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RESEARCH ARTICLE

Evaluation of Antioxidant Properties and Metabolic Enzyme Inhibitory Potential of *Medicago sativa* L.

Sultan PEKACAR[*], Ömer Furkan GÜVERTİ[**°], Burçin ÖZÜPEK[***], Didem DELİORMAN ORHAN[****]

Evaluation of Antioxidant Properties and Metabolic Enzyme Inhibitory Potential of Medicago sativa L.

SUMMARY

Medicago sativa L. (Alfalfa) is a perennial plant belonging to the Leguminosae family. The plant has a wide distribution in Turkey. It has traditionally been used to improve memory, relieve muscle pain, and treat central nervous system disorders due to its antidiabetic, antioxidant, and anti-inflammatory effects. Diabetes, whose prevalence is rapidly increasing, is a metabolic disease characterized by insulin deficiency and insulin resistance resulting from the autoimmune destruction of pancreatic β cells. In this study, the antioxidant capacity and inhibitory activities of methanol extracts prepared from the leaves and aerial part of M. sativa on metabolic enzymes were investigated. Additionally, the total phenolic, total flavonoid, and total tannin contents of the extract were evaluated. Upon evaluation of the results in the metal chelating capacity assay, the M. sativa leaf methanol extract (93.06±1.06%) demonstrated a good effect compared to the reference compound EDTA (100.01±0.16%). The total tannin content of the leaf extract was 67.71±2.74 (mg catechin equivalent/g extract), while this value was determined to be 129.64±4.21 (mg catechin equivalent/g extract) in the aerial parts. Based on the enzyme results, the leaf extract (IC_{50}: 159.95±1.52 µg/mL) exhibited a moderate effect on the α-amylase enzyme compared to the reference substance, acarbose (IC_{50}: 0.68±0.03 µg/mL). As for the pancreatic cholesterol esterase enzyme, the aerial extract (IC_{50}: 166.55 ± 3.74 µg/mL) exhibited moderate inhibition compared to the reference compound simvastatin (IC_{50}: 3.51 ± 0.86 µg/mL). In conclusion, further in vitro and in vivo studies on this species are needed.

Keywords: *Medicago sativa L., antioxidant, α-amylase, Leguminosae*

Medicago sativa L.'nin Antioksidan Özelliklerinin ve Metabolik Enzim İnhibisyon Potansiyelinin Değerlendirilmesi

ÖZ

Medicago sativa L. (Yonca) Leguminosae familyasına ait çok yıllık bir bitkidir. Bitki Türkiye'de geniş bir yayılış alanına sahiptir. Bitki geleneksel olarak hafızayı güçlendirmek, kas ağrılarını hafifletmek ve antidiyabetik, antioksidan, antienflamatuvar etkileri ile merkezi sinir sistemi bozukluklarının tedavisinde kullanılmaktadır. Prevalansı hızla artan diyabet, pankreas β hücrelerinin otoimmün yıkımına bağlı insülin eksikliği ve insülin direnci ile karakterize edilen bir metabolik hastalıktır. Bu çalışmada M. sativa'nın yaprak ve topraküstü kısımlarından hazırlanan metanol ekstrelerinin antioksidan kapasitesi ve metabolik enzimler üzerindeki inhibitör aktiviteleri çalışılmıştır. Buna ek olarak, ekstrenin total fenol, total flavonoid ve total tanen içerikleri değerlendirilmiştir. Sonuçlar değerlendirildiğinde, metal bağlama kapasitesi deneyinde M. sativa yaprak metanol ekstresi (%93,06±1,06) referans madde olan EDTA'ya (%100,01±0,16) kıyasla iyi bir etki göstermiştir. Yaprak ekstresinin total tanen içeriği 67,71±2,74 (mg kateşin eşdeğeri/g ekstre) olurken, toprak üstü kısımda bu oran 129,64±4,21 (mg kateşin eşdeğeri/g ekstre) olarak belirlenmiştir. Enzim sonuçlarına bakıldığında α-amilaz enzimi üzerinde referans madde olan akarboza (IC_{50}: 0,68±0,03 µg/mL) kıyasla yaprak ekstresi (IC_{50}: 159,95±1,52 µg/mL) ılımlı derecede etki göstermiştir. Pankreatik kolesterol esteraz enzimi üzerinde ise referans madde olan simvastatine (IC_{50}: 3,51±0,86 µg/mL) göre toprak üstü ekstresi (IC_{50}: 166,55±3,74 µg/mL) ılımlı derecede inhibisyon göstermiştir. Sonuç olarak, bu tür üzerinde daha fazla in vitro ve in vivo çalışma yapılması gerekmektedir.

