







Investigation of *in vitro* anti-hyperpigmentation, antidiabetic, neuroprotective and antioxidant potential of *Medicago murex* Willd. (Fabaceae)

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ABSTRACT

Background and Aims: Most of the *Medicago* species are considered good quality forage crops due to their rich protein content. This study reports antioxidant properties, total phenolic and flavonoid content, enzyme (acetylcholinesterase (AChE), tyrosinase, α -amylase and α -glucosidase) inhibition activities of methanol (MeOH), ethyl acetate (EA) and, aqueous (AQ) extracts of *Medicago murex* (*M. murex*).

Methods: The antioxidant activity of the different extracts of *M. murex* were evaluated using FRAP and CUPRAC assays. The contents of total phenolic and flavonoid were determined by the Folin-Ciocalteu and the Aluminium chloride (AlCl₃) colorimetric methods. Also, the enzyme inhibition activities were shown spectrophotometrically for AChE, α -amylase, α -glucosidase and tyrosinase enzymes.

Results: Total phenolic and flavonoid contents of different extracts were determined between 83.70-163.93 mg GAE/g extract and 24.48-26.05 μ g QE/g extract, respectively. AQ extracts were the most active extracts in FRAP while being the best inhibitor of the AChE enzyme. On the other hand, EA extracts were the most active extracts in CUPRAC methods and the best inhibitors for other enzymes.

Conclusion: Considering the findings, *M. murex* appears to be an important source of natural antioxidants and enzyme inhibitors, and it provides important baseline data for the food and pharmaceutical industries.

Keywords: *M. murex*, bioactive substances content, antioxidant potential, enzyme inhibition

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INTRODUCTION

Plants have been used for therapeutic purposes throughout history. There has been an increasing interest in bioactive compounds from the plant kingdom recently because they have fewer side effects, are cheaper than general synthetic drugs, and influence the production of free radicals (Martins, Barros & Ferreira, 2016). Apart from these facts, bioactive compounds have been reported to have protecting effects against extensive dietary-related diseases, including cancer, obesity, cardiovascular diseases, diabetes and various inflammatory conditions (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011).

Medicago spp., a member of the family Fabaceae, is one of the important forage plants around the world with economic value and includes 87 flowering plant species, some of which are consumed by humans (Gholami, De Geyter, Pollier, Goormachtig & Goossens, 2014). *Medicago* species synthesize various natural bioactive products to maintain symbiotic interactions and at the same time to deter pathogens and herbivores (Ghimire, Ghimire, Yu, & Chung, 2019). These products are most of the time natural bioactive compounds with a functional nutrient source and promising pharmaceutical properties for humans. *Medicago* species are traditionally consumed as salads, sandwiches, soups and tisane (Takyi, Kido, Rikimaru, & Kennedy, 1992), and also its leaves are used to reduce cholesterol level (Mølgaard, von Schenck, & Olsson, 1987). Despite these uses, *Medicago* spp. has been practiced in health problems, including diabetes, gastric ulcer, cancer and menopause symptoms due to its different therapeutic effects (Mortensen et al., 2009).

Medicago spp. contain bioactive compounds like flavonoids, ligands, carotenoids and phenolic acids that have beneficial effects on health (Xu et al., 2017). Especially are a major group of phenolic compounds and there is a great interest in these compounds because of their strong antioxidant activity against oxidative stress. Recently, some studies have identified the relationship between these compounds and cancer risk (Sudhakaran, Sardesai, & Doseff, 2019).

M. murex is a member of the *Medicago* genus and is an annual legume of Mediterranean origin, usually grown in acidic soils. It is also an important grazing plant that is commonly seen in the pastures and is widely distributed from the Mediterranean to Central Asia (Francis & Gillespie, 1981). Studies on *Medicago* spp. generally focus on Alfalfa (*Medicago sativa* L.). Other *Medicago* species, including *M. murex* have been ignored. The studies on *M. murex* are generally limited to molecular cytogenetics, phylogenetics, morphology and geographical distribution (Gillespie & McComb, 1991). In this study, *M. murex* plant was chosen as a bioactive compound source for the following reasons; i) easy cultivation, ii) worldwide distribution, and iii) used as a functional food in different countries. This research is the first study to reveal the therapeutic properties of *M. murex* such as anti-hyperpigmentation, antidiabetic, neuroprotective and antioxidant. The aim of this study was to investigate the antioxidant properties, enzyme inhibition activities and phenolic composition of different extracts of *M. murex* and to evaluate the potential of this species for food and pharmacology industries.

