Original Article

# Quantitative and Qualitative Analyses of the Constituents of the Hydroalcoholic Extract of *Quercus infectoria* Gall from Kermanshah and Evaluation of Its Antioxidant and Antibacterial Activities

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## Abstract

**Background and Purpose:** *Quercus infectoria* is a species of *Quercus* genus (Fagaceae) whose galls are known in traditional medicine for their antibacterial, analgesic, anti-inflammatory, and astringent effects. The present study aimed to carry out quantitative and qualitative analyses of the constituents of the hydroalcoholic extract of the *Q. infectoria* galls from Kermanshah and to evaluate its antioxidant and antibacterial activities. **Materials and Methods:** Following the extraction process using ethanol/water (70/30), phytochemical tests were done. Total phenol and flavonoid and antioxidant and antibacterial activities against specific strains of bacteria were evaluated. Some of the constituents of the extract were identified using high-performance liquid chromatography-photodiode array, and their amount was obtained. **Results:** The phytochemical tests proved that the extract contained alkaloid, flavonoid, tannin, saponin, and phenolic compounds. The amount of total phenolic and flavonoid compounds was 16.21 and 1.78 mg/g dried galls, respectively. The $IC_{50}$ value of the antioxidant constituents of the extract was 47 µg/mL. The results of the antimicrobial assay showed the high activity of the extract against *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *S. epidermidis*. The quantitative analysis of the extract confirmed the presence of gallic acid, rutin, quercetin, benzoic acid, and caffeic acid (12.30, 10.72, 5.00, 9.25, and 3.94 mg/g dried galls, respectively). **Conclusion:** Considering the results of this study, the extract of *Q. infectoria* galls could be used as a primary substance in treating bacterial infections and oxidative stress-related diseases.

**Keywords:** *Antibacterial, antioxidant, gall, HPLC-PDA, phytochemical screening, Quercus infectoria*

## Introduction

*Quercus infectoria* belongs to *Quercus* genus (Fagaceae) in which more than 200 different species have been identified. Being native to the Northern Hemisphere and widely distributed in evergreen forests as well as being economically valuable, *Quercus* is one of the most significant genera of the Fagaceae family that the phytochemical and biological studies of whose species are important.[1,2] It can also be used in pharmaceutical industries. Studies of different species have shown a wide range of antibacterial, anti-inflammatory, antigenotoxic, antitumorgenesis, antidiabetic, astringent, and analgesic effects and so forth.[3-5] Of different species of *Quercus* genus, six species could be found in Iran including, *Q. infectoria, Q. brantii, Q. libani, Q. macranthera, Q. castaneifolia,* and *Q. petraea*.

*Q. infectoria*, which is known as Mazu or Mazuj in Iran, is about 2-m tall and commonly found in Asia Minor, parts of Eastern Europe, and Iran. The galls of *Q. infectoria* (GQI), formed on the branches, are a result of the deposition of the eggs of a gall wasp called *Cynips gallae tinctoriae*.[6] In India, the galls are used to relieve toothache and gingivitis. It has also been observed that topical use of GQI reduces inflammation and swelling.[7] Other pharmacological applications are as antibacterial, antivirus, and astringent, which have made it an appropriate candidate for the treatment of diseases such as gastrointestinal ulcers and diarrhea.[4] A large number of research in different countries has been carried out so far to identify the constituents and effects of GQI, confirming the presence of phenolic constituents such as tannic acid (50–70%), gallic acid (>5%), and less flavonoid constituents such as quercetin and rutin, which have led to its above-mentioned therapeutic effects.[8,9] In this study, we aimed

**Received:** 15 Mar 2021
**Accepted:** 14 Aug 2021
**Published:** 17 Dec 2021

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**Access this article online**

**Website:** www.jrpsjournal.com

**DOI:**10.4103/jrptps.JRPTPS_36_21

**Quick Response Code:**



**How to cite this article:** Mohammadzadeh N, Ghiasian M, Faradmal J, Dastan D. Quantitative and qualitative analyses of the constituents of the hydroalcoholic extract of *Quercus infectoria* gall from Kermanshah and evaluation of its antioxidant and antibacterial activities. J Rep Pharma Sci 2021;10:287-93.

to investigate for the first time the quality and quantity of the constituents of GQI and its antioxidant and antibacterial activities from samples collected from Kermanshah in western Iran.

