that this plant has scavenging activity on free radical, which is result in hypertension, Ischemia, neurodegenerative and rheumatoid diseases [11].

According to the World Health Organization, traditional medicine are used as primary health care in developing countries. The new plants and bioactive compounds that have therapeutic properties, are important in traditional medicine [12-13]. Phenolic compounds are known antioxidants and are helpful in disease resistance [14-16]. The antioxidant molecules improve the body's cellular defense system against oxidative damage. The oxygen free radicals that can oxidize lipids, proteins, and even DNA, and antioxidant molecules are present in a balance. If this balance disturbed, the oxidative stress may occur. As a result of the oxidative stress, various diseases such as cancer, autoimmune disorders, aging, cardiovascular, and neurodegenerative diseases may develop [17-18].

Acetylcholinesterase is an enzyme responsible for acetylcholine hydrolysis by controlling acetylcholine concentration in the organism [19-20]. Acetylcholinesterase is known to have a more important role in the brain, studies conducted in recent years have shown that butyrylcholinesterase activity is increased and acetylcholinesterase activity is not altered in AD patients

***Corresponding author: Nuraniye Eruygur**, Department of Pharmacognosy, Faculty of Pharmacy, Selcuk University, Konya 42250, Turkey, E-mail: nuraniye.eruygur@selcuk.edu.tr

Burak Dincel: Department of Food Technology, Cumhuriyet University, Sivas 58140, Turkey

Nazire Gulsah Kutuk Dincel, Esra Ucar: Medicinal and aromatic plant Department, Cumhuriyet University, Sivas 58140, Turkey

[21]. Therefore, cholinesterase inhibitors are used for treatment of AD. However, cholinesterase inhibitors have some side effects. In recent years, much more attention has been focused on researching related biological activity of medicinal plants. There are some reports of cholinesterase and butyrylcholinesterase inhibitors from plant origin.

Diabetes mellitus (DM), is a life-long metabolic disease, develops as the result of insufficient production of insulin hormone by the pancreas gland in human body or the inability to use the hormone efficiently. As a result, people cannot use the sugar that is taken in from the food and blood sugar increases, suffering from hyperglycemia [22]. Therefore, regulating glucose level is important for controlling of the Type 2 diabetes. At present, the use of carbohydrate hydrolyzing enzymes namely α -amylase and α -glucosidase is one of the therapeutic strategies for control postprandial hyperglycemia by reducing the absorption of glucose [23-24]. However, carbohydrate metabolic enzyme inhibitors have some side effects such as vomiting, nausea, cramping, and abdominal pain [25]. Therefore, the effort to find alternative, more effective enzyme inhibitors without side effects from natural sources is increasing day by day.

In this study, the antioxidant, cholinesterase and α -glucosidase enzyme inhibitory activities of *M. sativa* L. collected in two growing period and phenolic substances that may be responsible for these activities were investigated. In this way, the beneficial effects of the plant will be revealed and a scientific work will be provided for the evaluation of the pharmaceutical and food industry.

2 Material and Method

Acetylcholinesterase (E.C.No.3.1.1.7) Type VI-S: from Electric Eel, Galanthamine, acetylthiocholine iodide, DTNB (dithiobis nitrobenzoic acid), potassium dihydrogen phosphate and dipotassium hydrogen phosphate obtained from Sigma, USA.

2.1 Plant material and preparation of extracts

The plant material was collected from the locality of Cumhuriyet University garden, Sivas in June 2016 before flowering and after flowering at two different season. The identification of the plant material was undertaken according to The Flora of Turkey by a senior botanist Dr. Erol Dönmez at Cumhuriyet University [26]. (Davis 1970). Samples are stored in the herbarium of the faculty

of science and literature, department of the Biology at Cumhuriyet University (CUFH: 18051). The leaves, stems and flowers air-dried separately and ground to a powder with laboratory type mill, and then each of the parts (10 g) subjected to cold maceration with 100 mL of 80% ethanol for 3 times and after filtering concentrated with rotary evaporator under vacuum.

2.2 Antioxidant assay

DPPH radical scavenging: 100 μ L of extracts with concentrations ranged between 10 and 1000 μ g/mL were mixed with 100 μ L of the DPPH radical solution (0.10 mM in methanol) in 96- well plate [27]. The plate was shaken with orbital shaker and left to stand for half hour in the dark at room temperature. The reduction in the color of purple DPPH solution was measured with Elisa reader at 517nm. Scavenging activity was calculated according to control absorbance containing only methanol and DPPH solution without test samples.

