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Original Article

# Bioactive Properties of Saffron-Fortified Glycolic Propolis Extracts

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## Abstract

**Introduction:** Propolis is a bee-derived natural product characterized by its complex chemical composition and pronounced antioxidant capacity. With the increasing consumer demand for functional foods, propolis has gained significant attention as a bioactive ingredient and is now widely incorporated into various formulations within the nutraceutical sector. Similarly, saffron, traditionally valued as a culinary spice, has recently attracted growing scientific interest due to its potential biological activities, particularly those associated with the modulation of central nervous system functions.

**Methods:** In this study, glycol-based propolis extracts containing 1% and 5% saffron were prepared. Their antioxidant properties were evaluated in terms of total phenolic content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP), and DPPH radical scavenging activity. The phenolic profiles of the extracts were also analyzed using HPLC-PDA with 26 phenolic standards.

**Results:** In the propolis extract, several phenolic and flavonoid compounds were identified, including p-hydroxybenzoic acid, caffeic acid, p-coumaric acid, ferulic acid, quercetin, trans-cinnamic acid, apigenin, rhamnetin, chrysin, pinocembrin, caffeic acid phenethyl ester (CAPE), and galangin. The incorporation of 1% saffron into the extract did not cause a notable alteration in the phenolic composition. However, supplementation with 5% saffron resulted in elevated concentrations of p-hydroxybenzoic acid, quercetin, apigenin, and rhamnetin.

**Conclusions:** This study suggests that the synergistic interaction between the bioactive constituents of propolis and saffron may enhance their overall biological efficacy. Nevertheless, the findings of the present study are limited to in vitro antioxidant assays. Therefore, further in vivo investigations are warranted to elucidate the underlying mechanisms of action and to optimize the formulation ratios for potential nutraceutical applications.

**Keywords:** Antioxidant, flavonoids, phenolic compounds, propolis, saffron

## 1. Introduction

Propolis is a natural bee product produced by honeybees using resins collected from plants (1). While it primarily consists of resins and beeswax, over 300 compounds have been identified in propolis, including vitamins B, C, and E, various minerals, phenolic acids, flavonoids, stilbene derivatives, terpenes, and amino acids. Among these, phenolic acids and flavonoids are particularly associated with the biological activities of propolis (2).

Historically, propolis has been used for its various therapeutic purposes such as embalming, wound care, and as an antiseptic (1). Today, due to its rich composition and broad spectrum of biological activities, propolis is being utilized in the development of nutraceutical products and in apitherapy. The World Health Organization (WHO) has stated that propolis can be safely used without interfering with medical treatments. Along with the growing global interest in functional foods, this has contributed to an increasing trend in the use of propolis as a food ingredient (3, 4).

However, several factors limit the use of propolis as a nutraceutical. Among these are its low water solubility, as well as its unpleasant taste and odor (3). Ethanolic extracts of propolis, which show better solubility than aqueous extracts, have been reported to exhibit higher antioxidant activity and a richer phenolic compound profile. Nevertheless, the potential toxicity of ethanol and its possible adverse health effects raise concerns among consumers, particularly regarding orally administered propolis drops. Therefore, the development of ethanol-free propolis extracts with high biological activity has become an important area of research (5, 6). In this context, glycol derivatives are being investigated as alternative solvents.

The phenolic content and antioxidant capacity of a water–polyethylene glycol (PEG) extract, prepared as an alternative to ethanolic propolis extracts, have been investigated. In one study, the antioxidant activities of ethanol and water–PEG extracts were found to be similar when assessed using ABTS and CUPRAC methods; however, ethanol extracts exhibited higher antioxidant activity based on DPPH and FRAP assays (6). Similarly, in another study comparing anhydrous PEG and ethanol extracts, no statistically significant difference was reported in terms of total polyphenol content (TPC). However, it was concluded that PEG was more effective in extracting polar compounds, whereas ethanol favored the extraction of more apolar phenolics. PEG is known to be a low-cost, non-toxic, and well-tolerated solvent. In fact, its use at certain concentrations is considered safe even in pediatric pharmaceutical formulations (5).