## Materials and Methods

### Chemicals

All chemicals were purchased from Sigma Chemical Company.

### Preparation of the hydroalcoholic extract of GQI

The samples of GQI were collected from Kermanshah province, identified, and registered under herbarium number 408 in the School of Pharmacy, Hamadan University of Medical Sciences [Figure 1 and Table 1]. Galls (700 g) were crushed into smaller pieces to make sure that there was no wasp residue in the galls. Sample extraction was performed on a shaker (maceration) using ethanol/water solvent (70/30) for 72 h and repeated three times. The extract was filtered with Whatman filter paper and concentrated with a rotary evaporator at a temperature of 50°C. An amount of 170 g dried extract was obtained and stored in a container at 4°C before being used in the tests.[10]

### Phytochemical analysis of the GQI extract

Phytochemical tests identifying alkaloids, flavonoids, tannins, terpenoids, saponins, glycosides, and phenols in the extract were performed to analyze the constituents qualitatively. The results of these tests provided a base for the following evaluations in the study.

#### *Alkaloids test*

An aliquot of 1 mL of extract (1% w/v) was mixed with 1 mL of 1% HCl in 95°C for 3 min. Dragendorff's reagent was add to the mixture to observe the reddish-brown color.

**Figure 1: *Q. infectoria* galls**

**Table 1: Macroscopic characteristics of GQI**

| **Shape** | **Round** |
|---|---|
| Color | Brown-maroon |
| Size | 2–3 cm |
| Thickness of cortex | 1–2 mm |
| Surface | Small thorn like rises |
| Texture | Hard, no hair |
| Odor | Not significant |
| Taste | Highly astringent |

*Flavonoids test*: An aliquot of 1 mL of extract (1% w/v) was mixed with few drops of 10% lead acetate solution. The positive result was based on appearing of yellow precipitate.

*Tannins test:* An aliquot of 1 mL of extract (1% w/v) was added to the mixture of 1% gelatin solution and 10% sodium chloride. Observing white precipitate is the sign of tannins presence.

*Terpenoids test*: An aliquot of 2 mL of chloroform was added to 1 mL extract (1% w/v). Few drops of concentrated $H_2SO_4$ were added carefully to the mixture. Formation of a reddish-brown ring is the indication of terpenoids.

*Saponins test*: Dried extract was added to warm distilled water and was shaken vigorously. The presence of a stable foam was considered as the effect of saponins.

*Glycosides test*: To 1 mL of extract (1% w/v), 2 mL of glacial acetic acid and few drops of 5% $FeCl_3$ solution were added, respectively. Afterwards, 1 mL of concentrated $H_2SO_4$ was carefully added to the mixture. The mixture was observed till the formation of a brown ring.

*Phenolic compounds test*: An aliquot of 0.5 mL of extract (1% w/v) was added to 1 mL of 5% $FeCl_3$. The dark green color of the solution was the indication of phenols.[11,12]

### Determination of total phenolic content

The amount of phenolic content was determined using colorimetry and Folin–Ciocalteu reagent. An aliquot of 20 µL of 10 mg/mL extract was mixed with 2 mL of distilled water and 500 µL of Folin–Ciocalteu reagent. After 3 min, 300 µL of $Na_2CO_3$ (7% w/v) was added and the solution was shaken for 2 h at room temperature. The absorbance of the solution at 765 nm was measured using a spectrophotometer. Likewise, the calibration curve was obtained using different concentrations of gallic acid and used to measure the amount of phenolic constituents of the extract.[13]

### Determination of total flavonoid content

The total amount of flavonoids was obtained using the colorimetry and aluminum chloride method. Six hundred microliters of ethanol, 200 µL of $AlCl_3$ (10% w/v) and 200 µL of 1 M sodium acetate were added to 200 µL of 10 mg/mL extract, respectively. Distilled water was filled up to obtain a final volume of 2 mL. After 1 h, the amount of absorbance was read at 415 nm. Likewise, the calibration curve was obtained using different concentrations of quercetin and used to measure the amount of flavonoid constituents of the extract.[14]