MATERIALS AND METHODS

Plant material

Aerial parts of *M. murex* were collected from Kargin Village (Aksaray-Turkey) in the vegetation period in February 2015 and were identified by botanist experts, Assist. Prof. Dr. Bulent Eskin and Dr. Mustafa Keskin. A voucher specimen (No. (AKSU) BE-2226) has been deposited at Aksaray University Herbarium, Turkey.

Preparation of extracts

For the extraction process of plant samples, the aerial parts of *M. murex* samples were dried at room temperature and weighed at 15 g each in order to make MeOH, EA and AQ extracts. MeOH and EA extracts were subjected to extraction using a Soxhlet apparatus for 6-8 h. AQ extract was carried out by steeping in boiling water for 30 min. After that, the extracts were filtered and evaporated. Total yields of dried samples were calculated and stored at -20 °C until analysis. Stock solutions were prepared from these extracts at a concentration of 2 mg/mL prior to analysis.

Profile of bioactive compounds

The content of two major groups of bioactives; phenols (TPC) and flavonoids (TFC) in obtained extracts was determined by using spectrophotometric methods. Expression of obtained results was done by equivalents of standards - gallic acid (TPC) and quercetin (TFC) (Uysal et al., 2017).

Determination of enzyme inhibition and antioxidant activity

The polarity and chemical properties of different solvents are known to affect the solubility of bioactive compounds. Therefore, solvents with different polarities (AQ (high polarity) > MeOH (less polar than AQ) > EA (moderately polar) were preferred. Potential biological activities of the obtained extracts with different solvents were performed: antioxidant, anti- α -amylase, anti- α -glucosidase, anti-AChE and anti-tyrosinase activity assays. Estimation of anti-enzymatic activity of the extracts was done by *in vitro* assays previously described by Uysal et al. (2017). The enzyme inhibitory actions of extracts were assessed as equivalents of kojic acid (KAE) for tyrosinase, galantamine for AChE and acarbose for α -amylase and α -glucosidase.

Antioxidant activity of different extracts was measured by ferric ion reducing antioxidant power (FRAP) and cupric ion reducing antioxidant capacity (CUPRAC) assays. Detailed description of applied assays were given previously (Uysal et al., 2017). The findings were given as standard compounds equivalent of Trolox (TE/g). The assay methods were given in our earlier work.

Statistical analysis

All analyses were performed in triplicate and the obtained results were expressed as mean \pm standard deviation (SD). The obtained data was analysed using SPSS 26 software (Chicago, IL, USA). One-way analysis of variance was conducted to see whether there is a statistical significance. $p < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

Phenol or polyphenol substances can be defined as chemical plant components containing a phenolic ring in structure. Ac-

According to the results given in Table 1, the TPC of the extracts of the plant were found to vary between 83.70 and 163.93 mg GAE/g extract. The highest phenolic content was seen in AQ extract followed by MeOH and EA extract. In previous studies on *Medicago* species Kicel & Olszewska (2015) showed that the TPC of different fractions obtained from *M. lupulina* were between 6.6 and 162.4 mg GAE and, while Çakmak et al. (2017) found the TPC of *M. rigidula* as 79.61 mg GAE. Based on the studies reported in the literature, different or same *Medicago* species may have different rates of phenolic substance content depending on different factors such as geographical area, climate or genetics (Baiano, Terracone, Viggiani, & Del Nobile, 2013).

The TFC of three different extracts of *M. murex* was determined by using the $AlCl_3$ colorimetric method. As stated in Table 1; the highest TFC was found in MeOH extract (26.05 µg QE/g) and also no flavonoid content was detected in EA extract. This may be due to the flavonoid compounds with different chemical properties and polarities that can be dissolved or insoluble in the *M. murex* plant in moderately polar solvents like EA. Besides, parameters such as the type of plant, the chemical nature of the extractable bioactive compounds and the solubilizing effect of solvents may affect the obtaining of phenolics from extracts (Adaramola & Onigbinde, 2016).

In a study on *M. rigidula*, the TFC of the plant was 27.38 µg QE/g extract (Çakmak et al., 2017). Rodrigues et al. (2013) investigated the TPC and TFC of some *Medicago* species and found that TFC ranged from 5.54 to 11.67 mg CEQ/g db. In the present study, considering the TFC being found in different extracts, one could conclude that *M. murex* contains a good amount of flavonoids. Besides that, no statistically significant correlation was found between TPC and TFC in the statistical analysis performed.