Anahtar Kelimeler: *Medicago sativa L., antioksidan, α-amilaz, Leguminosae*

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[*] ORCID: 0000-0002-7782-9832, Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Türkiye
[**] ORCID: 0009-0006-8258-7264, Department of Pharmacognosy, Faculty of Pharmacy, Adıyaman University, Adıyaman, Türkiye; Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Türkiye
[***] ORCID: 0000-0003-2159-9860, Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Türkiye
[****] ORCID: 0000-0003-3916-4048, Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Türkiye

[°] Corresponding Author; Ömer Furkan GÜVERTİ
E-mail: furkanguvertiomer@gmail.com

INTRODUCTION

The genus *Medicago* belongs to the Fabaceae family and is represented by approximately 83 species worldwide. Plants of the *Medicago* genus are adapted to various environmental conditions and are rich in alkaloids, flavonoids, naphthoquinones, and saponins (Wang et al., 2021). *Medicago sativa* L. (alfalfa) is a forage plant belonging to the Leguminosae family. This perennial herbaceous plant is widely cultivated in Mediterranean countries and is also capable of adapting to harsh winter conditions. *Medicago* species, frequently used as feed for animals, contain high levels of beneficial compounds as active ingredients and are highly valuable plants in terms of protein content. Due to the high protein concentration in *Medicago* species, people consume the plant in various forms (salad, soup, and tea) (İrtem Kartal & Şayak, 2019).

M. sativa is extensively cultivated in North Africa, Southern Europe, and Spain. It is widely used as a forage protein source for animals and is grown in 80 countries around the world, mainly in the United States, Argentina, and China, across an area of 30 million hectares. The plant is also readily cultivated in Türkiye (Zhang & Wang, 2025).

Traditionally, *M. sativa* has been employed for improving memory and treating kidney pain, cough, and muscle pain, in addition to being used for its rejuvenating, antidiabetic, antioxidant, anti-inflammatory, antifungal, antimicrobial, and diuretic effects, as well as in the management of central nervous system disorders. *M. sativa* has historically been utilized in homeopathy and in the treatment of disorders related to the central nervous system. Since the sixth century, Chinese medicine has used *M. sativa* for kidney stones, fever, urinary difficulties, and edema. In ancient Indian Ayurvedic medicine, it was used for ulcers, arthritis pain, and fluid retention. In Mexico, *M. sativa* is considered beneficial for enhancing memory and reducing muscle pain and inflammation. Native Americans utilized *M. sativa* in the treatment of arthritis, boils, scurvy, and intestinal and urinary disorders. In Iraq, it is traditionally used against arthritis. In Türkiye, the plant is used as a cardiotonic and in the treatment of arthritis and scurvy (Bora & Sharma, 2011).

The National Institutes of Health describes diabetes mellitus as "a chronic disease in which the body is unable to control blood sugar levels." Diabetes mellitus involves a complex pathogenic mechanism in which autoimmune destruction of pancreatic β-cells causes abnormalities associated with insulin deficiency and insulin resistance. Metabolic disorders related to insulin resistance arise from the metabolic effects of insulin on carbohydrates, fats, and proteins in living organisms (Triplitt, Solis-Herrera, Cersosimo, Abdul-Ghani, & Defronzo, 2015). Cholesterol has an important role in regulating the physicochemical characteristics and functions of the cell membrane. A proper membrane structure is essential for glucose-stimulated insulin secretion in pancreatic β cells, and alterations in cellular cholesterol levels may adversely influence this mechanism, resulting in β-cell dysfunction. The low-density lipoprotein receptor (LDL-R) is significantly involved in β-cell dysfunction caused by cholesterol accumulation. Since β-cell dysfunction is a hallmark of type 2 diabetes, cholesterol-induced impairment of β-cell function may contribute to the development and progression of the disease (Perego et al., 2019). The prevalence of type 2 diabetes continues to increase, and this increase is strongly associated with rising obesity rates. Approximately 90% of type 2 diabetes cases are estimated to be linked to excess body weight. Obesity causes a sustained elevation in plasma free fatty acid (FFA) levels both at rest and following a glucose load, and this is a significant factor in insulin resistance. The source of excess FFA in obese individuals is believed to be fatty acids produced from meals and lipolysis of adipose tissue (Verma & Hussain, 2017).

Antioxidant activity has an important role in diabetes. Research has shown that oxidative stress increases under diabetic conditions. The activity of

oxygen free radicals may initiate lipid peroxidation, which subsequently leads to protein glycation, enzyme inactivation, and alterations in the structure and function of collagen, basement membranes, and other membranes, thereby contributing to the long-term complications associated with diabetes. In diabetes, oxidative stress is generally associated with reduced antioxidant status, which may intensify the damaging effects of free radicals (Sabu & Kuttan, 2002).

In the present study, the antioxidant potential and inhibitory activities of methanol extracts obtained from the leaves and aerial parts of *M. sativa* were assessed. The inhibitory effects of the plant on α-glucosidase, α-amylase, pancreatic lipase, and pancreatic cholesterol esterase enzymes were examined, and the effects of the aerial and leaf parts were compared. These enzymes are important therapeutic targets in the management of type 2 diabetes and its associated metabolic disorders. Inhibition of α-glucosidase and α-amylase delays carbohydrate digestion and glucose absorption, thereby reducing postprandial hyperglycemia, whereas inhibition of pancreatic lipase and pancreatic cholesterol esterase may decrease the intestinal absorption of dietary lipids and cholesterol, contributing to the management of obesity and dyslipidemia, which are closely associated with type 2 diabetes (Triplitt et al., 2015; Verma & Hussain, 2017). Furthermore, total phenolic, total flavonoid, and total tannin contents were determined to identify and compare the chemical compositions of the extracts prepared from the aerial and leaf parts of the plant. Antioxidant activity was evaluated using DPPH radical scavenging activity, metal chelating ability, and reducing power assays.