Saffron (*Crocus sativus* L.) is a spice commonly used to impart color, flavor, and aroma of foods. In addition to its culinary use, saffron has also been traditionally applied in cosmetic and therapeutic practices. Notably, it has been used as a tonic in Persian traditional medicine (7). Today, saffron is particularly studied for its effects on the central nervous system, and for its potential antidepressant, anti-inflammatory, and antioxidant properties (8).

The biological activities of saffron are mainly attributed to its active compounds: safranal, crocin, and crocetin. Some preclinical studies have demonstrated the antidepressant properties of crocin and crocetin. Clinical studies have also reported that saffron reduces anxiety scores in patients compared to placebo (9). In a six-week study conducted with patients diagnosed with moderate depression, saffron administered at a dose of 60 mg/day showed comparable effects to fluoxetine treatment at 40 mg/day (10). Due to its low toxicity, significant effects on oxidative stress and inflammation, anxiolytic properties, and its ability to modulate mitochondrial function, saffron extracts and their constituents are considered promising nutraceutical compounds in this field (7).

In this study, glycol extracts of high-activity propolis enriched with different concentrations of saffron were prepared, aiming to obtain antioxidant-rich extracts with high nutraceutical value.

## 2. Methods

### 2.1. Materials

Propolis was obtained from a local beekeeper in Turkey. Saffron was sourced from Iran. Food-grade glycol was used for extract preparation. All chemicals and phenolic standards used for the analyses were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Preparation of Extracts

All extracts were prepared in a solvent system containing 50% glycol. The first extract served as the control and contained only 30% (w/v) propolis. The second extract contained 30% propolis and 1% (w/v) saffron, while the third extract contained 30% propolis and 5% (w/v) saffron. The extracts were subjected to ultrasonic treatment for 40 minutes, followed by incubation at 50 °C with shaking at 100 rpm for 24 hours.

### 2.3. Total Phenolic Content (TPC)

The method described by Slinkard and Singleton (11) was used to determine TPC. In the analysis, 680 µL of distilled water, 400 µL of 0.2 N Folin–Ciocalteu reagent, 20 µL of the sample, and 400 µL of 10% sodium carbonate solution were used. After incubation for 2 hours, absorbance was measured at 760 nm using a spectrophotometer (Evolution™ 201, Thermo Scientific, USA). Gallic acid was used as the standard, and results were expressed as milligrams of gallic acid equivalents per milliliter (mg GAE/mL).

### 2.4. Total Flavonoid Content (TFC)

The method described by Mohammadzadeh et al. (12) was used for the analysis. For the assay, 0.5 mL of sample extract, 2.15 mL of methanol, 0.05 mL of 10% aluminum nitrate, and 0.05 mL of 1 M ammonium acetate were used. After incubation for 40 minutes, absorbance was measured at 415 nm using a spectrophotometer. Quercetin was used as the standard, and results were expressed as milligrams of quercetin equivalents per milliliter (mg QE/mL).

### 2.5. Antioxidant Activity

The ferric reducing antioxidant power (FRAP) assay was performed according to the modified method described by Benzie and Strain (13). The FRAP reagent was freshly prepared for the test. In each test tube, 3 mL of FRAP reagent and 0.1 mL of sample were added. After incubation for 4 minutes, absorbance was measured at 593 nm. Results were expressed as milligrams of Trolox equivalents per milliliter (mg Trolox/mL).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay was conducted following the method described by Molyneux (14). A fresh DPPH solution (0.04 mg/mL) was prepared for the test. In a test tube, 0.75 mL of DPPH solution and 0.75 mL of sample were mixed. After 50 minutes of incubation, absorbance was read at 517 nm. Results were expressed as $SC_{50}$ (mg/mL) values.