### Evaluation of antioxidant capacity

The antioxidant capacity of the extract was measured using DPPH (2,2-dipheny1-1-picrylhydrazyl). DPPH is a free radical, which is seen purple in methanol solvent in the absence of antioxidants or turns to yellow once antioxidant is added to the solution. Solution of extract of 1000 mg/L was obtained by solving 10 mg of dried extract in 10 mL of methanol. Different concentrations of the extract were prepared using stock solution, mixed with 1 mL of 90 µM DPPH, and were reached a volume of 4 mL by adding methanol 95%. The final solutions were stored in a dark place for an hour. Using a spectrophotometer, the absorbance of both standard and blank solutions was read at 517 nm, and the antioxidant capacity of the extract was measured using the following equation:

$$RSC\% = 100 \times \left[ \frac{A_{blank} - A_{sample}}{A_{blank}} \right]$$

The $IC_{50}$ value for different concentrations of the extract was determined using radical scavenging capacity (RSC) value by plot of data as concentration vs. inhibition % and then the equation of obtained line. The butylated hydroxytoluene (BHT) compound was used as the positive control.[15]

### Antibacterial activity

In comparison to tetracycline and ampicillin (broad-spectrum antibiotics) and gentamicin (active mostly against Gram-negative bacteria) as standard, the antibacterial activity of the GQI extract was measured using disk diffusion method on microorganisms including *Bacillus pumilus* (PTCC1274), *B. subtilis* (ATCC 9372), *Staphylococcus aureus* (ATCC 25923), *B. cereus* (PTCC 1015), *Klebsiella pneumoniae* (ATCC 3583), *Enterococcus faecalis* (ATCC 15753), *Escherichia coli*) ATCC 25922), *S. epidermidis* (ATCC 12228), and *Pseudomonas aeruginosa* (ATCC 27852). To increase accuracy for each concentration, three plates of each bacteria were cultured, and the average zone of inhibition was measured. Microdilution assay was used to determine the minimum inhibitory concentration (MIC) for each antibiotic and GQI extract.[16-19]

### High-performance liquid chromatography (HPLC) analysis of the GQI extract

About 3 mg of the GQI extract was weighed and solved in 1 mL of methanol. The prepared solution was passed through a 0.45 µm filter. Then, 20 µL of the solution was injected into HPLC machine Shimadzu (LC-20AD XR) model and passed through Spherisorb OSD-2 (5 µm) 4.6 mm × 250 mm column with 1.0 mL/min flow rate [using methanol and water/acetic acid (1%) as mobile phase gradient]. The output of the column was detected by an SPD-M20A detector at 200–400 nm, and the chromatogram of the GQI extract was obtained in the stated conditions. The exact amount of the constituents in the chromatogram was calculated using calibration curves of standard compounds. Different concentrations of pure standards including gallic acid, rutin, quercetin, benzoic acid, and caffeic acid (25–100,000 µg/L) were prepared, filtered, and injected into HPLC column. The calibration curves were plotted using obtained chromatograms.[20]

### Statistical analysis

All experiments were repeated three times, and the results were expressed as mean ± SD. Data were analyzed by Excel 2016.

## Results

The results of the phytochemical analysis confirmed the presence of alkaloids, flavonoids, tannins, saponins, and phenols in the GQI hydroalcoholic extract [Table 2]. The total phenolic and flavonoid contents of the extract were obtained by standard calibration curve of gallic acid and quercetin, respectively. The total phenolic and flavonoid contents and DPPH radical scavenging activity of the GQI extract were 16.21 and 1.78 mg/g and 47 µg/mL, respectively [Table 3].

The results of the zone of inhibition diameter of the GQI extract in the antibacterial assay showed that the extract was more effective against *E. coli, K. pneumonia, S. aureus,* and *S. epidermidis* (MIC=7.5 mg/g) [Table 4].

HPLC analysis confirmed the presence of gallic acid, rutin, quercetin, benzoic acid, and caffeic acid in the GQI extract [Figures 2 and 3]. The amount of each constituent was measured by using the extract chromatogram and the standard calibration curve of each one and reported in Table 5.

