Turkish Journal of Biodiversity

Turk J Biod, December 2025, 8(2): 74-80
https://doi.org/10.38059/biodiversity.1843090

Journal homepage: http://dergipark.org.tr/biodiversity

e-ISSN:2667-4386

RESEARCH ARTICLE

Open Access

Quantitative analysis of *Medicago lupulina* stem and evaluation of antioxidant activity

Medicago lupulina gövdesinin kantitatif analizi ve antioksidan aktivitesinin değerlendirilmesi

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Article Info

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Article history
Received: December 16, 2025
Received in revised form: December 26, 2025
Accepted: December 26, 2025
Available online: December 27, 2025

Keywords: Quantitative analysis, *Medicago lupulina* stem, antioxidant activity

Anahtar Kelimeler: Kantitatif analiz, *Medicago lupulina* gövde, antioksidan aktivite

ABSTRACT

Plants have gained considerable interest in drug discovery owing to their rich content of bioactive secondary metabolites, which play a critical role in the pharmaceutical industry. A significant proportion of currently used therapeutic agents are derived from naturally occurring biologically active compounds. In this study, the phenolic composition of *Medicago lupulina* stem was quantified by LC–MS/MS, revealing salicylic acid as the predominant phenolic compound (78.4 µg/g extract). The antioxidant potential of the methanolic stem extract was evaluated using the DPPH and ABTS assays. The extract exhibited notable antioxidant activity, with IC_{50} values of 14.8 µg/mL and 8.7 µg/mL in the DPPH and ABTS assays, respectively. Identifying bioactive compounds in *M. lupulina* stems at high concentrations will significantly contribute to studies aimed at their isolation.

ÖZ

Bitkiler, ilaç endüstrisinde önemli rol oynayan zengin biyoaktif ikincil metabolit içerikleri nedeniyle ilaç keşfinde yoğun ilgi görmektedir. Günümüzde kullanılan terapötik ajanların önemli bir kısmı, doğal olarak oluşan aktif bileşiklerden elde edilmektedir. Bu çalışmada, *Medicago lupulina* gövdesinin fenolik bileşimi LC-MS/MS ile kantitatif olarak belirlenmiş ve ana ürün olarak salisilik asit (78,4 µg/g ekstrakt) tespit edilmiştir. Metanol ekstraktının antioksidan potansiyeli, DPPH ve ABTS testleri kullanılarak değerlendirilmiştir. Ekstrakt, DPPH ve ABTS testlerinde sırasıyla 14.8 µg/mL ve 8.7 µg/mL IC50 değerleriyle kayda değer antioksidan aktivite göstermiştir. *M. lupulina* saplarında yüksek konsantrasyonlarda bulunan biyoaktif bileşiklerin tanımlanması, bunların izolasyonuna odaklanan çalışmalara önemli katkı sağlayacaktır.

Citation:

To cite this article: Erenler R, Yıldız İ, Gecer E N, Hosaflioğlu İ (2025). Quantitative analysis of *Medicago lupulina* stem and evaluation of antioxidant activity. *Turk J Biod* 8(2): 74-80. https://doi.org/10.38059/biodiversity.1843090

1. INTRODUCTION

Plants have long played a vital role in folk medicine, serving as primary sources of treatment for various diseases across different cultures (Elmastas et al., 2004; Topçu et al., 1999). Traditional knowledge of medicinal plants has guided the discovery of numerous bioactive compounds with therapeutic potential (Aksit et al., 2014; Demirtas et al., 2013; Sahin Yaglioglu et al., 2013). Many modern pharmaceuticals have been developed directly from plant-derived compounds or inspired by their chemical structures (Newman & Cragg 2007). The vast diversity of secondary metabolites found in plants provides valuable opportunities for novel drug discovery (Bayir et al., 2014; Kaya et al., 2014; Türkmen et al., 2014). Therefore, plants remain indispensable resources in both traditional healing practices and modern drug development (Erenler et al., 2015).

The determination of bioactive compounds in plant extracts by LC–MS/MS is of great importance for both phytochemical research and drug discovery (Erenler et al., 2018). This technique enables the simultaneous, sensitive, and accurate identification and quantification of multiple compounds, even at low concentrations (Atalar et al., 2023). LC-MS/MS provides detailed structural information, facilitating the characterization of complex plant matrices (Erenler, Atalar, et al., 2023). Accurate profiling of bioactive constituents helps to correlate chemical composition with biological activity (Erenler, Karan, et al., 2023). Consequently, LC-MS/MS plays a crucial role in standardization, quality control, and the development of plant-based pharmaceuticals (Erenler, Yaman, et al., 2023).

