Journal of the Hellenic Veterinary Medical Society

Vol 76, No 4 (2025)


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doi: 10.12681/jhvms.35696



**To cite this article:**

Dadeghi-Dehkordi, Z., Ghorbanipour, M., Sazmand, A., Daraei Garmakhany, A., & Sari, A. (2025). Rosmarius officinalis and propolis extract: Antioxidant properties and Echicoccus granulosus sensu lato protoscoleces inhibition activity. *Journal of the Hellenic Veterinary Medical Society*, *76*(4), 9863–9876. https://doi.org/10.12681/jhvms.35696

J HELLENIC VET MED SOC 2025, 76 (4): 9863-9876
ΠΕΚΕ 2025, 76 (4): 9863-9876

Research article
Ερευνητικό άρθρο

# Rosmarius officinalis and propolis extract: Antioxidant properties and Echicoccus granulosus sensu lato protoscoleces inhibition activity

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**ABSTRACT:** Hydatidosis is an important zoonosis caused by the larval stage of several species of the genus *Echinococcus*. In humans, surgical removal of cysts is the treatment of choice, but leakage of cyst contents during surgery and formation of new cysts remain a concern. Currently, various solutions, such as hypertonic saline and silver nitrate, are used to inactivate the protoscolexes living in the cysts. Therefore, the search for new protoscolicidal formulations with higher efficacy and lower side effects is always in focus. The aim of this study was to optimize the extraction conditions of *Rosmarinus officinalis* and propolis extracts and to evaluate their antioxidant properties and lethal effects on protoscoleces of *Echinococcus granulosus* sensu lato *in vitro*. For this purpose, sheep livers with hydatid cysts were collected from a local slaughterhouse between September and December 2021. In the laboratory, the cysts were excised, and the fertility and viability of protoscoleces were evaluated. Extracts of *R. officinalis* and propolis were prepared using different ethanol concentrations (0, 50, and 100% v/v) and different extraction times (24, 48, and 72 hours). Response Surface Methodology (RSM) was performed to determine the optimal extraction conditions. Finally, different concentrations of the optimized extracts (200, 400, and 600 ppm) were prepared and added to protoscoleces to study their protoscolicidal activity. The results showed that 100 µl/mL of the extract of *R. officinalis* prepared in 66.6 min with 77.01% ethanol inactivated 86.66, 100, and 100% of protoscoleces after 5, 15, and 30 min of exposure, respectively. Similarly, 100 µL/mL of the propolis extract prepared under optimal extraction conditions (67.79 minutes with ethanol 43.58 v/v) killed 94.16, 100, and 100% of the protoscoleces after 5, 15, and 30 minutes of exposure, respectively. Data presented herein shows for the first time that the addition of water to the ethanol solvents of *Rosmarinus officinalis* and propolis increases the efficiency of phenolic compounds and antioxidant properties of the resulting extracts due to the increasing polarity of the solvent. In addition, *in vitro* experiments indicated that rosemary and propolis extracts can be used as suitable candidates for scolicidal agents. Evaluation of the efficacy of these extracts in an experimental *in vivo* study is recommended to better understand their protoscolicidal effects.

***Keyword:*** Hydatid cyst; optimum extraction conditions; parasitology; surgery

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*Date of submission:* **26-10-2023**
*Date of acceptance:* **3-11-2025**

## INTRODUCTION

Cystic echinococcosis (CE), AKA hydatid disease, is caused by the larval stage of *Echinococcus granulosus* sesu lato complex tapeworms. It is one of the most important and widely spread zoonotic helminthiasis, as more than one million people are affected with echinococcosis at any one time (Sazmand and Nourian, 2023). As intermediate hosts, humans become infected by ingestion of eggs excreted through dog feces into the environment, e.g., drinking contaminated water, consuming raw vegetables, or through contaminated hands (Darabi et al., 2022). CE is hyperendemic in Iran, and the affected individuals and livestock farmer communities suffer from considerable medical costs and economic losses (Sadjjadi, 2006). Clinical symptoms vary greatly and depend on the involved organ, e.g., lung, liver, bone marrow, and also the number and location of the cysts. If a cyst ruptures, it may entail different consequences, the most common of which are immunological reactions such as asthma and anaphylactic shock (Gouran et al., 2021). The hydatid cyst treatment is a complex procedure. Generally, surgical operation is the recommended approach in most of the cases, but because of aforementioned consequence of cyst rupture and subsequent recurrence (Mouaqit et al., 2013) however, employed scolicidal agents during hydatid cyst surgery e.g. hypertonic saline solution (15–30%), formalin (2%), silver nitrate (0.5%), povidone-iodine (10%), chlorhexidine (0.05%), and a combination of cetrimide (0.5%) and chlorhexidine (0.4%), may cause sclerosing cholangitis, stenosis of the biliary ducts, hepatic necrosis and hypernatremia (Fakharzadeh Jahromi et al., 2022; Álvarez-Santamarta et al., 2021). Hence, researchers are seeking solutions with greater efficacy and fewer side effects, preferably plant-based agents.