ABTS radical scavenging: The ABTS cation decolorization assay was conducted with the method of Re et al. [28]. The ABTS \bullet + working solution was prepared from the stock ABTS which are produced by reacting 2.46 mM potassium persulfate and 7 mM ABTS for 12-16 h, by diluting with methanol to give an absorbance 0.7 ± 0.02 at 734 nm. 100 μ L of extracts with different concentration was mixed with 100 μ L of ABTS \bullet + working solution in microwell plate and incubated at 25°C for 7 min. The absorbance value was measured at 734 nm with microplate reader.

Total phenolic content: The Folin-Ciocalteu method was used to determine total phenolic content and expressed mg of gallic acid equivalents GAE/g of dry extract [29].

Total flavonoid content: The total flavonoid content of the extract was determined with the aluminum chloride colorimetric method and expressed as mg of quercetin equivalents QE/g of dry extract [30].

2.3 Anti-cholinesterase inhibition assay

Acetylcholinesterase/butyrylcholinesterase inhibition assay was performed according to the Ellman method [31]. Briefly, the mixture consisting of 20 μ L of test sample/reference standard of various concentrations, 140 μ L of 100 mM phosphate buffer (pH 7.7), 10 μ L of DTNB and 20 μ L of enzyme (0.22 U/mL for acetylcholinesterase/ 0.1 U/mL for butyrylcholinesterase) was incubated for 5 min at 25°C. Following preincubation, 10 μ L of the substrate

(0.71 mM acetylthiocholine iodide/0.2 mM butyrylthiocholine chloride in phosphate buffer) was added to start the reaction and incubated again for 10 min. The developed yellow color was measured at 412nm (Epoch, USA). Galanthamine was used as positive control.

2.4 α -glucosidase inhibition assay

α -Glucosidase inhibitory activity was determined as previously described [32] by mixing 20 μ L extracts in varying concentration with 20 μ L of α -glucosidase (0.1 unit/mL in 250 mM phosphate buffer, pH= 6.8) in 96-well plate. After incubating at 37°C for 30 min, 40 μ L of 0.375 mM 4-nitrophenyl- α -D-glucopyranoside (pNPG) was added. After incubation period, the reaction was terminated by the addition of 80 μ L of 1M Na₂CO₃, and the absorbance was recorded at 405 nm with a microplate reader. Acarbose was used as positive control.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and Discussion

In this study, *in vitro* antioxidant and enzyme inhibitory activity of ethanol extract of *M. sativa* collected at two different growing stage were evaluated. The yield of extracts was found as 9.87%, 10.2%, 9.65%, 9.39%, and 7.48% respectively in leaves, stems before and after flowering and flower extracts.

The free radical scavenging activity assays reflect the ability of antioxidants to donate hydrogen. The IC₅₀ value for DPPH is much higher than for ABTS. According to the results, the ABTS radical scavenging ability of the extracts is better than the DPPH radical scavenging activity, this may be attributed to the predominantly polar compounds in the extracts. Flavonoids are phenolic compounds, and are well known to have potent antioxidant activity on many important chronic diseases, such as cancer, diabetes, Alzheimer's disease, and cardiovascular disease, which are caused by oxidative stress [33-34]. Total phenolic and flavonoid content was given in Figure 3. Among the extracts, total phenolic content was highest in stem extract after flowering (75.89 mg GAE/g extract), total flavonoid content was highest in stem extract before flowering (34.6 mg QE/g extract). In previous reports, total phenolic and flavonoid content for *M. rigidula* was found as 79.61 mg GAE/g and 27.38 μ g QE/g [35].

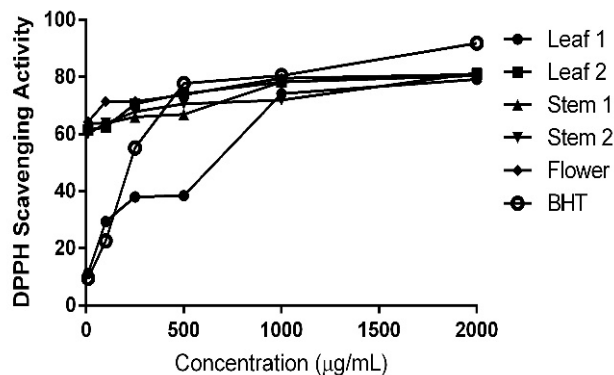


Figure 1: DPPH radical scavenging activity of *M. sativa* L. extracts at different growing period and standard compound BHT.

