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Research Article

Determination of antioxidant activity of *Medicago sativa* L. flowers by biosensor and quantitative analysis of bioactive compounds by LC-MS/MS

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Abstract — Natural products play a vital role in various aspects of human life, particularly in health, agriculture, and industry. These are substances derived from plants, animals, microorganisms, and minerals, and they have been used for centuries. Natural products have historically served as the foundation for many modern medicines. Over 50% of current pharmaceuticals are derived from or inspired by natural compounds. Plants are a rich and vital source of natural antioxidants that protect the body from oxidative stress caused by free radicals. The antioxidant effect of *Medicago sativa* L. flowers (MSF) was determined by sensory evaluation. Moreover, quantitative analysis of phenolics in MSF was presented. MSF scavenged DPPH radical by 41.71%. This value indicates that the plant exhibits a good antioxidant effect. Total phenolic content of MSF was determined to be 92.18 mg GA/g extract. MSF contained kaempferol (88.99), coumarin (24.42), and Kaempferol-3-glucoside (20.39 µg/g extract). Consequently, this plant's flowers contain the pharmaceutically critical natural compounds essential for the food and pharmaceutical industries.

1. Introduction

Biosensors are powerful and innovative devices that incorporate biological recognition elements and are widely used in various fields, including drug discovery, clinical diagnostics, biomedical applications, food safety, and security. These devices convert biological responses into measurable physical signals, enabling precise and rapid detection of target analytes [1]. This sensor utilized an immobilized glucose oxidase electrode and operated by electrochemically detecting either oxygen consumption or hydrogen peroxide production. This approach laid the foundation for modern biosensor technology [2]. Biosensor technologies range from sophisticated, high-throughput devices used by quantitative biologists to portable, user-friendly systems designed for non-specialist users in field applications. This versatility makes biosensors accessible across disciplines and operational environments. Current trends in biosensor research indicate rapid development; however, several challenges and limitations remain in ensuring the widespread and sustainable application of these technologies. Addressing these issues requires interdisciplinary collaboration among materials science, microelectronics, and biotechnology to enhance further the performance and reliability of biosensor devices [3].

Plants have been increasingly utilized for medicinal purposes due to their rich content of bioactive compounds [4-6]. The identification and quantification of active molecules in medicinal plants are crucial for understanding their therapeutic potential. LC-MS/MS is a very sensitive analytical technique that plays a key role in this process [7]. It offers several advantages, including high sensitivity and selectivity, accurate compound identification, quantitative analysis, rapid and efficient screening, and support for drug discovery and development, as well as quality control of herbal products [8, 9]. Recently, plants have been commonly employed for nanoparticle synthesis by green chemistry approaches [10-19]. These nanoparticles displayed considerable biological activities [20-27].

Plant secondary metabolites are natural compounds not directly involved in a plant's growth or reproduction. These bioactive substances, including alkaloids, flavonoids, terpenoids, phenolics, and glycosides, show significant pharmacological potential [28]. Due to their diverse biological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and cardioprotective effects, these compounds are frequently studied as lead structures in the development of new therapeutic agents [29-32]. Modern drug development often begins with the identification and isolation of these secondary metabolites, followed by chemical modification, pharmacological testing, and clinical trials to create effective and safe pharmaceutical products. Therefore, plant-derived compounds remain a rich and valuable source of new drugs for treating a wide range of diseases [33-35].

The progress of spectroscopy in the 19th century brought the study of natural products to the forefront of scientific research, as it enabled more precise analysis of their structure and properties [36-39]. A wide range of compounds exhibiting notable biological activity have been isolated and structurally elucidated [40-42]. Furthermore, the structural complexity and biological relevance of these natural compounds have motivated synthetic chemists to replicate and modify them through total and semi-synthesis [43-47].

Free radicals, which include reactive oxygen species such as superoxide, hydroxyl, peroxyl, and nitric oxide, are biologically significant molecules generated during oxidative processes in the human body [48]. The body owns several defense systems against oxidative stress. However, under certain conditions, such as exposure to environmental pollutants like cigarette smoke, alcohol, and ultraviolet (UV) radiation, these natural antioxidant defenses may become overwhelmed. When this happens, the excess free radicals can initiate chain reactions that damage the structure and function of cell membranes, contributing to various degenerative conditions [49, 50]. These include Alzheimer's disease, the aging process, acute liver toxicity, cardiovascular disease, diabetes mellitus, rheumatism, inflammatory responses, and DNA damage that may lead to cancer. Consequently, many antioxidant-based drug formulations are developed to help prevent and treat complex diseases [51].

This study presents the phytochemistry of Medicago sativa L. flowers (MSF), including a quantitative analysis of phenolic compounds. Moreover, antioxidant activity and total phenolic content were evaluated.

