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Quantitative analysis of phenolics in *Trifolium pratense* L. flowers and evaluation of antioxidant activity by sensory

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Trifolium pratense,
Natural compounds,
Quantification,
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Antioxidant activity.

Abstract – *Trifolium pratense* L. flowers (TPF) were collected and dried in shade in this study. After extraction in methanol, a diluted solution was applied to the liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) device to determine the bioactive compounds quantitatively. Isoquercitrin (38.64 mg/g extract), coumarin (13.66 mg/g extract), and catechin (12.52 mg/g extract) were verified as major products. Antioxidant activity of TPF was performed using a potentiometric PVC membrane sensor to evaluate 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and total phenolic content of TPF. TPF inhibited the DPPH radical as 31.4 ± 0.02% at the 500-ppm concentration. However, the DPPH activity of gallic acid was determined as 81.43 ± 0.07% and 92.44 ± 0.1% at the TPF concentrations of 12.5 ppm and 25 ppm, respectively. In addition, the total phenolic content was calculated to be 82.4 ± 0.15 mg gallic acid eq/g extract. It was observed that TPF has the potential to be an antioxidant and a valuable source of isoquercitrin, coumarin, and catechin.

Subject Classification (2020):

1. Introduction

Plants play a substantial role in drug development since they contain bioactive compounds called secondary metabolites [1-3]. Quantitative analysis of phenolics in plants is crucial for reflecting their significant roles in plant biology, agriculture, medicine, and industry [4,5]. Phenolic compounds are essential in plant growth, development, and defense mechanisms. Quantifying phenolics provides an understanding of how plants respond to various stresses such as drought, salinity, and pests, enabling the development of stress-resistant plant varieties. Phenolic content affects fruits' and vegetables' taste, color, and nutritional quality [6-8]. Determining phenolic compositions helps improve post-harvest storage and processing techniques, enhancing agricultural products' shelf life and quality [9-12]. Many phenolic compounds have strong antioxidant properties, contributing to the health benefits of plant-based foods [13,14]. Quantifying these compounds helps in assessing and promoting dietary sources of antioxidants. Phenolic-rich extracts are used in cosmetics for their antioxidant and anti-aging properties [15-17]. Natural compounds have inspired many synthetic chemists to synthesize them in laboratory conditions [18-23].

Reactive oxygen species (ROS) are chemically reactive molecules in cell signaling pathways that regulate cell proliferation and apoptosis [24,25]. ROS are produced by immune cells to destroy invading pathogens, playing

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a crucial role in the body's defense mechanism. Excessive ROS can cause oxidative damage to lipids, proteins, and DNA, contributing to cellular dysfunction and death [26]. Constant oxidative stress is correlated with the promotion of various chronic diseases, including cardiovascular diseases, neurodegenerative disorders, diabetes, and cancer. To alleviate the harmful effects of ROS, the body develops a complex antioxidant defense system, including enzymatic and non-enzymatic antioxidants [27]. The body's antioxidant system can become insufficient in certain situations, leading to an imbalance between the production of ROS and the body's ability to neutralize them [28]. Therefore, antioxidants are taken from natural products obtained from plants and are rich sources of antioxidants. These compounds help neutralize ROS and reduce oxidative stress, thereby protecting cells from damage [29]. Antioxidants are added to the food to prevent deterioration. However, synthetic antioxidants are restricted as food additives due to suspected carcinogenicity. Hence, the interest in natural products used in food as natural antioxidants has increased considerably [30].

Natural products play an important role in nanotechnology [31-35]. Nanoparticles can be synthesized from natural products [36-39]. Plant extracts act as reducing, capping, and stabilizing agents [40-44]. Many silver nanoparticles were synthesized by plant extracts that revealed considerable biological activities [45-54].

For the assessment of antioxidant assays, spectroscopic techniques like UV-Vis spectroscopy are widely used [55-57]. This conventional method is expensive, time-consuming, necessitates pre-treatment, and requires costly equipment [58]. The potentiometric method is new and efficient and has many advantages for measurement, such as applicability in heterogeneous solutions, simple design, inertness, robustness, integration into computer systems, fast response, and selectivity [59].