Bioactive natural compounds have inspired synthetic chemists to develop structurally related analogues for pharmaceutical research and drug discovery (Cakmak et al., 2006; Erenler et al., 2009; Lu et al., 2014; Okten et al., 2013; Sunkur et al., 2021). Advances in spectroscopic and chromatographic methods during the 19th century led to increased scientific interest in plants as sources of bioactive compounds (Elmastas et al., 2016).

Plant extracts have been widely and effectively employed in the green synthesis of nanoparticles due to their eco-friendly nature, cost-effectiveness, and sustainability (Erenler, Gecer, et al., 2021). In this approach, phytochemicals such as phenolic acids, flavonoids, terpenoids, alkaloids, proteins, and sugars present in plant extracts act as natural reducing, stabilizing, and capping agents during nanoparticle formation (Erenler, Temiz, et al., 2021; Gecer 2021). This eliminates the need for toxic chemicals and high-energy physical processes commonly used in conventional synthesis methods (Dag 2022; Genc et al., 2021). As a result, green-synthesized nanoparticles exhibit improved biocompatibility and reduced environmental impact (Erenler & Dag 2022; Erenler & Gecer 2022a). Nanoparticles produced via plant-mediated green synthesis have found extensive applications across various fields, including medicine, electronics, agriculture, and environmental remediation (Erenler & Gecer 2022b). In biomedical applications, these nanoparticles demonstrate promising antimicrobial, antibiofilm, antioxidant, anticancer, and drug-delivery capabilities (Erenler et al., 2022; Gecer & Erenler 2022).

Antioxidants are compounds that protect biological systems from oxidative damage by neutralizing free radicals and reactive oxygen species (Erenler, Adak, et al., 2017). They play a crucial role in preventing cellular damage associated with aging and various chronic diseases (Erenler, Meral, et al., 2017). Natural antioxidants, particularly those derived from plants, include phenolic acids, flavonoids, and other secondary metabolites (Koysu et al., 2018). These compounds contribute to the health-promoting effects of plant-based foods and medicinal plants (Dede et al., 2019). Consequently, antioxidants are of significant interest in pharmaceutical, nutraceutical, and biomedical research (Erenler et al., 2019; Genç et al., 2019).

Black medick (*M. lupulina* L.) is a member of the genus *Medicago* in the legume family (*Fabaceae*). Species of this genus are rich in proteins, amino acids, vitamins, and trace elements and are widely used in animal husbandry. *M. lupulina* is traditionally used in folk medicine for its anti-inflammatory, anti-ulcerogenic, antiseptic, diuretic, antispasmodic, and antitumor effects (Amer et al., 2013).

In this study, the quantitative analysis of phenolic compounds in *M. lupulina* was determined, and antioxidant activity was evaluated. This study is unique in that it is the first to quantitatively and in detail define the phenolic compounds in the stem of *M. lupulina* using LC-MS/MS. Furthermore, the combined assessment of the relationship between the determined phenolic profile and antioxidant activity is unique to this plant and represents a significant innovation to the scientific community. The findings reveal the potential of *M. lupulina* as a natural antioxidant source, providing a unique and valuable contribution to research in natural product-based pharmaceuticals and functional foods. Moreover, this study will serve as a resource for research in the isolation of major compounds.

2. MATERIALS AND METHODS

2.1. Chemical

All chemicals were of analytical grade and used as received, without additional purification, with a purity greater than 99%. The DPPH radical, gallic acid, dibutyl phthalate, and sodium tetraphenylborate (NaTPB) were provided by Merck KGaA (Darmstadt, Germany).

2.2. Plant material

M. lupulina L. was collected from the Iğdır University campus and identified by Dr Belkız Muca Yiğit of Iğdır University. A specimen was deposited in the University Herbarium (No: INWM 117).

2.3. Quantitative analysis

The phenolic profile of *M. lupulina* stem extract was analyzed by LC–ESI–MS/MS using an Agilent Technologies 1260 Infinity II system equipped with an SBC18 column (3.0 × 100 mm, 2.7 µm). The stem extract was prepared by dissolving the sample in methanol (1.0 mL), then adding hexane and centrifuging for 15 min. After phase separation, an aliquot of the methanolic phase (100 µL) was diluted with methanol (450 µL) and water (450 µL). The solution was filtered through a 0.22 µm membrane filter and injected into the LC–MS/MS system. The flow rate was set to 0.40 mL/min, and the injection volume was 5.12 µL. The mobile phase consisted of water containing 0.1% formic acid and 5.0 mM ammonium formate (A) and methanol containing 0.1% formic acid and 5.0 mM ammonium

formate (B). The gradient elution program was as follows: 20% B (1–5 min), 50% B (6–15 min), 80% B (16–22 min), and 5% B (23–30 min). The column temperature was maintained at 40 °C. Nitrogen was used as the nebulizing gas at a flow rate of 11 L/min, with a capillary voltage of 4000 V, nebulizer pressure of 15 psi, and gas temperature of 300 °C (Başar & Erenler 2024).