2. Experimental

2.1. Chemical and instrumentation

All chemicals used were of analytical grade and employed without any further purification, with a purity exceeding 99%. DPPH radical, gallic acid, dibutyl phthalate, sodium tetraphenylborate (NaTPB), bis(2-ethylhexyl) adipate (DEHA), polyvinyl chloride (PVC), and tetrahydrofuran (THF) were obtained from Merck KGaA (Darmstadt, Germany). The electrode potentials were measured using a potentiometric system with a sensitivity of ±0.1 mV, equipped with a computer controller and a high-impedance multi-channel setup.

Measurements were conducted relative to an Ag/AgCl reference electrode (Thermo-Orion) containing a 3.0 M KCl aqueous solution saturated with AgCl.

2.2. Plant material

Medicago sativa L. flowers were collected from Şehit Bülent Yurtseven Campus field. Botanical identification was carried out by Dr. Belkız Muca Yiğit, Iğdır University. A sample was deposited in the University Herbarium (No: INWM00000116).

2.3. PVC Membrane Biosensors

The biosensors were developed in two main stages. In the first stage, a solid contact layer was prepared by coating the tips of copper wires with a mixture of graphite, epoxy, and hardener. This mixture was formulated by combining tetrahydrofuran (THF, 3.0 mL) with graphite (50%), epoxy (35%), and hardener (15%). The copper wires were then dipped into this mixture to ensure proper viscosity and uniform coating. After coating, the cables were left to dry in the dark for 24 hours. The second stage involved preparing the membrane surface. Polyvinyl chloride (PVC), gallic acid, and a plasticizer were mixed in a watch glass, followed by the addition of 1.0 mL of THF to achieve a homogeneous mixture. This blend was conditioned at room temperature for 4 hours. Using this method, DPPH-selective and FCR-selective PVC membrane biosensors were fabricated, and the activity of the plant extract was subsequently evaluated [52].

2.4. DPPH Free Radical Scavenging Effect by PVC Membrane Biosensor

The DPPH$^{\bullet}$ free radical scavenging activity of *Medicago sativa* L. flowers was evaluated using a biosensor. A total of 10 mL of TPF extract (500 mg/L) was mixed with 10 mL of DPPH• solution (100 µg/mL). The potential was recorded by immersing the DPPH-selective PVC membrane biosensor (DPPH-SPMB) into the solution. Gallic acid served as the standard reference compound. The experiment was conducted in triplicate, and DPPH$^{\bullet}$ scavenging activity was calculated using Equation (2.1)

$$\%\text{Activity} = \frac{[(\text{A1} - \text{A0})] - (\text{A2} - \text{A0})]}{\text{A1} - \text{A0}}$$

A0 is the potential of the plant sample, A1 is the potential of the standard DPPH solution, and A2 is the activity remaining in the medium after 30 min [53].

2.5. Total phenolic content

A gallic acid-Folin-Ciocalteu (FCR) calibration curve was constructed to determine the gallic acid equivalents corresponding to the amount of FCR reduced by the plant extract. The redox potential was established by recording the potentiometric responses of these FCR solutions. To evaluate the total phenolic content (TPC) of TPF, a solution of TPF (50 mL, 0.25 mg/mL) was prepared and analyzed using the calibration curve. A mixture was prepared by combining 5.0 mL of MSF with 40.0 mL of deionized water and 5.0 mL of FCR solution (0.5 mmol/L), followed by vortexing. After the reduction reaction, the potential of the MSF mixture was measured. The total phenolic content was then calculated and expressed as gallic acid equivalents (GAE) [52].

2.6. Quantitative Analysis of Phenolic Compounds

The quantitative analysis of bioactive compounds in *Medicago sativa* L. flowers was performed using Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS), employing an Agilent Technologies 1260 Infinity II system coupled with a 6460 Triple Quadrupole mass spectrometer. For sample preparation, 50 mg of the flower material was dissolved in 1.0 mL of methanol, followed by the addition of 1.0 mL of hexane. The mixture was then subjected to ultrasonic extraction at 10,000 rpm for 15 minutes [54].

2.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.0.1. Following confirmation of data normality and homogeneity, differences between the means of the standards and samples within the same group were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistically significant differences among means were indicated by different letters (a, b, c) within the same column. Results were expressed as mean ± standard deviation (SD). Both the antioxidant and total phenolic content assays were conducted in triplicate ($p<0.05$).

3. Results and Discussion

Antioxidant activity, as assessed by the DPPH radical scavenging assay, was evaluated using a novel potentiometric PVC membrane sensor developed by the Isıldak research group [53]. At a concentration of 500 ppm, MSF inhibited the DPPH radical by 41.71%, whereas gallic acid exhibited a much stronger effect, inhibiting 92.4% of the DPPH radical at just 25.0 ppm. This indicates a significant difference in antioxidant capacity between MSF and the standard gallic acid, with MSF showing statistically lower activity. Based on this, MSF can be considered to have good antioxidant activity against the DPPH radical (Table 1).

Table 1. Antioxidant activity and total phenolic contents (mg GA/g plant extract)

Sample	Conc. ppm	DPPH˙ effect (%)	Total phenolic
MSF	500	41.71 ± 0.08^{a}	92.18 ± 0.11^{a}
Gallaic acid	25	92.44 ± 0.1^{b}	555.625 ± 0.05^{b}

GA: Gallic acid. Different letters (a,b,c) indicate the significant differences in the mean in the column.