Medicinal plants have been used since ancient times all over the world to treat and prevent certain conditions and diseases. Previously, the scolicidal effects of different nature-based substances such as garlic, ginger, *Artemisia multiflora,* and thyme have been tested (Ali et al., 2020; Mohammadi et al., 2018). In a systematic review study, 52 plant species belonging to 22 families were identified as the scolicidal agents globally, among which Lamiaceae (25%) and Apiaceae (11.3%) families, as well as plant compounds berberine, thymol, and thymoquinone, were the most widely used (Khaleghi-Miran et al., 2015). Rosemary (*Rosmarinus officinalis*; RO) is a member of the Lamiaceae family, a native plant of the Mediterranean countries. It is a stable, herbaceous plant with a wooden stem, whose used parts are leaves and flowering branches (Eshraghi and Valafar, 2008). RO is widely used in traditional medicine as blood blood-clotting, tonic, and anti-bloating compound, as it is rich in phenolic substances with antioxidant, antimicrobial, and antiviral activities, with inhibition of hepatotoxicity, and having antioxidant properties (Nieto et al. 2018; Xie et al. 2017). Furthermore, supplementation of poultry diet with RO is beneficial for broiler and quail performance and immune system (Rostami et al., 2015; Rostami et al., 2017; Sarmad et al., 2020). Studies have attributed these activities to compounds such as carnosol, rosmanol, and their acid forms or flavonoids (Ghasemzadeh Rahbardar et al, 2020), phenolic compounds, such as terpenes, antioxidant activator, 1,8-cineol, and camphor antimicrobial activator (Rašković et al., 2014; Rostami et al., 2017; Nieto et al., 2018).

Propolis is one of the most beneficial products of the hives, and besides its importance for honey bees inside the hive, its medical properties, including antimicrobial and anti-inflammatory properties, treatment of autoimmune diseases, and tumor control, have been shown (Šuran et al. 2021; Zullkiflee et al. 2022; Bhatti et al. 2024).

The aims of this study were 1) to achieve the optimal extraction conditions of RO and propolis in terms of solvent type (aqueous, hydroalcoholic, alcoholic) and extraction time, according to different antioxidant tests, 2) to assess protoscolicidal activity of the obtained optimum extracts, and evaluate the minimum exposure time for protoscolicial activity.

## MATERIAL AND METHODS

Rosemary and propolis were purchased from the local market in Hamedan city, western Iran. The chemicals used for the extraction process included ethanol, Folin Ciocalteu's reagent, sodium carbonate, gallic acid, trichloroacetic acid, ferric chloride, DPPH (2,2-Diphenyl-1-picrylhydrazyl), phosphate buffer, potassium cyanide buffer, ammonium molybdate, sulfuric acid, sodium phosphate, as well as highly-purified solvents. The equipment employed was a simple mixer (Sunny, Iran), an oven (Fan Azma Gostar, Iran), a laboratory scale with 0.0001 accuracy (Sartorius, Germany), a UV-visible spectrophotometer (PG instrument, England), a centrifuge (Sigma, Germany), an RV10 rotary evaporator (IKA, Germany), a pH meter (Denur, Germany),

a water bath (Fan Azma Gostar, Iran), and a tube shaker (Labinko Pars Khazar, Iran). Protocols used in this study were reviewed and approved by the Ethical Committee of Bu-Ali Sina University (code: IR.BASU.REC.1398.023).