Herein, quantitative analysis of phenolic compounds was carried out in *Trifolium pratense* L. flowers by LC-ESI/MS/MS, and a potentiometric PVC membrane sensor determined antioxidant activity and total phenolic content.

2. Materials and Methods

2.1. Plants Materials

Trifolium pratense was obtained from Iğdır University Campus in July 2023 and identified by Dr. Belkız Muca Yiğit, Iğdır University. A voucher specimen was deposited in the herbarium of Iğdır University (No: INWM00000113).

2.2. PVC Membrane Biosensor

The biosensors were designed in two stages. The first one is the preparation of solid contact, in which the end of the copper wires was coated with graphite, epoxy, and hardener. The mixture was prepared by adding THF (3.0 mL), graphite (50%), epoxy (35%), and hardener (15%). Then, the copper wires were immersed into the solid-contact mixture to get the appropriate viscosity and coating. After the copper wires were covered with solid contact, they were kept dark for 24 hours to dry. The second stage includes the preparation of the membrane surface. PVC, gallic acid and plasticizer were mixed in a watch glass, and THF (1.0 mL) was added to homogenize. Afterward, the homogenized mixture was conditioned at rt for 4.0 hours. DPPH-selective and FCR-selective PVC membrane biosensors were prepared, and the activity of plant extract was carried out [59]. The schematic representation is given in Figure 1.