MATERIALS AND METHODS

Plant material

The *M. sativa* plant used in this study was collected in 2023 from Amasya, Türkiye (Tokat-Amasya road). The collected plant was identified by Prof. Dr. Osman Tugay (Department of Pharmaceutical Botany, Faculty of Pharmacy, Selçuk University, Konya, Türkiye). Specimens of the plant are stored at the Herbarium of the Faculty of Pharmacy, Gazi University (Herbarium Number: GUE 3949). The plant was dissected to separate the leaves and the aerial parts. These parts were then left to dry in the shade at room temperature. The dried plant material was ground into powder using a mechanical grinder.

Chemicals

All chemical reagents and solvents used were of high purity and were obtained from Sigma-Aldrich (USA). The standards used in the analyses were also obtained from the same company.

Extraction method

The samples obtained from the leaves and aerial parts of the plant were weighed separately as 10 g and extracted with 200 mL of methanol at room temperature for 24 hours. The extraction procedure was performed three times. Following each extraction step, the mixtures were filtered through 0.5 μm filter paper. The collected filtrates were concentrated under reduced pressure at 45 °C using a rotary evaporator in order to eliminate the solvent.

Antioxidant activity

DPPH radical scavenging effect

A 1 mM solution of 1,1-diphenyl-2-picrylhydrazine (DPPH) was mixed with the extracts. The resulting mixture was incubated in the dark at room temperature for 30 minutes. Following incubation, absorbance was recorded at 520 nm. Ascorbic acid served as the reference standard in the assay. All experiments were performed in triplicate. The DPPH radical scavenging activity was calculated according to the following formula (Deliorman Orhan, Orhan, Özçelik, & Ergun, 2009):

$$(\text{Inhibition}\%) = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{control}}] \times 100$$

Ferric reducing power

A 0.1 mol/L sodium phosphate buffer (pH 7.2) was added to the extracts and the positive control querce-

tin, followed by the addition of 1% (w/v) $K_3Fe(CN)_6$ solution. The mixtures were subsequently incubated at 37 °C in an oven. After incubation, 10% trichloroacetic acid solution was added. Absorbance measurements were carried out at 700 nm using an ELISA microplate reader. Afterwards, 0.1% (w/v) $FeCl_3$ solution was added, and the absorbance was measured again. The difference between the two absorbance readings was calculated. All assays were conducted in triplicate (Pekacar et al., 2025).

Metal chelating capacity

A 2 mM $FeCl_2$ solution was mixed with the extracts, and the samples were incubated at room temperature for 5 minutes. Subsequently, a 5 mM ferrosin solution was added, and the mixtures were allowed to stand at room temperature for an additional 10 minutes. Absorbance values of the extracts and the reference compound ethylenediaminetetraacetic acid (EDTA) were determined at 562 nm using an ELISA microplate reader. All experiments were carried out in triplicate. Metal chelating capacity was calculated according to the following formula (Bulut, Toslak, Muhammed, Kahraman, & Yilmaz, 2025):

$$\% \text{ Activity} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{control}}] \times 100$$

Enzyme inhibition assays

***α*-Glucosidase enzyme inhibition activity**

α-Glucosidase type IV enzyme (EC 3.2.1.20, Sigma) was dissolved in 0.5 M phosphate buffer solution at pH 6.5. *p*-Nitrophenyl-*α*-D-glucopyranoside (PNG) was utilized as the substrate. The substrate was dissolved in phosphate buffer to obtain a concentration of 20 mM. The enzyme solution and extracts were mixed and incubated on a microplate at 37 °C for 15 minutes, followed by the addition of 20 mM PNG substrate to the wells. The mixtures were then incubated again at 37 °C for 35 minutes. Acarbose (Bayer, Türkiye) was employed as the reference standard. The experiments were performed in triplicate. Absorbance values were determined at 405 nm using an ELISA microplate reader. *α*- Glucosidase enzyme inhibition was calculated according to the following formula (Özüpek et al., 2025):

$$\text{Inhibition (\%)} = [1 - (\text{Absorbance (sample)} / \text{Absorbance (control)})] \times 100$$

***α*-Amylase enzyme inhibition activity**

The *α*-amylase type I-A enzyme (EC 3.2.1.1, Sigma) was dissolved in sodium phosphate buffer (pH 6.9). Potato starch prepared in phosphate buffer at a concentration of 2.5% (w/v) was used as the substrate. A 3,5-dinitrosalicylic acid (DNS) solution served as the color reagent. The 96 mM DNS solution was prepared using 5.31 M sodium potassium tartrate in 2 M NaOH. Acarbose was used as the reference standard in the assay. Sodium phosphate buffer (pH 6.9) and *α*-amylase enzyme were added to the samples, and the mixtures were incubated at room temperature for 5 minutes. After this step, substrate was added, and the mixtures were incubated again at 37 °C for 15 minutes. Subsequently, DNS solution was added, and the samples were incubated in an oven at 80 °C for 40 minutes. Absorbance values were measured at 540 nm using an ELISA microplate reader. A standard maltose calibration curve ($y = 1.2545x - 0.0712$, $r^2 = 0.9913$) was then prepared. All experiments were conducted in triplicate. *α*-Amylase enzyme inhibition was calculated according to the following formula (Nair, Kavrekar, & Mishra, 2013):

$$\text{Inhibition (\%)} = [1 - (\text{Average maltose (sample)} / \text{Average maltose (control)})] \times 100$$