Antioxidant activity

In the present study, the FRAP results of the different extracts obtained from *M. murex* are given in Table 1 as equivalent to Trolox. The iron-reducing antioxidant power of extracts was found quite close to each other, and for MeOH, EA and AQ were measured as 81.94, 85.81 and 91.41 mg TE/g, respectively. A previously reported study, FRAP test was applied to the MeOH extract of *M. lupulina*, and it was observed that MeOH extract had 0.2 mmol Fe^{+2} /g dry extract activity but when Trolox and quercetin used as a standard were found as 9.2 and 36.7 0.2 mmol Fe^{+2} /g dry extract activity (Kicel & Olszewska,

2015). In another study investigating different *Medicago* species, the highest FRAP activity (with 120.84 µmol TE/g) was determined in *M. segitalis*, while the lowest FRAP activity (with 58.05 µmol/g) was determined in *M. minima* (Rodrigues et al., 2013). Also, a correlation analysis was conducted between FRAP and TPC/TFC of *M. murex* extracts in this study. Based on the obtained results, there is a strong correlation between FRAP and TPC and TFC. In the previously reported studies, it was mentioned that there is a correlation between total phenolic and flavonoid substance and FRAP (Babbar, Oberoi, Upal, & Patil, 2011).

The results of the applied CUPRAC test to determine the reducing power of copper ions are given in Figure 1. Accordingly, at the highest concentration (400 µg/mL), the EA extract had an absorbance of 0.510 abs, followed by MeOH (0.358 abs) and AQ extract (0.313 abs). In a study on *M. rigidula*, it was found that the absorbance values vary between 0.058 to 0.733 in the same concentration range (50, 100, 200 and 400 µg/ml) (Çakmak et al., 2017). There was a correlation between TPC and CUPRAC in our study based on the statistical evaluation of obtained results, as some authors have previously stated (Fidriany, Budiana, & Ruslan, 2015). Besides, a strong correlation was determined between TFC and CUPRAC, and it was observed that the correlation coefficient decreased when copper ions concentration increases: $r = 0.997$, $r = 0,424$, $r = 0,321$ and $r = 0,218$ correlation coefficients were obtained for 50, 100, 200 and 400 of CUPRAC concentrations, respectively.

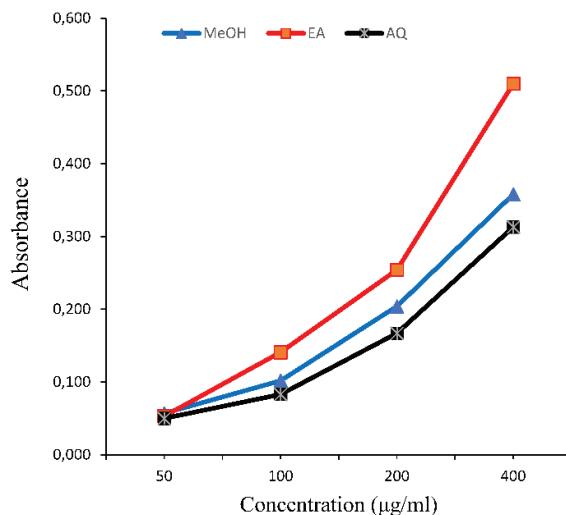


Figure 1. Cupric ion reducing power activity of *M. murex*.

Table 1. The TPC, TFC and FRAP activity of different extracts obtained from *M. murex*.

Extracts	Yield (%)	TPC ^a (mg GAE/g)	TFC ^b (µg QE/g)	FRAP ^c (mg TE/g)
MeOH	12.09	95.98±0.64*	26.05±2.16	81.94±1.74
EA	3.77	83.70±3.36	-	85.81±0.05
AQ	11.80	163.93±0.64	24.48±0.35	91.41±2.22

^a TPC expressed as gallic acid equivalent (mg GAE g⁻¹ extract);
^b TFC expressed as quercetin equivalents (µg QE g⁻¹ extract);
^c FRAP expressed as trolox equivalents (mg TE g⁻¹ extract); * Values expressed are means ± S.D. of three parallel measurements.