Quantitative analysis of phenolics in MSF established that MSF contained kaempferol, coumarin, and Kaempferol-3-glucoside as major products (Table 2, Figure 1).

Flavonoids are polyphenolic compounds predominantly present in fruits and vegetables and serve as key constituents in various herbal formulations. Epidemiological studies in both humans and animals suggest that flavonoids may help reduce the risk of several diseases. One of the most widely studied flavonoids is kaempferol. Kaempferol is usually found in fruits, vegetables, and traditional medicinal sources, and is produced by plants through enzymatic processes. Both kaempferol and its derivatives demonstrate antiproliferative, antioxidant, anti-inflammatory, and antineoplastic properties. Additionally, kaempferol has shown cytotoxic effects against pancreatic and breast cancer cells [55].

Quantitative analysis of phenolic of *Echinacea purpurea* was carried out and caffeic acid was found as a chief compound [56]. Another study presented that the rosmarinic acid was the majör compound of *Origanum onites* [57]. Phytochemical analysis of *Mentha spicata* was carried out by LC-TOF/MS and rosmarinic acid was determined as major product (32.05 mg/100 g dried plant) [58].

Table 2. Quantitative analysis of natural compounds in *Medicago sativa* L. flowers by LC-MS/MS (µg/g extract)

	Compound	Retention time	Conc
1	Protocatechuic acid	5.467	0.6744
2	Hydroxybenzaldeyde	7.697	0.3710
3	Caffeic Acid	7.891	0.5601
4	Caffein	8.498	0.0797
5	Vanillin	8.678	0.6296
6	o-coumaric acid	9.495	5.1055
7	Salicylic Acid	9.871	11.2371
8	Trans-ferulic acid	10.182	5.4649
9	Coumarin	11.173	24.4249
10	Isoquercitrin	11.735	3.1168
11	Kaempferol-3-glucoside	13.282	20.3877
12	Fisetin	13.293	0.5593
13	Chrysin	14.255	0.0114
14	Trans-cinnamic acid	14.340	1.7890
15	Quercetin	14.931	1.3784
16	Naringenin	15.041	0.7461
17	Kaempferol	16.523	88.9912
18	Biochanin A	17.909	0.0346

Coumarins represent a well-established class of naturally occurring compounds recognized for their wide range of biochemical and pharmacological effects. Certain members of this group have demonstrated the ability to impact various mammalian cellular functions significantly. In recent years, interest in developing coumarins as antioxidant agents has increased considerably. These compounds offer a promising path toward discovering new antioxidants with distinct mechanisms of action. A large number of coumarins have been extracted from natural sources, particularly green plants, providing valuable insights for designing and synthesizing more effective analogues [59].

The antioxidant mechanism of molecules refers to the way these molecules neutralize or reduce the harmful effects of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can damage lipids, proteins, and DNA. Antioxidants prevent or delay oxidative stress and cellular damage. Here are the main antioxidant mechanisms of molecules:

Evaluating the antioxidant activity of plants using sensory-based methods is important for identifying bioactive compounds that contribute to flavor, aroma, and potential health benefits. Such assessments can guide the selection of plant materials for functional food, nutraceutical, and pharmaceutical applications [60].

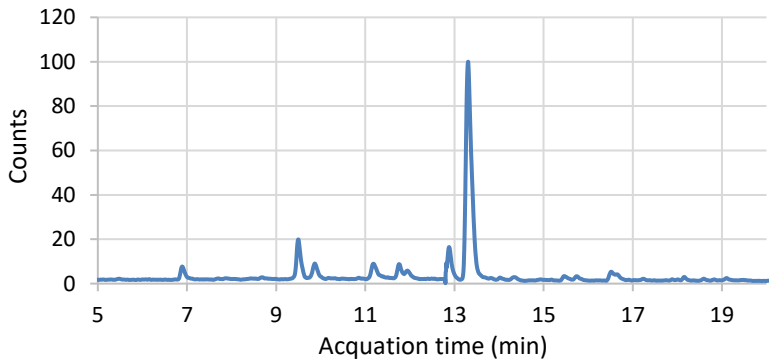

Figure 1. The MRM chromatogram of *Medicago sativa* L. flowers

4. Conclusion

Bioactive compounds in the flowers of *Medicago sativa* were determined quantitatively. Kaempferol, coumarin, and Kaempferol-3-glucoside were found as major products *in Medicano stiva* flowers. These compounds are pharmaceutically and nutritionally active. These flowers could be a valuable source for the corresponding compounds. In addition, *Medicano stiva* flowers exhibited antioxidant activity, making them suitable for use as antioxidant agents in food. Further studies should be conducted to isolate these active compounds. Additionally, various biological activity tests should be conducted on this plant.

Conflicts of Interest

The authors declare that there is no conflict of interest for this article.

Authors' Contributions

All authors contributed equally to this work. The author read and approved the final version of the paper.

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