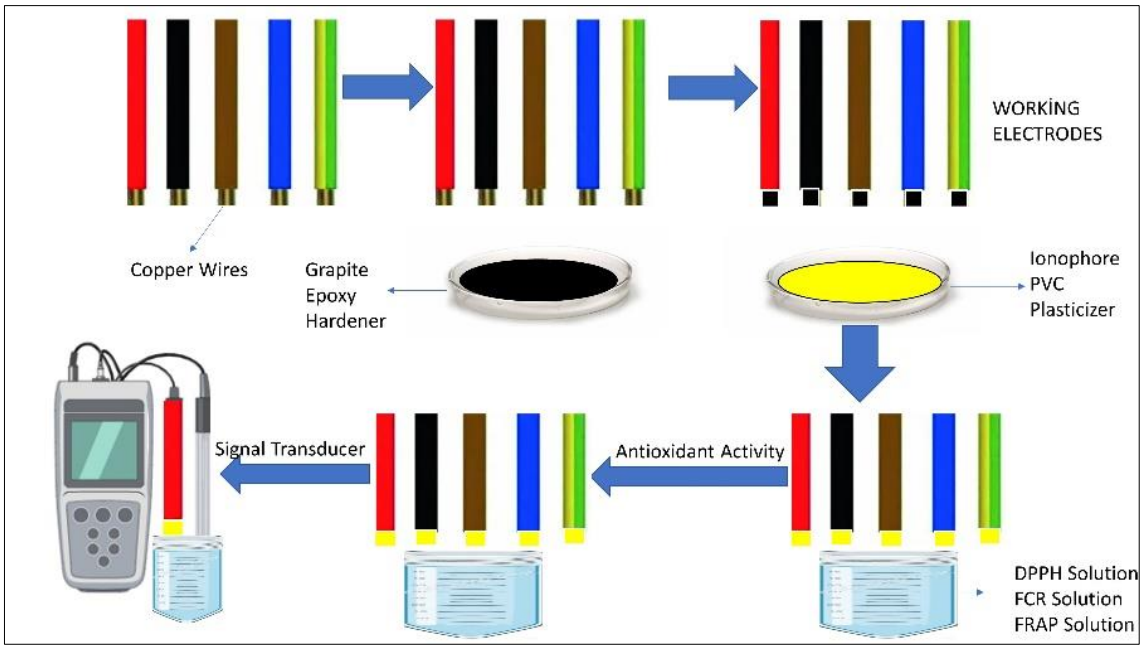

Figure 1. The measurement scheme of antioxidant activity

2.3. DPPH Free Radical Scavenging Effect by PVC Membrane Biosensor

DPPH• free radical effect of TPF was carried out using the biosensor. TPF extract (10 mL, 500 mg/L) was treated with DPPH• solution (10 mL of 100 µg/mL). The potential was measured by immersing the DPPH-SPMB into the solution. Gallic acid was used as a standard. The experiment was repeated three times. DPPH activity was calculated using the following equation (2.1) [59].

$$\%\text{Activity} = \frac{[(E1-E0)-(E2-E0)]}{E1-E0} \times 100 \quad (2.1)$$

Here, E_0 is the potential value of the plant sample, E_1 is the potential value of the standard DPPH solution, and E_2 is the potential value of the DPPH• activity remaining in the medium after a 30-minute reaction [59].

2.4. Total phenolic content analysis

The gallic acid- Folin-Ciocalteu calibration curve was plotted to reveal the gallic acid equivalent to the amount of FCR reduced by the TPF extract. The gallic acid solution was prepared (1.0 - 0.75 - 0.50 - 0.375 - 0.375 - 0.250 - 0.125 - 0.050 - 0.025 mg mL^{-1}). The potential was generated by measurement of the potentiometric responses of these FCR solutions. The TPF solution was prepared (50 mL, 0.25 mg/mL) to determine the total phenolic content of the TPF using the calibration curve. TPF (5.0 mL) was mixed with the deionized water (40.0 mL) and FCR solution (5.0 mL, 0.5 mmol/L) and vortexed. The potential of TPF was measured after the reduction reaction. Total phenolic content was calculated concerning GA equivalent using the equation (E= 0.0513 [GA] + 8.771) R^2 = 0.9996 obtained from the FCR - gallic acid calibration graph [60].

2.5. Quantitative Analysis of Phenolic Compounds by LC-ESI-MS/MS

The bioactive compounds in TPF were determined quantitatively using the Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) (An Agilent Technologies 1260 Infinity II, jointed 6460 Triple Quad mass spectrometer) instrument. The sample (50 mg) was dissolved in methanol (1.0 mL), and hexane (1.0 mL) was added. Then, the mixture was subjected to the ultrasonic bath at 10000 rpm for 15 minutes. A sample from the methanol phase (100 µL) was mixed with methanol and water

(each 450 µL). After filtration of the mixture, it was transferred to the vial. Poros hell 120 EC-C18 column was used. An Electrospray ionization (ESI) source was employed with positive and negative ionization modes to detect the compounds' mass-to-ion ratio (m/z). The injection volume was kept at 4.0 µL. The water, including formic acid (0.1%), ammonium format (5.0 mM) A, methanol consisting of formic acid (0.1%), and ammonium format (5.0 mM) B were used as mobile phase. The gradient program was set as 80% for 0-5 min, 70% for 6-15 min, 15% for 16-20 min, 10% for 21-25 min, and 26-30 min 95% was applied in the mobile phase A. The flow rate was 0.4 mL/min, and the gas flow (Nitrogen) was 11 L/min [61].

2.6. Statistical Analysis

GraphPad Prism (8.0.1) was used for statistical analysis. After approving the normality of distribution and homogeneity of the data, the differences of the means of the standard and sample in the same column were submitted to analysis of variance (one-way ANOVA), followed by Tukey's test. Different letters (a, b, c) reveal the significantly different mean in the column. The results were indicated as mean ± standard deviation (SDs). The antioxidant assay and total phenolic content assay were executed in triplicate. The statistical significance level was accepted at $p < 0.05$.