Enzyme inhibitory effects

Metabolic diseases are among the most common diseases, especially diabetes, Alzheimer's disease (AD) or skin pigmentation disorders and enzyme inhibition studies are a novel and up-to-date alternative in the treatment of these diseases (Zengin et al., 2019). Therefore, potential enzyme inhibitor research of phytochemicals in plants is becoming more common. In this study, MeOH, EA and AQ extracts of *M. murex* were

Table 2. Enzyme inhibitory potentials of *M. murex*.

Extracts	AChE Inhibition (mg GALAE/g extract)	α -Amylase Inhibition (mmol ACAE/g extract)	α -Glucosidase Inhibition (mmol ACAE/g extract)	Tyrosinase Inhibition (mg KAE/g extract)
MeOH	1.84±0.06	0.82±0.03	4.28±0.31	17.92±0.18
EA	1.17±0.14	1.01±0.02	6.34±0.17	28.29±1.02
AQ	2.02±0.16	0.41±0.02	2.70±0.16	4.23±0.56

Values expressed are means \pm S.D. of three parallel measurements. GALAE: Galantamine equivalent; ACAE: Acarbose equivalent; KAE: Kojic acid equivalent.

investigated to evaluate for potential inhibitory activity against key enzymes related to diabetes, AD and hyperpigmentation problems and the obtained results are given in Table 2.

Control of AD is largely dependent on the inhibition of AChE. The AChE is the principal therapeutic target in the treatment of AD because of its ability to hydrolyse neurotransmitter acetylcholine. The elevation of AChE causes acetylcholine deficiency in the brain and hence, inhibition of AChE plays an important role in alleviating the symptoms of AD (Hassan, Raza, Abbasi, Moustafa, & Seo, 2019). Based on our findings, the highest activity in AChE enzyme inhibition was found in AQ extract (2.02 mg GALAE/g) and the lowest value was found in EA extract (1.17 mg GALAE/g). Also, all three extracts were found to be active against AChE.

The α -amylase and α -glucosidase enzymes, which are effective on glucose metabolism, showed inhibition activity as 1.01 mmol ACAE/g and 6.34 mmol ACAE/g extract, respectively, and the highest ratio was observed in EA extract for both enzymes. α -amylase and α -glucosidase are carbohydrate hydrolysing enzymes and inhibition of both enzymes has a considerable role in the regulation of hyperglycemia. Nowadays, hypoglycemic drugs have many side effects, including gastrointestinal disorders. Excessive α -amylase inhibition has been found to disrupt the normal digestion process and cause different gastrointestinal problems such as bloating, diarrhea, and abdominal pain (Alam, Shafique, Amjad, & Bin Asad, 2019). While the tested extracts showed a weak activity on the α -amylase enzyme, they showed a moderate activity on the α -glucosidase enzyme. At the same time, α -amylase enzyme inhibition activities were found to be statistically significant ($p < 0.05$) with both TPC and TFC. In a study on the enzyme inhibition in MeOH extract of *M. rigidula*, AChE, amylase, α -glucosidase and tyrosinase enzyme inhibitions were determined as 2.05 mg GALAE/g, 0.73 mmol ACAE/g, 1.30 mmol ACAE/g and 16.36 mg KAE/g extract, respectively (Çakmak et al., 2017).

Hyperpigmentation management in today's common diseases is largely dependent on the tyrosinase enzyme, an enzyme-containing copper, and the tyrosinase enzyme also plays an important role in melanin synthesis (Zengin et al., 2019). The use of tyrosinase inhibitors is increasing in the pharmaceutical and cosmetic industries, especially because of their skin whitening and anti-pigmentation effects after sunburn. In the present study, EA extract showed higher tyrosinase inhibitory activity than MeOH and AQ extracts (Table 2).

CONCLUSION

In this study, besides antioxidant and enzyme inhibition activities of *M. murex*, total phenolic and flavonoid substance contents were also studied. According to the obtained results, the TPC was recorded highest in the AQ extract, while the TFC was recorded in the MeOH extract. In addition, the FRAP value was found the highest in AQ extract as well as maximum CUPRAC value was recorded in EA extract. All of the extracts of the *M. murex* plant showed inhibitory activity against all the studied enzymes. Especially α -glucosidase enzyme inhibition has been found to be at high levels compared to the α -amylase enzyme which works together with α -glucosidase in glucose metabolism. High inhibition of α -amylase and α -glucosidase enzymes, especially in EA extract, may be considered as an indicator that the plant has an important potential in the treatment of type 2 diabetes. However, more research is needed to understand the mechanisms and effects of *M. murex* biological activity.

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