## Discussion

The properties of GQI, which were mentioned in the previous studies such as being astringent, antioxidant, and analgesic, can be attributed to the presence of alkaloids, flavonoids, tannins, saponins, and phenols. However, in this study, unlike the previous researches, some compounds such as terpenoids and glycosides were not found. This difference can be caused by the habitat of the plant and the conditions of the growing environment.[21,22]

The amount of total phenolic content and the value of the DPPH radical scavenging activity of the GQI extract in the samples obtained by the Soxhlet method were 99 mg/g and 92%, respectively. The difference in the amount of the compounds of the same species could be ascribed to the

**Table 2: Phytochemical screening of the GQI hydroalcoholic extract**

| Chemical constituent | *Q. infectoria* |
|---|---|
| Alkaloid | + |
| Flavonoid | + |
| Tannin | + |
| Saponin | + |
| Terpenoid | − |
| Glycoside | − |
| Phenol | + |

+ = presence of compounds, − = absence of compounds, Nt = not tested

**Table 3: Total phenol, flavonoid, and GQI's DPPH radical scavenging activity**

| Sample | Total phenol (mg/g dried galls) | Total flavonoid (mg/g dried galls) | Radical scavenging $IC_{50}$ (µg/mL) |
|---|---|---|---|
| *Q. infectoria* (hydroalcoholic extract) | 16.21 | 1.78 | **47** |
| Butylated hydroxytoluene (BHT) | | | **26** |

ND = not determined

**Table 4: Determination of zone of inhibition and MIC value of GQI**

| | Sample | | | |
|---|---|---|---|---|
| **Microorganism** | **Hydroalcoholic extract** | **Tetracycline[c]** | **Gentamicin[d]** | **Ampicillin[e]** |
| *B. pumilus* | 11[a] (15)[b] | Nt | Nt | 15 (1.5) |
| *B. subtilis* | 12 (>15) | 21 (3.2) | −(Nt) | 14 (1.5) |
| *S. aureus* | 18 (7.5) | 20 (3.2) | −(Nt) | 13 (1.5) |
| *B. cereus* | 12 (15) | Nt | Nt | 19 (1.5) |
| *K. pneumoniae* | 17 (7.5) | Nt | Nt | 14 (1.5) |
| *E. faecalis* | 12 (>15) | Nt | Nt | 15 (1.5) |
| *E. coli* | 18 (7.5) | −(Nt) | 23 (3.2) | 12 (1.5) |
| *S. epidermidis* | 17 (7.5) | 34 (1.6) | −(Nt) | 19 (1.5) |
| *P. aeruginosa* | 12 (>15) | Nt | Nt | 13 (1.5) |

[a]Zone of inhibition (in mm) includes diameter of the disc (6 mm), (−): inactive, (7–13): moderately active, (> 14): highly active, Nt: not tested
[b]Minimum inhibitory concentration volumes as mg/mL
[c]Tested at 30 µg/disc
[d]Tested at 10 µg/disc
[e]Tested at 30 µg/disc