3. Results and Discussion

Antioxidant activity, including DPPH radical scavenging assay, was executed by a novel potentiometric PVC membrane sensor developed by the Isıldak et al. [59]. TPF inhibited DPPH radical by 31.4% at the 500-ppm concentration. Whereas gallic acid inhibited the DPPH radical by 92.4% at 25.0 ppm. Compared to the standard gallic acid, there is a significant difference between gallic acid and TPF. TPF has lower activity than the standard gallic acid statistically. TPF can be considered to have moderate activity on the DPPH radical (Table 1). In the reported study, *Origanum onites* inhibited the DPPH radical by 50.5% at 40 ppm, *Thymus praecox* inhibited the DPPH by 99.55 at 40 ppm, and *Origanum bilgeri* activity was reported as 52.25% at the same concentration [59]. In total phenolic content analysis, TPF was determined to include total phenolic with the value of 82.44 (mg GA/g plant extract). There is an agreement between the total phenolic content and antioxidant activity. Quantitative analysis of bioactive compounds was carried out by LC-MS/MS. Isoquercitrin (38.64 mg/g extract), coumarin (13.66 mg/g extract), and catechin (12.52 mg/g extract) were established as major products (Table 2, Figure 2). Signals marked with an asterisk (*) in Figure 2 indicate compounds not found in the standards.

Table 1. Antioxidant activity and total phenolic content

Sample	Conc. (ppm)	DPPH˙ scavenging effect (%)	Total phenolic mg GA/g plant extract)
TPF	500	31.43 ± 0.02^{a}	82.44 ± 0.15^{a}
Gallic Acid	25	92.44 ± 0.1^{c}	555.625 ± 0.05^{c}
Gallic Acid	12.5	81.43 ± 0.07^{b}	325.625 ± 0.05^{b}

GA: Gallic acid. Different letters (a,b,c) indicate the significantly different of the mean in the column

Flavonoids are a class of plant-derived polyphenolic compounds widely recognized for their beneficial health effects. These compounds are known for their anti-inflammatory, antimicrobial, anticancer, and antiallergic activities. Flavonoids are found in various fruits, vegetables, tea, and other plant-based foods, contributing to the health benefits of a diet rich in these foods. Flavonoids play a crucial role in plant biology, including UV filtration, symbiotic nitrogen fixation, and floral pigmentation [62].

Isoquercitrin is a type of flavonoid, specifically a flavonol glycoside, found in many plants. It is a quercetin molecule bound to a glucose molecule. Isoquercitrin is known for its potent antioxidant properties and

contributes to the overall health benefits attributed to flavonoids. It was reported that Isoquercitrin exhibited biological activities such as antioxidant, anti-inflammatory, antimicrobial, and anticancer properties [63].

Table 2. Quantitative analysis of standard compounds' in *Trifolium pratense* flowers by LC-MS/MS (mg/g extract)

No	Compound	RT	Quantity
1	Catechin	6.904	12.520
2	4-hydroxybenzaldehyde	7.697	0.272
3	Caffeic Acid	7.891	0.494
4	Caffeine	8.498	0.113
5	Vanillin	8.678	0.496
6	p-coumaric acid	9.495	1.249
7	Salicylic Acid	9.871	1.145
8	Coumarin	11.173	13.668
9	Isoquercitrin	11.735	38.642
10	Kaempferol-3-glucoside	13.282	1.766

RT: Retention time

Coumarin is a naturally occurring fragrant organic compound found in many plants, notably in the tonka bean, vanilla grass, sweet woodruff, and some species of cinnamon. It is known for its sweet, vanilla-like aroma and is used in the fragrance and flavor industry [64].

Catechin is a type of natural phenolic compound and antioxidant belonging to the flavonoid family, specifically a subgroup known as flavan-3-ols. Catechins are widely found in various foods and beverages, with particularly high concentrations in tea, cocoa, and certain fruits. Catechins help neutralize free radicals, reducing oxidative stress and cellular damage. Regular consumption of catechin-rich foods is associated with improved heart health, including reduced blood pressure, improved blood lipid profiles, and decreased risk of heart disease. Catechins have been studied for their potential to inhibit cancer cell growth and induce apoptosis in various cancer types. Catechins may protect brain health, potentially reducing the risk of diseases like Alzheimer's and Parkinson's [65].