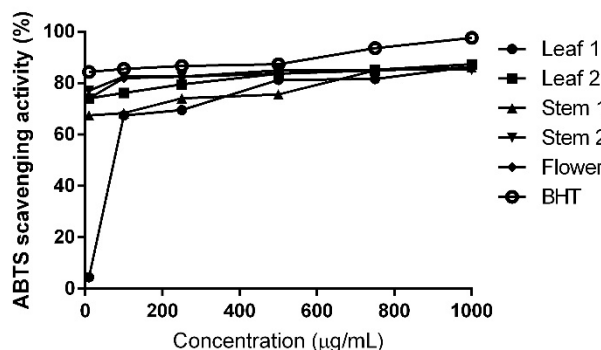


Figure 2: ABTS radical scavenging activity of *M. sativa* L. extracts at different growing period and standard compound BHT.

Acetylcholinesterase induces the hydrolysis of acetylcholine, a neurotransmitter in the brain, to choline and acetate. Brain degenerative diseases arise due to the decrease in the amount of acetylcholine. Alzheimer's disease (AD) is a neurodegenerative disease characterized by decline in brain function in old age. Acetylcholinesterase inhibitors are used in the treatment of AD disease to prevent hydrolysis of acetylcholine [36]. Acetylcholinesterase and butyrylcholinesterase inhibition activity results are shown in table 1 with IC₅₀ (μ g/mL). Among the extracts, the leaf extract after flowering was more potent than others. All the extracts have weak anti-AChE activity except for the post-flowering leaf extract, compared to standard compound- galanthamine, but for the anti-BChE activity, all the extract demonstrated very weak activity, the IC₅₀ value was much higher than that of the standard substance.

The inhibition of α -glucosidase enzyme is an increasingly effective treatment strategy for diabetes mellitus. α -glucosidase enzyme inhibition activity results were given in Figure 4. All of the extract were

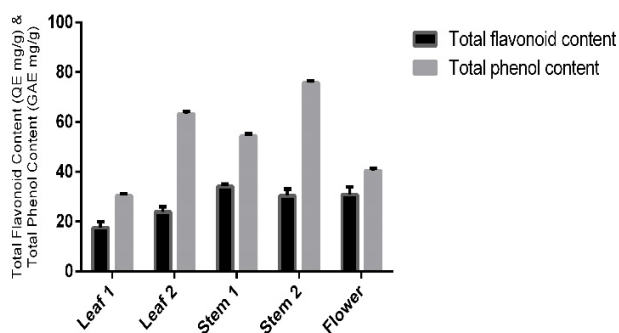


Figure 3: Total phenol and flavonoid content of *M. sativa* L. extracts at different growing period.

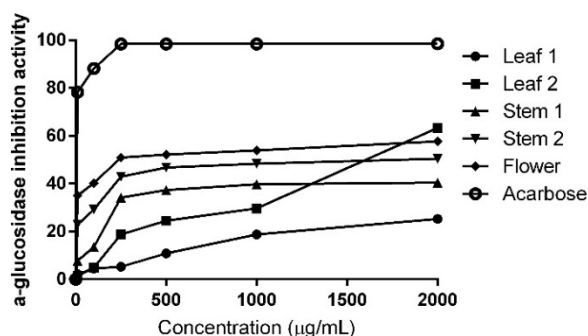


Figure 4: α-glucosidase enzyme inhibition activity of acarbose and *M. sativa* L. extracts at different growing period.

Table 1: Acetylcholinesterase and butyrylcholinesterase inhibitory activity of different part extracts of *M. sativa* L.

Plant extracts and reference compounds	AChE		BChE	
	IC ₅₀ (μg/mL)	R ²	IC ₅₀ (μg/mL)	R ²
Leaf 1*	561.29	0.991	1297.83	0.955
Leaf 2*	21.57	0.961	1215.13	0.955
Stem 1*	190.64	0.948	1723.07	0.971
Stem 2*	353.41	0.958	1752.79	0.965
Flower*	499.39	0.977	1794.77	0.934
Galanthamine	32.36	0.958	39.11	0.981

*Not: leaf 1 and stem 1 is before flowering, leaf 2 and stem 2 is after flowering

exhibited concentration dependent inhibition activity on α-glucosidase, the leaf extract after flowering was more active than others at the concentration of 2 mg/mL, but at the lower concentration, the flower extract was more active. As far as we know, no activity studies of enzyme inhibition have been done on *M. sativa* extracts. In this context our work will be the basis for the work to be carried out on this species.