Pancreatic lipase enzyme inhibition activity

Pancreatic lipase type II (EC 3.1.1.3, Sigma) was employed in the study. The enzyme solution was prepared in a buffer containing 1 mM EDTA (pH 6.8) and 10 mM 4-morpholinopropanesulfonic acid. The extracts and the reference compound orlistat were mixed with Tris buffer composed of 100 mM Tris-HCl and 5 mM $CaCl_2$ (pH 7.0). The mixtures were incubated at 37 °C for 15 minutes. Afterwards, 5 mM 4-nitrophenyl butyrate (*p*-NPB) was added to the

wells, and the mixtures were further incubated at 37 °C for 30 minutes. Absorbance values were measured at 405 nm using an ELISA microplate reader. Pancreatic lipase enzyme inhibition was calculated according to the following formula (Güverti, Pekacar, Özüpek, & Orhan, 2025):

Inhibition (%) = [1 – (Absorbance (sample) / Absorbance (control))] × 100

Pancreatic cholesterol esterase enzyme inhibition activity

Porcine pancreatic cholesterol esterase (EC 3.1.1.13, Sigma) was used in this study. The enzyme solution was prepared with 100 mM NaCl and 100 mM buffer solution at pH 7. Phosphate buffer (50 µL) was added to the extracts. Taurocholic acid (12 mM) and 5 mM p-NPB substrate were subsequently added to the mixture. The mixtures were incubated at 25 °C for 5 minutes. Following incubation, porcine pancreatic cholesterol esterase enzyme (0.1 µg/mL) was introduced into the mixtures. Absorbance measurements were carried out at 405 nm for 15 minutes using an ELISA microplate reader in a kinetic assay. Simvastatin was employed as the reference standard. Pancreatic cholesterol esterase enzyme inhibition was calculated according to the following formula (Zor, Pekacar, & Orhan, 2022):

Inhibition (%) = 100 × [(Absorbance (control) – Absorbance (sample)) / Absorbance (control)]

Chemical composition of the plant extract

Total phenolic content

Prepared extracts were mixed with Folin- Ciocalteu reagent (10% w/v). Subsequently, 7.5% (w/v) sodium carbonate solution was added to the mixtures. The samples were incubated in the dark at room temperature for 30 minutes. After incubation, absorbance values were recorded at 735 nm using an ELISA microplate reader (SpectraMax i3x, Molecular Devices, USA). Total phenolic content was expressed as mg gallic acid equivalent (GAE) per g extract (Sengul et al., 2009). The calibration equation was calculated as y (Abs.) = 6.3692x (Conc.) – 0.03114, with an R^2 value of 0.9896. Total phenolic content was calculated according to the following formula:

Total phenolic content (mg GAE/g extract) = Gallic acid equivalent (mg) / Extract weight (g)

Total flavonoid content

Aluminum chloride, ethanol, and sodium acetate solutions were added to the extracts, and the mixtures were incubated at room temperature for 30 minutes. Total flavonoid content was calculated as mg quercetin equivalent (QE) per g extract. Absorbance measurements were carried out at 415 nm using an ELISA microplate reader (Özüpek, Pekacar, & Orhan, 2023). The calibration curve equation was determined as y (Abs.) = 1.3685x (Conc.) – 0.003, and the R^2 value was found to be 0.9993. Total flavonoid content was calculated according to the following formula:

Total flavonoid content (mg QE/g extract) = Quercetin equivalent (mg) / Extract weight (g)

Total tannin content

Methanol and 1% vanillin-sulfuric acid solution were added to the extracts. The resulting mixtures were incubated at 50 °C for 45 minutes. Absorbance values were measured at 500 nm. The results were expressed as mg catechin equivalent (CATE) per g dry extract. A calibration curve was generated for the calculation of the results (Broadhurst & Jones, 1978). The calibration equation was determined as y (Abs.) = 1.2356x (Conc.) – 0.0593, while the r^2 value was calculated as 0.9469. Total tannin content was calculated according to the following formula:

Total tannin content (mg CATE/g extract) = Catechin equivalent (mg) / Extract weight (g)

Statistical analysis

All analyses were carried out in triplicate. Results are presented in the article as mean ± standard deviation (SD). Linear regression analyses and related calculations were performed using Microsoft Excel. Statistical significance of IC_{50} values was evaluated by GraphPad ANOVA ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

RESULTS AND DISCUSSION

As a result of our study, the yield of the *M. sativa* leaf extract was calculated to be 15.05% (w/w), while the yield of the *M. sativa* aerial part extract was 20.50% (w/w).