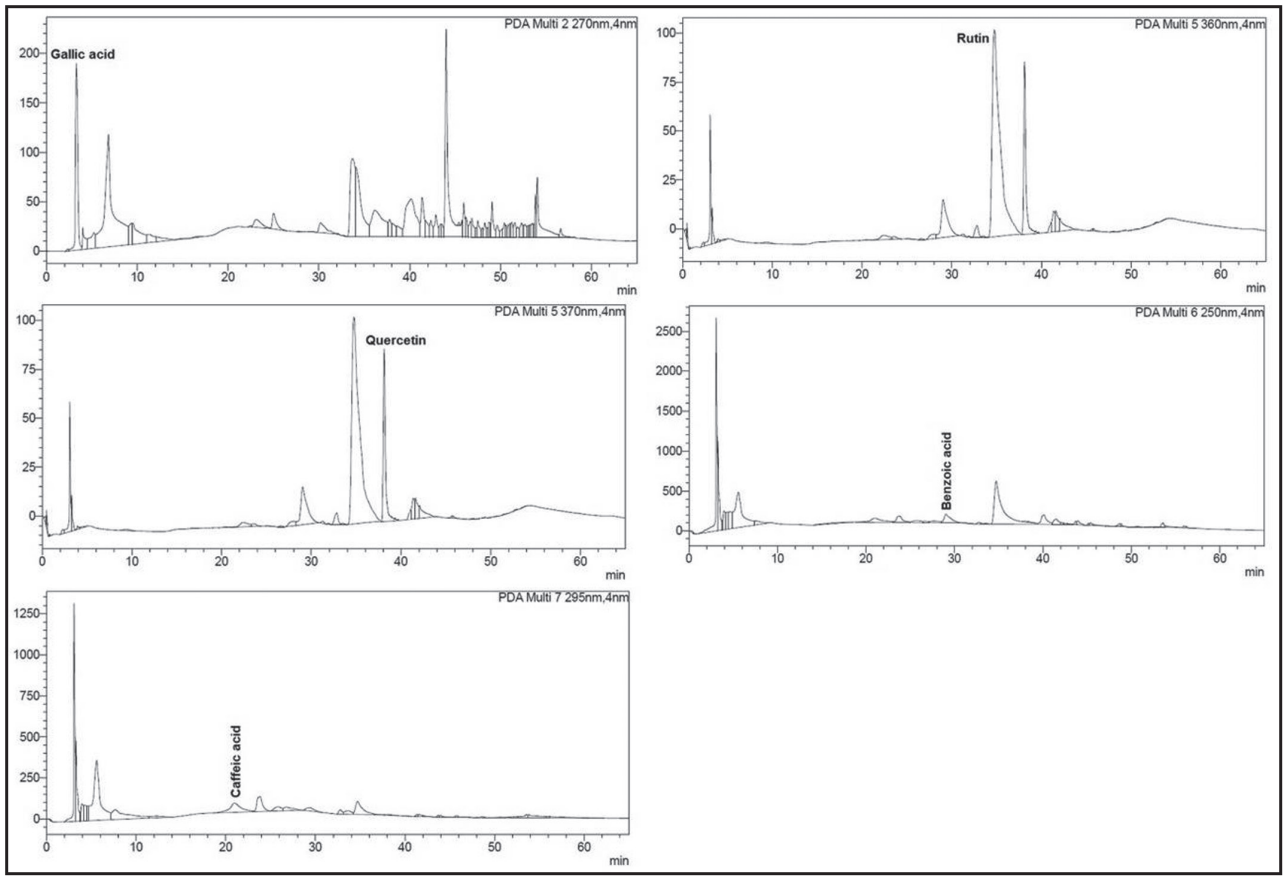


**Figure 2: HPLC-PDA chromatogram of GQI extract**

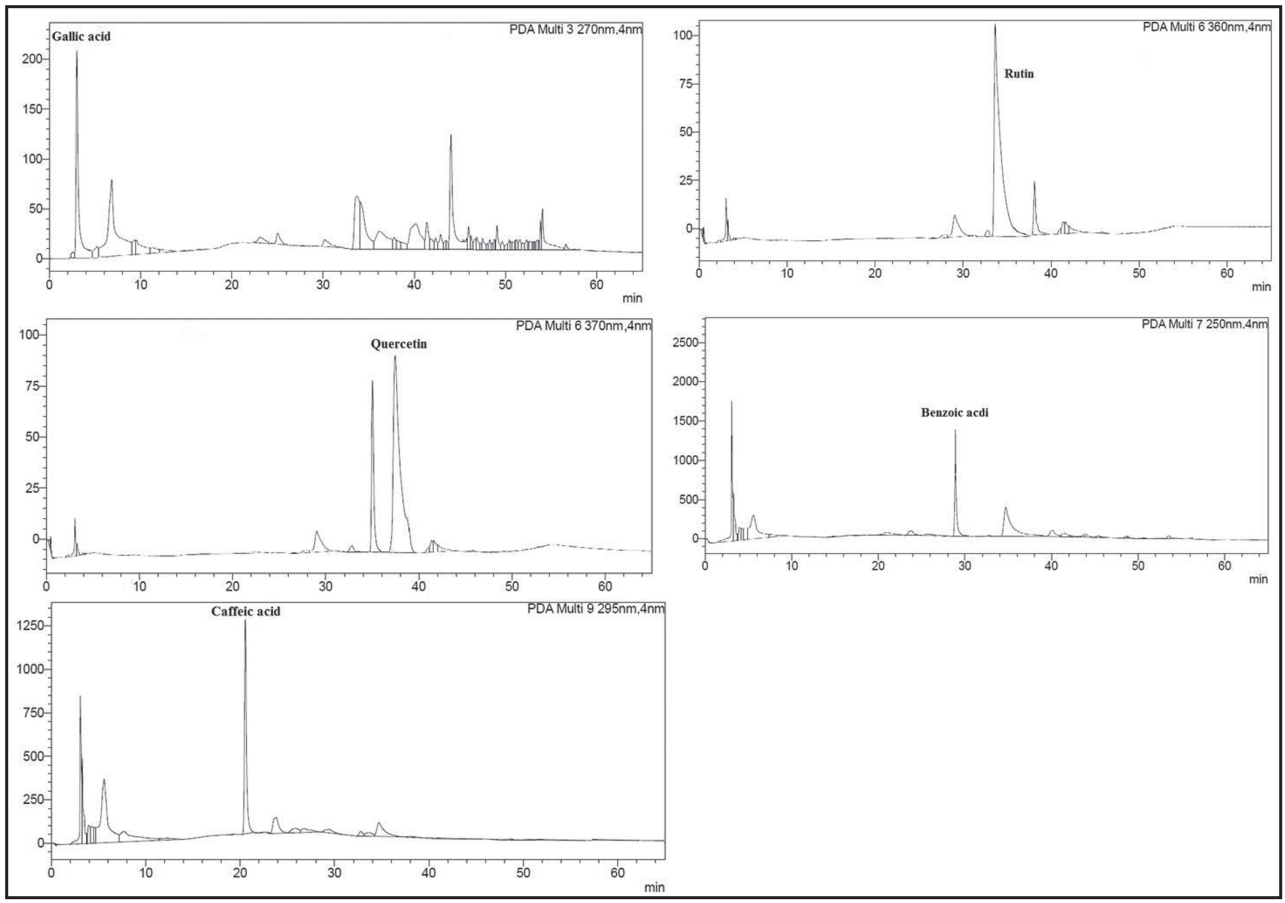


**Figure 3: The standard addition in HPLC-PDA chromatogram of GQI extract**

**Table 5: Compounds identified in GQI and their quantities**

| Chemical constituent | Wavelength (nm) | RT (min) | RT of standards (min) | Amount of constituents (mg/g dried galls) | LOD (ppb) | LOQ (ppb) |
|---|---|---|---|---|---|---|
| Gallic acid | 270 | 3.30 | 3.28 | 12.30±0.9 | 500 | 1000 |
| Rutin | 360 | 35.00 | 35.75 | 10.72±0.7 | 20 | 50 |
| Quercetin | 370 | 38.31 | 38.82 | 5.00±0.3 | 20 | 50 |
| Benzoic acid | 250 | 29.12 | 28.99 | 9.25±0.6 | 10 | 25 |
| Caffeic acid | 295 | 20.99 | 21.21 | 3.94±0. 2 | 50 | 100 |

different sources of collection and the method of extraction.[23] The antioxidant property of the extract is attributed to the presence of a high amount of phenolic compounds such as gallic acid and flavonoid compounds such as rutin and quercetin.[24-27] The antioxidant activity of these compounds is due to the radical scavenging activity of their phenol groups. Antioxidant compounds can be useful in the prevention or adjunctive therapies in many oxidative stress-related diseases including cancers and chronic inflammatory conditions.[28] The antioxidant property of this plant shows its high potential to be developed as a treatment for such diseases.

Although the MIC of the extract is more than that of a broad-spectrum antibiotic such as tetracycline, it can still be used as adjuvant therapy with tetracycline or other antibiotics to reduce their dosage, duration of treatment, and bacterial resistance.