### 2.6. Phenolic Profile

Equal volumes were taken from each extract to prepare the samples. First, the pH of the samples was adjusted to 2 with 1 N HCl. Subsequently, liquid-liquid extraction was performed twice using 15 mL each of ethyl acetate and diethyl ether (10 mL in the first extraction and 5 mL in the second extraction) at 200 rpm for 15 min. The organic phases were collected and evaporated using a rotary evaporator (IKA®-Werke RV 05 Basic). The residue was dissolved in 2 mL of methanol and then injected into the instrument for analysis. Twenty-six phenolic standards were analyzed using reversed-phase high-performance liquid chromatography (RP-HPLC, Shimadzu Corporation LC 20AT) equipped with a photodiode-array (PDA) detector, as described by Kara and Birinci (15). A C18 column (5 µm, 4.6 mm × 250 mm; GL Sciences) was used in the analyses. The injection volume was 20 µL, the column temperature was maintained at 30 °C, the flow rate was set at 1 mL/min, and measurements were performed at four different wavelengths (250, 280, 320, and 360 nm). In the method used, 70% acetonitrile (ACN)–ultrapure water (reservoir A) and 2% acetic acid (AcH)–ultrapure water (reservoir B) were used as the mobile phase. A gradient program with a total analysis time of 50 minutes was employed. Calibration curves were prepared for all 26 standards, with linear ranges of X–Y µg/mL and correlation coefficients ($R^2$) > 0.99. Within the analytical procedure, standard compounds were examined at four distinct wavelengths: 250, 280, 320, and 360 nm. The developed method for phenolic compound quantification was based on measuring each standard at its characteristic absorption wavelength. Specifically, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, rutin, ellagic acid, and daidzein were analyzed at 250 nm; gallic acid, catechin hydrate, epicatechin, syringic acid, trans-cinnamic acid, naringenin, hesperetin, chrysin, and pinocembrin at 280 nm; chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, apigenin, and caffeic acid phenethyl ester (CAPE) at 320 nm; while myricetin,

luteolin, quercetin, rhamnetin, and galangin were detected at 360 nm.

### 2.7. Statistical Analysis

Statistical analysis were performed using the SPSS Statistics 22.0 software. Results are presented as mean ± standard deviation. Data were analyzed using the ANOVA method and Tukey's test. A level of $p< 0.05$ was considered statistically significant. All analyses were performed in triplicate, and the results were expressed as mean ± standard deviation.

## 3. Results and Discussion

The antioxidant properties of the prepared extracts were evaluated in terms of TPC, TFC, FRAP, and DPPH radical scavenging activity (Table 1). The TPC value of the control extract containing 30% propolis was found 18.34±0.68 mg GAE/mL. In the Yildiz (16) study, the TPC value of 20% propolis-glycol (w/v) extract was determined as 49.78±2.55 mg GAE/mL. According to the TPC results, the addition of 1% saffron did not cause a statistically significant change in the antioxidant properties of the extract. However, a significant increase in TPC content was observed with the addition of 5% saffron. Previous studies comparing the antioxidant activities of various saffron extracts reported the highest TPC value (29.20 mg GAE/g) in an extract prepared with 80% ethanol and incubated for 24 hours at room temperature under shaking conditions (17). Another study demonstrated optimal antioxidant activity in extracts obtained through a combination of ultrasonic and microwave extraction using a 50% methanol/water solvent system, reporting a TPC value of 31.56 mg GAE/g (18).

**Table 1.** TPC, TFC and antioxidant properties of extracts.

| | 30% propolis | 30% propolis+ 1% saffron | 30% propolis+ 5% saffron |
|---|---|---|---|
| **TPC (mg GAE/mL)** | 18.34±0.68$^{a}$ | 18.75±0.82$^{ab}$ | 19.74±0.14$^{b}$ |
| **TFC (mg QE/mL)** | 2.90±0.06$^{a}$ | 3.12±0.02$^{b}$ | 4.48±0.10$^{c}$ |
| **FRAP (mgTrolox/mL)** | 12.69±0.10$^{a}$ | 13.18±0.45$^{a}$ | 14.94±0.47$^{b}$ |
| **DPPH-$SC_{50}$ (mg/mL)** | 0.066±0.002$^{b}$ | 0.049±0.0001$^{a}$ | 0.049±0.0001$^{a}$ |

Letters indicate statistical difference in the same row ($p < 0.05$).