2.4. DPPH assay

The antioxidant activity of the plant extract and standards was evaluated using the DPPH radical-scavenging assay. The samples were allowed to react with a methanolic DPPH solution, during which a gradual decrease in absorbance was observed due to the reduction of DPPH radicals. Stock solutions of DPPH and the samples were freshly prepared. Sample solutions at concentrations of 5-50 µg/mL (3.0 mL) were mixed with the DPPH solution and incubated for 30 min in the dark at room temperature. The absorbance of the reaction mixtures was then recorded at 517 nm (Erenler & Geçer 2025).

2.5. ABTS assay

The ABTS radical-scavenging assay is a commonly used method for evaluating the antioxidant capacity of samples. In this study, the ABTS radical cation ($ABTS^{\bullet+}$) was generated by reacting ABTS with sodium persulfate in the dark at room temperature for 6 hours. The resulting solution was subsequently diluted with Na_3PO_4 buffer (pH 7.4). Antioxidant activity was determined by reacting the $ABTS^{\bullet+}$ solution with the samples at various concentrations and monitoring the decrease in the absorbance of the radical (Karan & Erenler 2024).

2.6. Statistical analysis

Statistical analysis of antioxidant activity was performed using GraphPad Prism 8.00. A one-way ANOVA with Tukey's multiple comparison test was used ($P< 0.05$). The experiments were repeated in triplicate.

3. RESULTS AND DISCUSSION

Phenolic compounds in the *M. lupulina* stem extract were quantified by LC-MS/MS, and the results revealed a diverse phenolic profile with varying concentrations (Table). Among the identified compounds, salicylic acid was the most abundant phenolic acid (78.28 µg/g), followed by rutin (57.61 µg/g) and syringic acid (29.82 µg/g). In addition, moderate amounts of flavonoids, including luteolin (19.71 µg/g), kaempferol (18.71 µg/g), and hesperidin (15.71 µg/g), were detected (Figure 1).

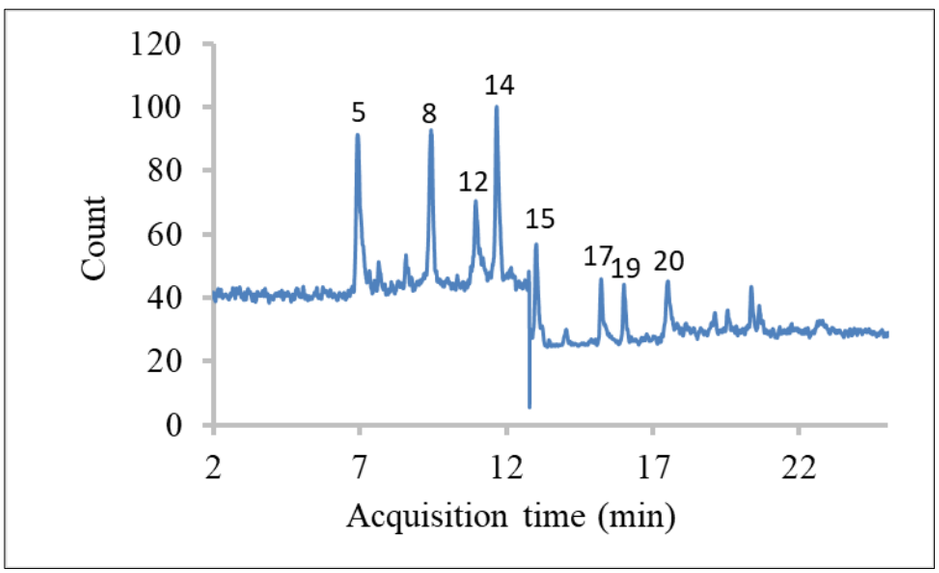

Figure 1. LC-MS/MS chromatogram of *M. lupulina* stem

The predominance of salicylic and syringic acids suggests that phenolic acids constitute a significant fraction of the stem extract. These compounds are well known for their antioxidant, anti-inflammatory, and antimicrobial properties, which may contribute to the biological activities of *M. lupulina*. Salicylic acid, in particular, plays a vital role in plant defense mechanisms and has been associated with vigorous radical-scavenging activity. The relatively high content of rutin further enhances the extract's antioxidant potential, as rutin is a potent flavonoid with documented free-radical quenching and metal-chelating abilities (Wani et al., 2017).