**Preparation of rosemary and propolis extracts**

After cleaning the rosemary plant of dust, the leaves and stems were cut and ground. The purchased propolis was also stored in the refrigerator until further use. To prepare the extract, purified water solvents, pure ethanol, and water ethanol mixture (50% V/V) were used in a ratio of 5:1. The solvent and rosemary/ propolis mixture were placed on a stirred heater at 40°C for different time periods (24, 48, and 72 hours). During the experiment, the level of solvent was maintained constant by adding 40°C fresh solvent. After the completion of different extraction times, the extracts were sieved using Wattmann No. 40 filter paper and condensed by rotary evaporator, then kept in sterile containers in a refrigerator.

**Evaluation of phenolic compounds of the extracts**

Total phenolic compounds were evaluated using Folin-Ciocalteu's reagent (Yildirim et al., 2001). At first, 0.5 mL of each extract was diluted with 2.25 mL of distilled water, then 250 µL of the mentioned reagent was added, and after 5 minutes of incubation in a dark environment, 2 mL of 7.5% sodium carbonate solution was added to the mixture, and the tubes were stored in a 40°C water bath for 30 minutes. The optical density (OD) of the samples was measured by a spectrophotometric device at 760 nm wavelength. The total phenolic compounds of the extracts were calculated using a standard curve based on milligrams of Gallic acid per gram of the sample.

**Measurement of antioxidant activity**

Antioxidant activity was measured by $Fe^{+3}$ to $Fe^{2+}$ reduction ability via Feric Reducing Power of PlasmaTest (FRAP) as described previously (Yildraim et al, 2001). One mL of extract was mixed with 2.5 mL phosphate buffer and 2.5 mL of potassium ferricyanide. The solution was placed at 50°C for 30 minutes. Then, 2.5 mL trichloroacetic acid was added to the mixture. The samples were centrifuged for 10 minutes, and the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride. Finally, absorbance was read using a spectrophotometer at 593 nm, and the gallic acid was used as a standard for drawing the standard graphs (Aghajani et al., 2023).

**Measurement of free radical scavenging activity**

For antioxidant activity, we used 2,2-diphenyl-1-picrylhydrazyl (DPPH). At first, one mL of methanolic DPPH solution was mixed with 3 mL of the extracts, and the resulting mixture was stored for 30 minutes at room temperature in a dark place. Then, the optical absorption was read at 517 nm, and finally, the activity was calculated according to the DPPH inhibition percentage according to the equation below (Aghajani et al., 2021, 2023).

$$\text{Percentage of active DPPH radical inhibition} = [OD_{DPPH}\ (\text{control}) - OD_{DPPH\ \text{with extracts}}]/OD_{DPPH}\ (\text{control}) \times 100 \quad (1)$$

**Determination of total antioxidant capacity**

To measure the antioxidant activity by total antioxidant capacity, 500 mL of reagent solution (sulfuric acid, sodium phosphate, ammonium molybdate) was prepared initially. Next, 0.5 mL of the extract was mixed with 5 mL of reagent solution, then placed in a water bath (95°C) for 90 minutes. After cooling down to room temperature, the OD of the samples was read at 695 nm using a spectrophotometer. Gallic acid was used to draw the standard graph (Ghorbanipour et al., 2023).

**Effect of extracts on protoscoleces of *Echinococcus granulosus***

From September to January 2021, sheep lungs and livers infected with hydatid cysts were collected from the industrial slaughterhouse of Hamedan city and transferred to the lab. Under aseptic conditions, the contents of the cysts were transferred to Falcon tubes by syringes, the germinal layer was transferred into a sterile laboratory plate, and washed using physiological saline solution to obtain the maximum number of protoscoleces. All tubes were centrifuged for 5 minutes (2500×rpm) to pellet the protoscolices, and the supernatant was discarded. The fertility of the larvae (20 µL) was examined using a 0.1% solution of eosin dye (20 µL) under a light microscope. Dead protoscoleces would stain red in color, but living ones are colorless (Mathialagan et al., 2017).