The results of the study showed that *M. sativa* leaf methanol extract at a concentration of 2 mg/mL exhibited moderate DPPH radical scavenging activity (76.67±2.70%) compared to the reference compound gallic acid (94.78±0.27%). Regarding metal chelating capacity, the *M. sativa* leaf extract (93.06±1.06%) again demonstrated a good effect compared to the reference substance EDTA (100.01±0.16%). In the reducing power results, while our reference compound quercetin had a value of 1.36±0.01, the *M. sativa* leaf extract had a value of 0.08±0.01 (Figure 1.).

Figure 1. DPPH radical scavenging activity, reducing power, and metal chelating capacity values of *M. sativa* leaf and aerial part extracts

When the phytochemical contents of the extracts were evaluated, the total phenolic content was higher in the *M. sativa* leaf extract (29.70±1.03 mg GAE/g) than in the aerial part (18.92±1.51 mg GAE/g), while the total flavonoid content (44.30±1.91 mg QE/ g) and total tannin content (129.64±4.21 mg CAT/g) were higher in the *M. sativa* aerial part extract compared to the leaf extract (28.27±1.70 mg QE/g) and (67.71±2.74 mg CAT/g), respectively (Figure 2.).

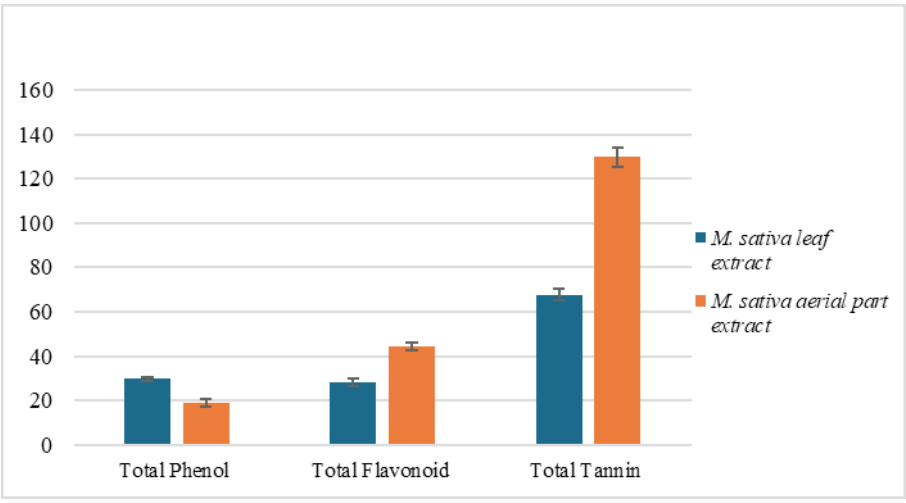

Figure 2. Total phenolic, flavonoid, and tannin contents of *M. sativa* leaf and aerial part extracts

In a study performed by Rana et al. (2010), the *in vitro* antioxidant and free radical scavenging activities of a methanol extract obtained from *M. sativa* roots were evaluated. According to the DPPH radical scavenging assay results, the methanol extract of the plant demonstrated an IC_{50} value of 100.381 µg/mL, whereas the reference compound showed an IC_{50} value of 9.9982 µg/mL. Regarding metal chelating activity, the methanol extract exhibited an IC_{50} value of 115.79 µg/mL, while the reference compound presented a value of 2.017 µg/mL (Rana et al., 2010).

In a study conducted by Krakowska et al. (2017), extracts prepared from various parts of *M. sativa*, including flowers, leaves, stems, roots, and seeds, were obtained using maceration, ultrasound-assisted extraction (UAE), accelerated solvent extraction (ASE), and supercritical fluid extraction (SFE) methods to evaluate extraction yield, phenolic compound, flavonoid, and saponin contents, as well as antioxidant potential. The concentrations of these compound groups were measured spectrophotometrically and further verified by High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS). Among the tested extracts, the 70% ethanol flower extract showed the highest phenolic content (48.4±4.6 mg gallic acid equivalent/g dry matter) together with the strongest DPPH radical scavenging activity (69.6±3.3%). The highest total flavonoid content (139.0±7.1 mg rutin equivalent/g dry weight) was detected in the leaf extract obtained by SFE. This extract was also reported to contain a high amount of saponins (622.2±30.3 mg oleanolic acid equivalent (OAE)/g dry weight). According to the findings, ethanol extracts obtained particularly from the flowers and leaves of *M. sativa* may serve as promising natural antioxidant sources for use in nutraceuticals, food additives, and cosmetic products (Krakowska, Rafińska, Walczak, Kowalkowski, & Buszewski, 2017).

In 2020, Zagórska-Dziok et al. (2020) carried out a study to assess the antioxidant properties and cytotoxic effects of water-glycerin extracts prepared from the seeds and aerial parts of *M. sativa*. The study focused on determining total phenolic and total flavonoid contents. The quantities of these compounds were calculated using gallic acid and quercetin calibration curves for total phenolic and total flavonoid contents, respectively. Analyses were conducted using six different extract concentrations (0.25%, 0.5%, 1%, 2.5%, 5%, and 10%). The findings for total phenolic and total flavonoid contents demonstrated a marked increase with increasing concentration. The highest phenolic and flavonoid levels were observed in the 10% water-glycerin extract. At this concentration, the total phenolic content was found to be 73.5±0.53 mg GAE/g in the

aerial part extract and 37.60±1.82 mg GAE/g in the seed extract. At the same concentration, total flavonoid content values were determined as 43.38±0.65 mg QE/g for the aerial part extract and 21.35±0.63 mg QE/g for the seed extract. When comparing seed and aerial part extracts, it was determined that the aerial part extract is richer in phenolic compounds, particularly at high concentrations, and that the difference in phenolic content reaches approximately 90% at a 10% concentration. In terms of flavonoid content, this difference was calculated to be approximately 15% at a 10% concentration and approximately 35% at a 5% concentration.