The quantitative analysis of *Medicago sativa* flowers was investigated using LC-MS/MS and kaempferol (88.99 µg/g extract) was determined as major compound. The antioxidant

activity of the methanol extract of these flowers was evaluated using a biosensor, and the extract exhibited high antioxidant activity (Erenler et al., 2025).

The presence of flavonoids such as luteolin and kaempferol is noteworthy, as these compounds are widely reported to exhibit antioxidant, antibacterial, and antibiofilm activities. Hesperidin, although detected at a lower concentration, is also recognized for its anti-inflammatory and antioxidant effects. The co-occurrence of these phenolic acids and flavonoids suggests a synergistic phytochemical profile that may underlie the observed antioxidant activity of the *M. lupulina* stem extract (Bounaas et al., 2025).

Table 1. Quantitative analysis of phenolic compounds by LC-MS/MS (µg/g extract)

No	Compound	RT (Retention time)	Yield
1	Gallic acid	3.23	3.61
2	Chlorogenic acid	7.11	1.91
3	Hydroxybenzaldeyde	7.60	3.24
4	Caffeic Acid	7.77	1.23
5	Syringic acid	8.41	29.82
6	Vanillin	8.66	4.23
7	o-Coumaric acid	9.39	2.92
8	Salicylic acid	9.54	78.38
9	t-Ferulic acid	10.12	6.21
10	Sinapic acid	10.77	3.91
11	p-Coumaric acid	11.54	1.61
12	Hesperidin	11.84	15.71
13	Isoquercitrin	11.81	2.81
14	Rutin	12.39	57.61
15	Kaempferol-3-glucoside	13.29	2.64
16	Fisetin	13.44	2.86
17	Naringenin	15.07	4.61
18	Hesperetin	15.87	3.24
19	Kaempferol	16.12	18.71
20	Luteolin	17.88	19.71

The antioxidant potential of the *M. lupulina* stem extract was evaluated using DPPH and ABTS radical scavenging assays. In the DPPH assay, the extract exhibited notable radical-scavenging activity, with an IC_{50} of 14.8 µg/mL. In contrast, the standard antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) showed IC_{50} values of 8.71 and 12.68 µg/mL, respectively. Similarly, in the ABTS assay, the extract's IC_{50} was 13.0 µg/mL, whereas BHA and BHT were more potent, with IC_{50} values of 8.69 and 11.83 µg/mL,

respectively (Figure 2). There is a coherence between this study and the reported studies. Many plant extracts were reported to exhibit considerable antioxidant activity (Kamah et al., 2025; Saygi et al., 2025).

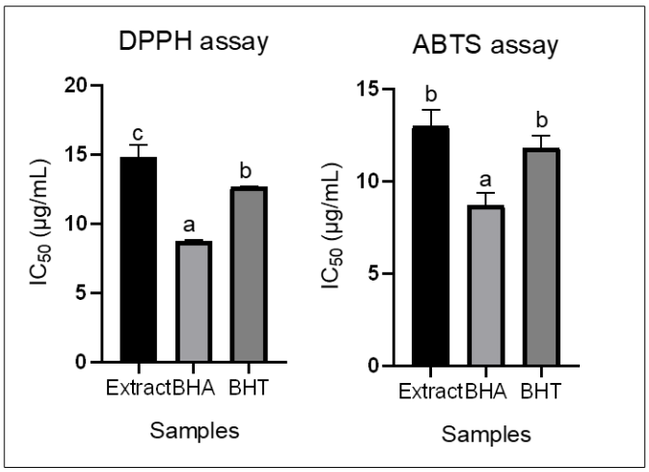

Figure 2. Antioxidant activity of *M. lupulina* stem extract

4. CONCLUSION

In the present study, the phenolic composition and antioxidant activity of *M. lupulina* L. stem extract were systematically investigated. LC-MS/MS analysis revealed a rich phenolic profile, with salicylic acid identified as the predominant compound, highlighting the stem as a valuable source of bioactive phenolic acids. The methanolic stem extract demonstrated considerable antioxidant potential in both the DPPH and ABTS assays, as evidenced by low IC_{50} values comparable to those of standard synthetic antioxidants. These findings indicate that the strong antioxidant activity of the extract is closely associated with its phenolic constituents. The results suggest that *M. lupulina* stems, often underutilized, have significant potential as a natural source of antioxidant compounds. The identification of phenolics at relatively high concentrations provides a scientific basis for further studies to isolate, characterize, and evaluate individual bioactive compounds. Such investigations may facilitate the development of natural antioxidant agents for pharmaceutical and nutraceutical applications.

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