**Statistical analysis and optimization**

Response surface methodology was used to optimize the extraction conditions regarding solvent concentration and extraction time. For this purpose, the central composite design (CCD) with 3 levels and 5 repeats at the center point was used to investigate the effect of extraction conditions on antioxidant

properties of the prepared extracts (+1, 0, -1). In this study, the range of independent variables of the solvent concentration (X1) and extraction time (X2) was obtained from the primary tests (Table 1). The experimental treatments were randomized to minimize the effects of unpredictable changes in the observed responses. Design Expert software ver. 6.0.2 was used to draw 3D charts and optimize the data. Protoscolicidal activity of the extracts was carried out based on a completely randomized design with 4 replications. SAS software (2001) was used for data analysis. The mean of measured traits was compared using Duncan's multiple range test at 5% probability level (Table 1).

## RESULTS AND DISCUSSION

### Study of antioxidant and phenolic compounds of rosemary

#### Total phenolic compounds of the extracts

The trend of changes in total phenolic compounds of rosemary plant extracts under different extraction conditions (extraction time and different concentrations of ethanolic solvent) is presented in Figure 2. Accordingly, the total phenolic compounds increased by increasing the extraction time, and upon increasing the concentration of the ethanolic solution, the amount of the extracted phenolic compounds also increased. As observed, extraction time substantially affects the extraction rate of total phenolic compounds, because over time the solvent has the opportunity to penetrate the plant tissue and sufficient time is provided for phenolic compounds to be released into the surrounding solvent, which was consistent with the results obtained by (Shabanian et al., 2021; Ghorbanipour et al., 2023).

Madani et al. (2023) studied the Iranian *Allium sativum* (garlic) controversum solvent extracts as a valuable source of bioactive compounds such as antioxidants. Results showed that the hydroalcoholic extract had the highest total phenols, and the aqueous extract of *Allium sativum* controversum showed the highest radical scavenging activity (11.85 ± 0.81 mg/g).

**Table 1**. Independent variables and levels used to optimize the antioxidant properties of extracts o *Rosmarinus officinalis* and propolis of under the influence of different extraction conditions

| Independent variables | Levels and limits of variables | | |
|---|---|---|---|
| | -1 | 0 | +1 |
| Extraction time (hr) | 24 | 48 | 72 |
| Solvent concentration (v/v) | 0 | 50 | 100 |

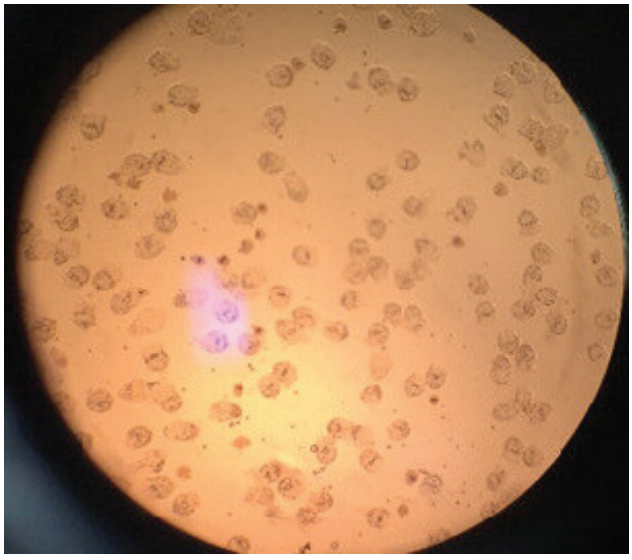

**Figure 1.** Protoscoleces exposed to the eosin dye (dead protoscoleces absorb eosin and color in red, while alive larvae remain colorless).

Noshirvani et al. (2024) in their study investigated the synergistic effect of the combination of three plant extracts, including green walnut hulls, potato peel, and date pulp, on the oxidation of sunflower oil over 15 days of storage at 70 $^0$C. The total polyphenol, flavonoid compounds, and the antioxidant efficiency were studied by evaluating the DPPH scavenging assay, and the IC50 of three extracts was measured. Finally, the effectiveness of plant extracts on the oxidation of sunflower oil was determined by measuring p-anisidine (AV), peroxide (PV), thiobarbituric acid (TBA-V), and total oxidation (TOTOX). Results showed that the total polyphenols and flavonoids ranged from (TP: 1084.36, 1076.59, and 414.71mg GAE/100 g extract; and TF: 549.9 mg CE/100 g extract, 475.28, 304.18mg CE/100 g extract) for green walnut hulls, potato peel, and date pulp extracts, respectively. The DPPH assay indicated that TBHQ is almost 2, 3, and 24 times more effective than green walnut hulls, potato peel, and date pulp extracts, respectively. According to the obtained results, the combination of plant extracts indicated high antioxidant effects, which were competitive with the synthetic antioxidant TBHQ.