In the subsequent stage of the study, the free radical scavenging activities of the seed and aerial part extracts were assessed by the DPPH method. Measurements were recorded at 5-minute intervals for 30 minutes using six different concentrations between 0.25% and 10%. The findings indicated that the free radical scavenging activity of all extracts increased in a concentration-dependent manner. The strongest DPPH radical scavenging effect was detected in the water-glycerin seed extract at the highest tested concentration of 10%, where the percentage of reduced DPPH radicals reached nearly 40% after 30 minutes. At the lowest concentration of 0.25%, this value was determined to be approximately 3%. The free radical scavenging level in the aerial part extract was found to be lower than that of the seed extract at all concentrations and was determined to be approximately 20% at a 10% concentration after 30 minutes. Overall, a positive correlation was observed between concentration and antioxidant potential in both plant parts, and it was demonstrated that free radical scavenging capacity increased as concentration increased (Zagórska-Dziok, Ziemlewska, Nizioł-Łukaszewska, & Bujak, 2020).

In our study, when examining the enzyme inhibition results of the plant, it was found that both the leaf extract (IC_{50}> 200) and the aerial part extract (IC_{50}> 200) exhibited inhibition of the α-glucosidase enzyme compared to the reference compound orlistat (IC_{50}: 0.04±0.01 µg/mL). However, since this inhibition level was low, IC_{50} values could not be calculated. Regarding the α-amylase enzyme, the *M. sativa* leaf extract (IC_{50}: 159.95±1.52 µg/mL) demonstrated an effect, albeit a lower one, compared to the reference compound acarbose (IC_{50}: 0.68±0.03 µg/mL). According to the pancreatic lipase enzyme results, the aerial part of the plant exhibited a weak inhibitory effect with an inhibition value of 25.26±1.20%, while the leaf extract showed no inhibitory effect on this enzyme. For the reference compound, orlistat, this value was found to be 63.89±1.26%. Regarding the pancreatic cholesterol esterase enzyme, the aerial part extract (IC_{50}: 166.55±3.74 µg/mL) was moderately effective compared to the reference compound simvastatin (IC_{50}: 3.51±0.86 µg/mL), whereas the leaf extract was found to be ineffective (Table 1.).

Table 1. Inhibitory activity of *M. sativa* leaf and aerial part extracts on α-glucosidase, α-amylase, pancreatic lipase, and pancreatic cholesterol esterase enzymes

Extracts and References	200 µg/mL Inhibition % ± S.D. (IC_{50}: µg/mL ± S.D.)			
	α-Glucosidase	α-Amylase	Pancreatic lipase	Pancreatic cholesterol esterase
Leaf extract	21.19±2.77^{***a} (IC_{50}> 200)	68.24±6.91^{***a} (IC_{50}: 159.95±1.52)	-	-
Aerial part extract	42.10±2.66^{***a} (IC_{50}> 200)	45.67±0.28^{***a} (IC_{50}> 200)	25.26±1.20^{***b} (IC_{50}> 200)	51.99±5.93^{***c} (IC_{50}: 166.55±3.74)
ACA^{a}/ORL^{b}/SIM^{c}	101.10±1.04^{***a} (IC_{50}: 0.04±0.01)	100.72±2.19^{***a} (IC_{50}: 0.68±0.03)	63.89±1.26^{***b} (IC_{50}: 15.13±1.89)	96.19±0.12^{***c} (IC_{50}: 3.51±0.86)

-: No measurable inhibition, SS: Standard Deviation, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ ^{a}ACA: Acarbose, ^{b}ORL: Orlistat, ^{c}SIM: Simvastatin

A study performed by Khaleel et al. (2005) examined the hypocholesterolemic and antiatherosclerotic activities of *M. sativa*. In the study, seeds obtained from the Egyptian market were germinated at the Experimental Station of the Faculty of Pharmacy, Cairo University (location A). Seeds collected from Fayoum Governorate were cultivated at the same site (location B). The effects of ground seeds from location B, prepared as Extract I (25 L of 30% ethanol–5 kg plant material) and Extract II (20 L water–4 kg plant material), administered at concentrations of 1% and 2%, were investigated against hypercholesterolemia and atherosclerosis. All *M. sativa* extracts, except 1% Extract II, produced significant decreases in plasma total cholesterol (TC) and LDL-cholesterol (LDL-C) levels. The strongest effect was observed in the group treated with 2% Extract I, where TC and LDL-C levels were reduced by 85.1% and 88%, respectively. This reduction was found to be more significant than the reduction caused by gemfibrozil at the same dose in the same animal model (73% and 74%). In histopathological evaluation, it was determined that *M. sativa* extracts administered at 1–2% doses significantly inhibited aortic wall thickening associated with hypercholesterolemia, and the appearance and thickness of the aortic wall approached normal levels. It was determined that all *M. sativa* extracts possess antiatherosclerotic activity, as evidenced by the near-normalization of aortic cross-sections when used in conjunction with a cholesterol-rich diet (Khaleel, Gad, El-Maraghy, Hifnawy, & Abdel-Sattar, 2005).