4 conclusion

The capability of alfalfa extracts to scavenge free radicals as well as enzyme inhibition activity, indicating that they may be useful in preparing pharmaceutical formulations to treat various oxidative stress related chronic disease and diabetes mellitus associated with α-glucosidase as well as brain degenerative disease related with cholinesterase inhibition.

Acknowledgement: Experimental studies were carried out in the high technology research laboratory of Cumhuriyet University. The authors sincere thanks to botanist Dr. Erol Dönmez for identification of the plant materials.

Conflict of interest: Authors state no conflict of interest.

References

- [1] Graham P.H., Vance C.P., Legumes: Importance and constraints to greater use. *Plant Physiology*, 2003, 131, 872-877.
- [2] Oleszek W., Price K.R., Colquhoun I.J., Jurzysta M., Ploszynski M., Fenwick G.R., Isolation and identification of Alfalfa (*Medicago Sativa* L.) root saponins: their activity in relation to a fungal bioassay, *J Agric Food Chem.*, 1990, 38, 1810-17.
- [3] Soto-Zarazúa M.G., Bah M., Costa A.S.G., Rodrigues F., Pimentel F.B., Rojas-Molina I., Rojas A., Oliveira M.B.P.P., Nutraceutical potential of new Alfalfa (*Medicago sativa*) ingredients for beverage preparations, *Journal of Medicinal Food*, 2017, 20, 1039-1046.
- [4] Stochmal A., Piacente S., Pizza C., De Riccardis F., Leitz R., Oleszek W., Alfalfa (*Medicago Sativa* L.) Flavonoids. 1. apigenin and luteolin glycosides from aerial parts, *J Agric Food Chem.*, 2001, 49, 753-758.
- [5] Ramírez R.G., Neira-Morales R.R., Ledezma-Torres R.A., Garibaldi-González C.A., Ruminant digestion characteristics and effective degradability of cell wall of browse species from Northeastern Mexico. *Small Ruminant Research*, 2000, 36, 49-55.
- [6] Karadavut U., Palta C., Tezel M., Aksoyak S., Determination of some physiological characters in Alfalfa (*Medicago sativa* L.), *J Agricultural Faculty SDU*, 2011, 6, 8-16.
- [7] Al-Dosari, Mohammed S., *In vitro* and *in vivo* antioxidant activity of Alfalfa (*Medicago sativa* L.) on carbon tetrachloride intoxicated rats. *Am J Chin Med.*, 2012, 4, 779-93.
- [8] Gomathi R., Banu S., Usha K., Phytochemical analysis and free radical scavenging potential of *Medicago sativa* Linn seeds. *International Research Journal of Pharmacy*, 2016, 7, 71-76.
- [9] Gray M., Flatt P.R., Pancreatic and extra-pancreatic effects of the traditional anti-diabetic plant, *Medicago sativa* (Lucerne). *Br J Nutr.*, 1997, 78, 325-34.
- [10] Bora K.S., Sharma A., Evaluation of antioxidant and cerebroprotective effect of *Medicago sativa* Linn. against