In the present study, the control extract containing 30% propolis showed an increase approximate of 1.4 mg GAE/mL in TPC upon the addition of 5% saffron (0.05 g/mL). When normalized to the amount of saffron added, this corresponds to about 28 mg GAE/g. Moreover, in the extraction of whole saffron flowers, the total polyphenol content (TPC) was found to be 4.1 mg GAE/mL for the extract with an S:L ratio of 0.10 g/mL, whereas the extract with an S:L ratio of 0.30 g/mL exhibited a TPC of 9.6 mg GAE/mL (19). These results are in line with those reported in the literature.

Regarding TFC, the addition of both 1% and 5% saffron resulted in a statistically significant increase compared to the control extract. The observed enhancement in TFC with increasing saffron concentration may be attributed to the enrichment of the extract with the natural flavonoid components of saffron. Yıldız (16) reported the TFC of the propolis extract prepared with glycol as 6.81 mg QE/mL. In contrast, Hafshejani et al found that TFC of ethanolic propolis extracts (10% propolis (w/v), 72 hours, 40°C) ranged from 4.80–100.03 mg QE/mL (20). Moreover, Mahood et al. (21), the TFC of a 70% methanolic saffron extract was reported as 5.967 mg catechin equivalents per gram of dry weight and 241.797 mg QE/g (dry weight). Additionally, Belyagoubi et al. (22) reported a TFC value of 3.77 mg QE/g for a 70% ethanolic saffron extract.

The FRAP assay results of extracts were found between 12.69 to 14.94 mg Trolox/mL and presented in Table 1. The FRAP assay results showed that the addition of 1% saffron did not cause a significant increase in the ferric reducing antioxidant power of the extract, whereas the addition of 5% saffron led to a statistically significant enhancement. In a study investigating the antioxidant content of seven different propolis samples obtained from urban beekeeping, FRAP values were found to range from 10.93 to 29.55 mg Trolox/mL. In addition, the study also mentioned that factors such as geographical origin, harvest time, plant sources, season and climatic conditions affect the composition (23).

Changes in DPPH activity of the extracts are also shown in Table 1. As well seen, the $SC_{50}$ of the propolis extract was found 0.066 mg/mL and both saffron enriched extracts were detected 0.049 mg/mL. Regarding DPPH radical scavenging activity, an increase in antioxidant activity was observed

with 1% saffron addition; however, higher saffron concentrations did not further enhance the activity.

The $SC_{50}$ value of 11 different ethanolic propolis extracts was reported to vary between 4.62 and 1031.57 mg/mL (20). On the other hand, Rahaiee et al. (17) reported that the DPPH radical scavenging activities ($SC_{50}$) of ethanolic and methanolic saffron extracts ranged between 0.037 and 0.346 mg/mL. It should not be overlooked that the differences in bioactive properties of propolis and saffron, such as TPC, TFC and antioxidant activity could be affected by factors such as climate, harvest region, cultivation extraction method/ parameters and solvent (16, 24, 25).

Flavonoids such as quercetin, galangin, and apigenin, along with phenolic acids like caffeic and p-coumaric acids, represent the predominant phenolic constituents of propolis (26, 27). However, saffron and its floral by-products are rich in compounds such as apigenin, quercetin, kaempferol, rutin and *p*-hydroxybenzoic acid (28-30). These compositional similarities suggest that the incorporation of saffron into propolis formulations could enhance the extract's phenolic diversity and overall bioactivity. The increase in TPC, TFC and antioxidant activity observed in the 5% saffron-enriched extract may be attributed to phenolics sourced from saffron, including apigenin, quercetin, and kaempferol. From a biological standpoint, such compounds have been extensively reported for their potent antioxidant, antimicrobial, and anti-inflammatory properties, as well as neuroprotective effects, including the inhibition of amyloid aggregation associated with Alzheimer's disease (31, 32). Therefore, adding saffron to propolis not only improves the TPC, TFC, and antioxidant potential of the extract, but may also extend its spectrum of biological functions, supporting its potential application as a multifunctional nutraceutical ingredient.