In a study carried out by Mehranjani et al. (2007), the effects of an aqueous ethanol extract of *M. sativa* on blood glucose levels and renal histopathological alterations were stereologically evaluated in streptozotocin (STZ)-induced diabetic rats. Thirty-two male Wistar rats were randomly divided into four groups: control, control + extract, diabetic, and diabetic + extract (n=8). Diabetes was induced through intraperitoneal administration of STZ at a dose of 90 mg/kg. The animals received treatment with *M. sativa* aqueous ethanol extract at a dose of 550 mg/kg for 4 weeks. At the end of the treatment period, the left kidneys were removed for stereological examination. The findings demonstrated that treatment with the aqueous ethanol extract of *M. sativa* significantly reduced blood glucose levels in diabetic rats. Final body weight increased significantly in the diabetic, control, and control + extract groups compared to initial body weight; however, this increase was not observed in the diabetic + extract group. Kidney weights were comparable among the groups, except for a significant increase detected in the diabetic + extract group relative to the control + extract group. Total kidney volume and cortical volume did not differ significantly between groups, whereas medullary volume was significantly elevated in the diabetic + extract group compared with the control + extract group. Moreover, total glomerular volume was significantly higher in diabetic rats than in the control group. In rats treated with *M. sativa* aqueous ethanol extract (550 mg/kg), blood glucose levels decreased from 231±24 mg/dl to 198±10 mg/dl in the diabetic + extract group ($p = 0.01$). Body weight increased from 154±15.9 g to 191±56.2 g, although this change was not statistically significant ($p = 0.067$). Total glomerular volume in the diabetic + extract group was measured as 4.98±0.18 mm^3 and was reported to be comparable to that of the diabetic group (5.27±0.23 mm^3). Overall, although administration of *M. sativa* extract significantly lowered blood glucose levels, no marked effect on nephropathy-associated complications was observed during this short-term study (Mehranjani et al., 2007).

A study conducted by Amraie et al. (2015) aimed to examine the effects of an aqueous extract of *M. sativa* on blood glucose and serum lipid levels in alloxan-induced diabetic rats. In this study, 32 female Wistar rats were randomly allocated into four groups: a control group, a diabetic control group, and two diabetic groups treated with aqueous alfalfa extract at doses of 250 mg/kg and 500 mg/kg. Diabetes was induced in the diabetic groups by intraperitoneal administration of alloxan monohydrate. The treatment

groups received oral administration of the aqueous *M. sativa* extract for 21 days. At the end of the experiment, blood glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and liver enzyme levels (ALT and AST) were measured. The results indicated that the aqueous extract of *M. sativa* had a significant beneficial effect on blood glucose and lipid profiles in alloxan-induced diabetic rats. Blood glucose levels were 242±14 mg/100 mL in the diabetic control group, while they decreased to 182±11 mg/100 mL in the 250 mg/kg group and 168±16 mg/100 mL in the 500 mg/kg group. Triglyceride levels were 231.87±13.37 mg/100 mL in the diabetic control group, whereas they decreased to 144.5±11.90 mg/100 mL in the 500 mg/kg treatment group. Total cholesterol levels also decreased from 120.12±11.36 mg/100 mL to 80.75±3.57 mg/100 mL, and LDL levels were significantly reduced to 21.62±2.61 mg/100 mL and 14.25±3.19 mg/100 mL in the treated groups, respectively. In contrast, HDL levels increased from 10.75±1.03 mg/100 mL to 28.87±9.50 mg/100 mL in the treated groups. In addition, extract treatment reduced the adverse effects of diabetes on the liver and pancreas and improved the structure of the islets of Langerhans. Overall, aqueous *M. sativa* extract significantly reduced blood glucose, cholesterol, triglyceride, and LDL levels while increasing HDL levels, and also decreased ALT and AST levels. Histopathological findings indicated regeneration of damaged liver tissue and an increase in the diameter of pancreatic islets (Amraie et al., 2015).