The medicinal effects of salicylic acid (SA) have been known for years. SA is the phenolic compound plants synthesized and contained in many regulatory pathways. SA has been shown to regulate cell growth, stomatal aperture, respiration, seed germination, fruit yield, nodulation in legumes, and the expression of senescence-related genes. Moreover, it is mostly known for its central role in defense responses [66].

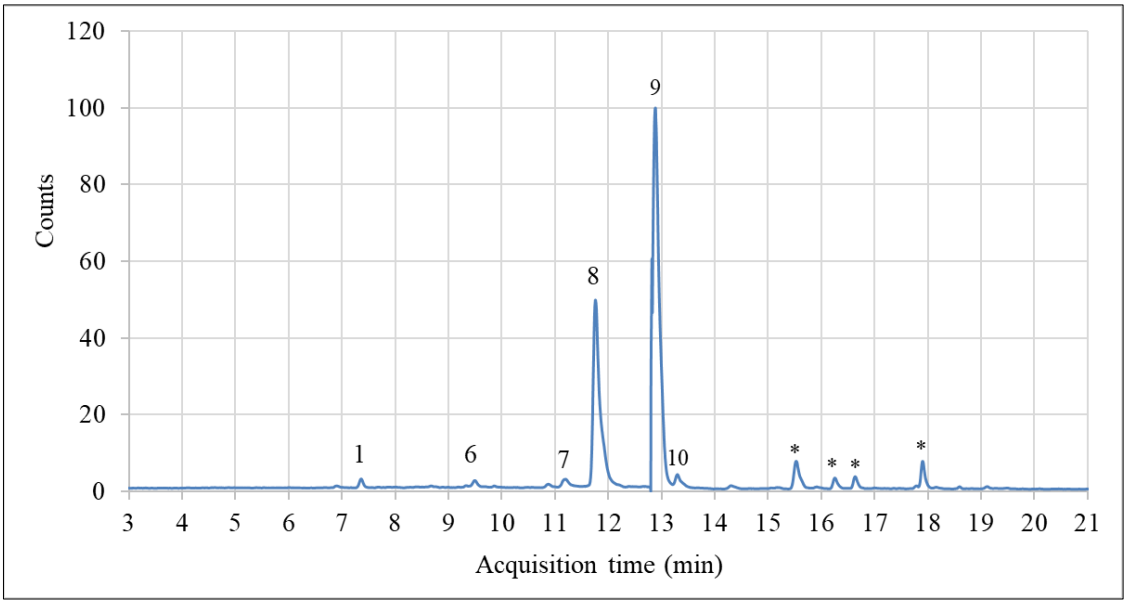

Figure. 2. The MRM chromatogram of *Trifolium pratense* flowers

4. Conclusion

The phytochemistry of *Trifolium pratense* flowers was determined. Quantitative analysis of phenolic compounds in *Trifolium pratense* flowers demonstrated that the corresponding plant included significant compounds for food and pharmaceuticals. This study proved that this plant could be a valuable source of important compounds, including isoquercitrin, coumarin, and catechin. In addition, a new, efficient, sensitive, and fast technique was utilized to determine the antioxidant activity and total phenolic content of TPF. It was presented that there was a correlation between the total phenolic content and antioxidant activity. TPF revealed moderate antioxidant activity in comparison to the standard gallic acid. Due to the importance of antioxidants in food and drugs, investigation of the antioxidant activity and total phenolic content of TPF will make significant contributions to related fields. This study will inspire scientists to study natural products to isolate high concentrations of bioactive compounds from this plant. This is the first report to determine the antioxidant activity and total phenolic content by sensory. Moreover, this study first presented the quantitative analysis of phenolic compounds in TPF.

Author Contributions

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

Conflict of Interest

All the authors declare no conflict of interest.

Ethical Review and Approval

No approval from the Board of Ethics is required.

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