- ischemia and reperfusion insult, J Evid Based Complement Altern Med., 2011, 2011, 792167.
- [11] Rana M.G., Katbamna R.V., Dudhrejiya A.D., Jivani N.P., Sheth N.R., *In vitro* antioxidant and free radical scavenging studies of alcohol extract of *Medicago Sativa* L. Rom. J. Biol-Plant Bioll., 2010, 55, 15-22.
 - [12] Tenkerian C., El-Sibai M., Daher C.F., Mroueh M., Hepatoprotective, antioxidant, and anticancer effects of the *Tragopogon porrifolius* methanolic extract. Hindawi Publishing Corporation, 2015, <http://dx.doi.org/10.1155/2015/161720>.
 - [13] Fabricant D.S., Farnsworth N.R., The value of plants used in traditional medicine for drug discovery, Environ Health Perspect, 2001, 109, 69-75.
 - [14] Al-Rimawi F., Rishmawi S., Arikat S.H., Khalid M.F., Warad I., Salah Z., Anticancer activity, antioxidant activity, and phenolic and flavonoids content of wild *Tragopogon porrifolius* plant extracts. Hindawi Publishing Corporation, 2016, <http://dx.doi.org/10.1155/2016/9612490>.
 - [15] Silva S., Gomes L., Leitão F., Coelho A.V., Boas L.V., Phenolic compounds and antioxidant activity of *Olea europaea* L. Fruits and leaves. Food Science and Technology International, 2006, 12, 385-396.
 - [16] Servili M., Montedoro G., Contribution of phenolic compounds in virgin olive oil quality. European Journal of Lipid Science and Technology, 2002, 104, 602-613.
 - [17] Waris G., Ahsan H., Reactive oxygen species: role in the development of cancer and various chronic conditions. Journal of Carcinogenesis, 2006, 5, 14.
 - [18] Evans M.D., Dizdaroglu M., Cooke M.S., Oxidative DNA damage and disease: induction, repair and significance. Mutation Research, 2004, 567, 1-61.
 - [19] Schwarz S., Loesche A., Lucas S.D., Sommerwerk S., Serbian I., Siewert B., Pianowski E., Csuk R., Converting maslinic acid into an effective inhibitor of acetylcholinesterases. Eur J Med Chem., 2015, 103, 438-445.
 - [20] Sens L., De Oliveira A.S., Mascarello A., Brighente I.M.C., Yunes R.A., Nunes R.J., Synthesis, Antioxidant activity, acetylcholinesterase inhibition and quantum studies of thiosemicarbazones. J Braz Chem Soc., 2018, 29, 343-352.
 - [21] Greig N.H., Lahiri D.K., Sambmurti K., Butyrylcholinesterase: an important new target in Alzheimer's disease therapy. Int Psychogeriatr., 2002, 1, 77-91.
 - [22] Ponnusamy S., Ravindran R., Zinjarde S., Bhargava S., Kumar A.R., Evaluation of traditional Indian antidiabetic medicinal plants for human pancreatic amylase inhibitory effect *in vitro*. Evid Based Complement Alternat Med., 2011, 2011, 515647.
 - [23] Tiwari A.K., Rao J.M., Diabetes mellitus and multiple therapeutic approaches of phytochemicals: Present status and future prospects. Curr Sci., 2002, 83, 30-8
 - [24] Ghadyale V., Takalikar S., Haldavnekar V., Arvindekar A., Effective control of postprandial glucose level through inhibition of intestinal alpha- glucosidase by *Cymbopogon martinii* (Roxb.) Evid Based Complement Alternat Med., 2012; 2012, 1-6.
 - [25] Telagari M., Hullatti K., *In-vitro* α -amylase and α -glucosidase inhibitory activity of *Adiantum caudatum* Linn. and *Celosia argentea* Linn. extracts and fractions. Indian J Pharmacol., 2015, 47, 425-429.
 - [26] Davis P.H., Flora of Turkey and the East Aegean Islands, 1970, 3, 488, Edinburgh University press, Edinburgh, UK.
 - [27] Wang H., Gao X.D., Zhou G.C., Cai L., Yao W.B., *In vitro* and *in vivo* antioxidant activity of aqueous extract from *Choerospondias axillaris* Fruit. Food Chemistry, 2008, 106, 888-95.
 - [28] Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free radical biology & medicine, 1999, 26, 1231-37.
 - [29] Ainsworth E.A., Gillespie K.M., Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. nature protocols., 2007, 2, 875-76.
 - [30] Chandra S., Khan S., Avula B., Lata H., Yang M.H., Elshohly M.A., Khan I.A., Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: a comparative study. Evid Based Complement Alternat Med., 2014, 2014, 1-9.
 - [31] Ellman G.L., Courtney K.D., Andres V., Featherstone R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol., 1961, 7, 88-95.
 - [32] Yang Z., Wang Y., Wang Yi., Zhang Y., Bioassay-guided screening and isolation of α -glucosidase and tyrosinase inhibitors from leaves of *Morus Alba*. Food Chemistry, 2012, 131, 617-25.
 - [33] Ceylan Y., Usta K., Usta A., Maltas E., Yıldız S., Evaluation of antioxidant activity, phytochemicals and ESR Analysis of *Lavandula stoechas*. Acta Physica Polonica A., 2015, 128, 483-87.
 - [34] Boutemak K., Safta B., Ayachi N., Study of the anti-inflammatory activity of flavonic extract of *Globularia alypum* L. Acta Physica Polonica A., 2015, 128, 239-40.
 - [35] Çakmak Y.S., Kaya M., Zengin G., Investigation of antioxidant and enzyme inhibition activities and phenolic compound of *Medicago Rigidula* (L.). Marmara Pharmaceutical Journal, 2017, 21, 522-522.
 - [36] Hasbal G., Ozden T.Y., Can A., Antioxidant and antiacetylcholinesterase activities of *Sorbus Torminalis* (L.) Crantz (Wild Service Tree) Fruits. J Food Drug Anal., 2015, 23, 57-62.