M. sativa has long been used traditionally in the management of diabetes, prompting scientific studies to evaluate its antihyperglycemic and antihyperlipidemic effects. Accordingly, Seida et al. (2015) investigated the effects of *M. sativa* shoots in an STZ-induced diabetic rat model. Dried shoots (4.3 kg) were extracted using 70% methanol via the percolation method, and 500 g of the resulting crude extract was suspended in water and fractionated successively with petroleum ether, chloroform, ethyl acetate, and n-butanol. Diabetes was induced in adult male Wistar rats by intraperitoneal injection of STZ at 55 mg/kg. The animals were divided into normal, diabetic control, rosuvastatin-treated, metformin-treated, and various extract-treated groups. Administration of the total methanolic extract (500 mg/kg), petroleum ether fraction (32.5 mg), and butanol fraction (60 mg) for 4 weeks significantly reduced triglyceride, total cholesterol, LDL, and VLDL levels compared with rosuvastatin ($p<0.05$). In the STZ-induced diabetic rats, the most pronounced reduction in fasting blood glucose was observed in the metformin group, from 314.10 mg/dL to 129.08 mg/dL after 28 days. Among the *M. sativa*-treated groups, the most effective result was reported in the total methanolic extract group, although overall changes in glucose levels varied across fractions, and the petroleum ether and chloroform fractions did not produce significant reductions in fasting blood glucose (Seida, El-Hefnawy, Abou-Hussein, Mokhtar, & Abdel-Naim, 2015). Since this study involved an *in vivo* diabetes model induced with STZ, its results cannot be directly compared with those of our study.

Paun et al. (2024) conducted a study evaluating the antioxidant and antidiabetic properties of polyphenol-rich extracts from *M. sativa* (aerial part) and *Solidago virgaurea* (aerial part). In the study, aqueous-ethanol (50% v/v) extracts were obtained from both plants using ASE and laser extraction methods. The α-amylase inhibitory activity of concentrated extracts obtained from *M. sativa* using the ASE method was determined, with an IC_{50} value of 23.90±1.20 µg/mL, which was comparable to that of the reference compound acarbose (IC_{50}: 24.20±1.60 µg/mL). The concentrated extract obtained via laser extraction also exhibited strong inhibition of the α-amylase enzyme, with an IC_{50} value of 26.80±1.10 µg/mL. When evaluated for α-glucosidase inhibitory activity, the *M. sativa* concentrated extracts were found to exhibit significantly stronger inhibition compared to acarbose. The IC_{50} values for the concentrated extract obtained via ASE and the concentrated extract obtained via laser extraction were determined to be 24.20±0.90 µg/mL

and 25.70±1.10 µg/mL, respectively, which are significantly lower than acarbose's IC_{50} value of 66.50±4.20 µg/mL ($p<0.05$). In contrast, both extracts exhibited weaker inhibitory activity compared to rutin (IC_{50}: 8.60±0.70 µg/mL), one of the potent natural inhibitors. The findings indicate that concentrated extracts derived from *M. sativa* possess strong inhibitory potential, particularly against the α-glucosidase enzyme, and that this effect may be associated with the extracts' polyphenol and flavonoid contents ($p<0.05$) (Paun et al., 2024).

CONCLUSION

In conclusion, this study evaluated and compared the antioxidant activity, total phenolic, total flavonoid, and total tannin contents, as well as the inhibitory effects of methanol extracts obtained from the leaves and aerial parts of *M. sativa* against α-glucosidase, α-amylase, pancreatic lipase, and pancreatic cholesterol esterase enzymes. The findings indicated that, at a concentration of 2 mg/mL, the leaf extract showed the highest metal chelating activity among all antioxidant assays, while the aerial part extract also demonstrated relatively strong metal-chelating capacity compared with other antioxidant test results and the reference compound. Both extracts exhibited moderate inhibitory activity against α-glucosidase and α-amylase enzymes. In terms of anti-obesity potential, the aerial part extract showed a low level of effectiveness. For antihyperlipidemic activity, the aerial part extract demonstrated partial inhibitory activity against pancreatic cholesterol esterase.

When compared with previously reported literature data, the results obtained in this study were consistent with earlier findings on *Medicago* species. It has been reported that *M. sativa* and related species are rich in phenolic constituents and possess notable antioxidant properties. Furthermore, this study presents, for the first time, data on the inhibitory effects of extracts prepared from the aerial and leaf parts of *M. sativa* on pancreatic lipase and pancreatic cholesterol esterase enzymes, thereby contributing new information to the literature. The relatively weak inhibition observed for enzymes involved in lipid metabolism suggests that the antihyperlipidemic effect of the plant may be associated more with antioxidant mechanisms rather than direct enzyme inhibition.

In conclusion, it has been demonstrated that *M. sativa* extracts exhibit moderate-to-high activity and selective enzyme modulation related to carbohydrate metabolism. Although the direct inhibitory effect on enzymes associated with lipid metabolism is limited, the plant's rich phenolic and flavonoid content makes it a potential natural source of bioactive compounds for metabolic diseases. Identifying the compounds responsible for the potential in the plant, increasing the number of *in vivo* studies, and examining structure-activity relationships *in silico* will contribute to this field.

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AUTHOR CONTRIBUTION STATEMENT

Concept (SP, ÖFG, BÖ, DDO), Design (SP, ÖFG, BÖ, DDO), Supervision (SP, ÖFG, BÖ, DDO), Resources (SP, ÖFG, BÖ, DDO), Materials (SP, ÖFG, BÖ, DDO), Data Collection and/or Processing (SP, ÖFG, BÖ, DDO), Analysis and/ or Interpretation (SP, ÖFG, BÖ, DDO), Literature Search (SP, ÖFG, BÖ, DDO), Writing (SP, ÖFG, BÖ, DDO), Critical Reviews (SP, ÖFG, BÖ, DDO).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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