In this study, the phenolic contents of the extracts were analyzed using the HPLC-PDA method, targeting 26 phenolic compounds (Table 2). In the propolis extract, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, *t*-cinnamic acid, apigenin, rhamnetin, chrysin, pinocembrin, CAPE, and galangin were detected. The addition of 1% saffron did not significantly alter the phenolic composition; however, enrichment with 5% saffron resulted in elevated levels of p-hydroxybenzoic acid, quercetin, apigenin, and rhamnetin. This outcome can be attributed to the naturally high abundance of these constituents in saffron (28-30).

Comparable results have been reported in previous studies. Analysis of the phenolic components of a 70% methanolic saffron extract revealed quercetin and epicatechin as the primary compounds (22) whereas ethanolic extracts were rich in gallic acid, kaempferol, quercetin, and pyrogallol (21). In another investigation, rutin, safranal, and picrocrocin were detected in all samples of 15 saffron samples from 11 different countries, while chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, luteolin, and apigenin were identified in varying amounts (29). These findings corroborate the present results, highlighting the role of saffron-derived phenolics in enriching the propolis matrix.

Although the composition of propolis varies with its botanical and geographical origin, Turkish propolis samples are known to contain abundant polyphenolic compounds such as pinocembrin, chrysin, CAPE, galangin, apigenin, quercetin, caffeic acid, p-coumaric acid, and trans-cinnamic acid. These compounds are thought to be responsible for the important biological activities of propolis, including antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory effects (15, 33, 34). The synergistic presence of both propolis – and saffron-derived phenolics in the enriched extracts may thus potentiate their overall biological efficacy.

**Table 2.** Phenolic profile of the extracts

| | Phenolic Content | 30% propolis | 30% propolis+ 1% saffron | 30% propolis+ 5% saffron |
|---|---|---|---|---|
| µg phenolic /mL sample | Gallic Acid | <LOD | <LOD | <LOD |
| | Protocatechuic Acid | <LOD | <LOD | <LOD |
| | Chlorogenic Acid | <LOD | <LOD | <LOD |
| | Catechin Hydrate | <LOD | <LOD | <LOD |
| | p-OH Benzoic Acid | 10.12 | 10.38 | 17.74 |
| | Epicatechin | <LOD | <LOD | <LOD |
| | Caffeic Acid | 305.64 | 297.15 | 287.48 |
| | Syringic Acid | <LOD | <LOD | <LOD |
| | Vanillic Acid | <LOD | <LOD | <LOD |
| | Rutin | <LOD | <LOD | <LOD |
| | Ellagic Acid | <LOD | <LOD | <LOD |
| | p-Coumaric Acid | 177.20 | 178.70 | 169.07 |
| | Ferulic Acid | 212.17 | 207.30 | 201.01 |
| | Myristin | <LOD | <LOD | <LOD |
| | Daidzein | <LOD | <LOD | <LOD |
| | Luteolin | <LOD | <LOD | <LOD |
| | Quercetin | 62.22 | 65.08 | 80.44 |
| | t-Cinnamic Acid | 239.34 | 240.65 | 220.86 |
| | Naringenin | <LOD | <LOD | <LOD |
| | Apigenin | 108.71 | 109.53 | 120.78 |
| | Hesperetin | <LOD | <LOD | <LOD |
| | Rhamnetin | 260.67 | 266.12 | 294.03 |
| | Chrysin | 1423.67 | 1446.66 | 1469.59 |
| | Pinocembrin | 2168.90 | 2171.55 | 2214.25 |
| | CAPE | 798.52 | 819.69 | 821.27 |
| | Galangin | 1479.81 | 1483.87 | 1586.23 |

LOD: Limit of detection

## 4. Conclusion

In this study, the effect of saffron supplementation on the biological properties of propolis extract was investigated. From the results obtained in the current study, it was determined that increasing the proportion of added saffron has the potential to enhance both the phenolic composition and antioxidant capacity of the propolis extract. These findings suggest that the synergistic interactions between the bioactive constituents of propolis and saffron may augment their overall biological efficacy. However, the current study is limited to *in vitro* antioxidant evaluations; therefore, further in vivo investigations are necessary to elucidate the underlying mechanisms of action and to optimize formulation ratios. Overall, this research provides valuable insights into the potential health benefits and practical applications of saffron-enriched propolis extracts in the development of functional and nutraceutical products.

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