Previous studies have demonstrated the antibacterial, antifungal, anti-inflammatory, antioxidant, and wound healing effects of gallic acid, rutin, quercetin, benzoic acid, and caffeic acid *in vitro* and *in vivo*.[25,27,29-34] Recent studies showed antimalarial activity, healing effect on diabetic ulcers, and positive effects in the treatment of gingivitis and plaque that also confirms the presence of the above-mentioned compounds.[35-37] The presence of these constituents in the GQI extract makes it an appropriate candidate for future clinical studies.

## Conclusion

The results of the present study confirm that the GQI hydroalcoholic extract can be used as a primary substance in treating a wide range of health problems due to the presence of gallic acid, rutin, quercetin, benzoic acid, and caffeic acid,

which have already been reported in other studies to be antibacterial, astringent, and anti-inflammatory. Furthermore, it is recommended to use this study's measurement of the amounts of rutin, quercetin, benzoic acid, and caffeic acid for standardization of GQI formulations in future studies. Also, the fact that local people use the GQI as a home remedy for burns could be ascribed to its antioxidant and antibacterial properties and containing phenolic, flavonoid, and tannin compounds, which can be further investigated in the future clinical studies.

### Acknowledgments

This study was financially supported by the Research Administration of Hamadan University of Medical Sciences, Hamadan, Iran (Grant Number 9606073726).

### Financial support and sponsorship

Nil.

### Conflicts of interests

The authors claim that there is no conflict of interest.

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