Sik et al. (2022) showed that all fruit extracts con-

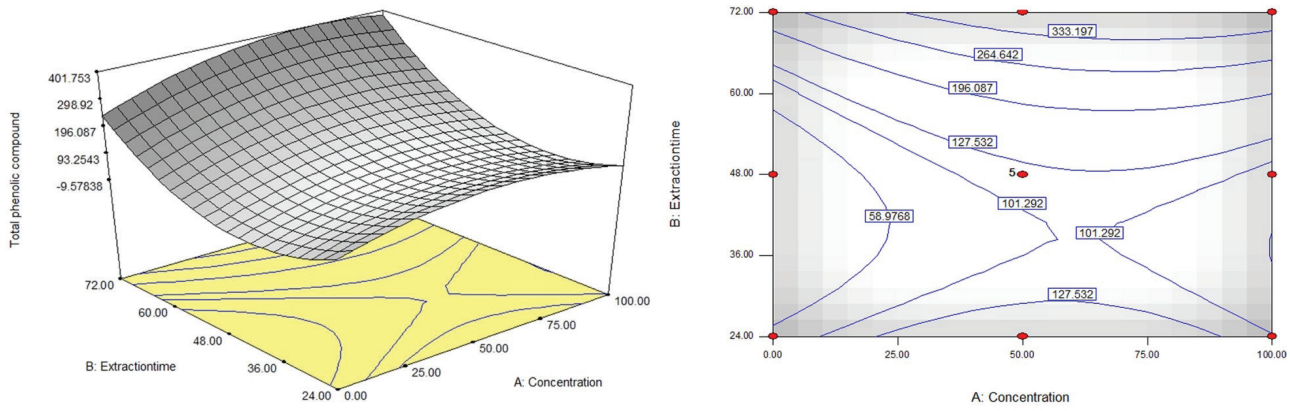


**Figure 2.** 3D and 2D diagram of changes in total phenolic compounds (mg Gallic acid per 100 g of *Rosmarinus officinalis* of extracts of *Rosmarinus officinalis* under the influence of ethanol solvent concentration and extraction time (extraction temperature of 40°C).

tained phenolic compounds, and the content of these ingredients varied according to the ethanol concentration of the solvent. For quince, a 438% (from 52.2 to 281 mg GAE/100g) increase in the TPC is indicated when the ethanol concentration was increased from 0 to 50%. A similar tendency was observed for yellow-skinned-greengage (88.0%, from 39.9 to 75.0 mg GAE/100 g) and bilberry (91.3%, from 98.3 to 188 mg GAE/100 g). Moreover, a significant TPC increase was recorded for purple-skinned greengage (193%, from 72.1 to 211 mg GAE/100 g), European crab apple (165%, from 21.1 to 55.9 mg GAE/100g), and red-skinned greengage (50.5%, from 47.7 to 71.8 mg GAE/100 g) when the ethanol concentration was increased from 0 to 80%. Our results are partly consistent with those obtained by Dumitraşcu et al (2019), who found that 50–70 EtOH–$H_2O$ mixtures were the best solvent systems for phenolics from cornelian cherry fruits.

In Mathialagan et al.'s (2017) study, the ultrasonic purification of the garlic extract was examined, and the antioxidant activity of the extracts was investigated by the UAE method. The results showed that with increasing the temperature and processing time, the Ferric reducing ability of the extract increased; however, at high temperatures and prolonged durations of the extraction process, UAE decreased (Mathialagan et al., 2017). In the study of Moshiri et al. (2020), Ajowan seed extract was prepared under different conditions of ethanol solvent extraction regarding concentrations (0, 50, and 100%), extraction time (0.25, 12, and 24 hours), and extraction temperature (20, 50, and 80 °C). The results showed an increase in extraction efficiency by adding water to the extracts. Also, it was found that by increasing extraction time, higher phenolic and antioxidant compounds were extracted, but the higher extraction temperature led to lower antioxidant properties, due to thermal degradation of phenolic compounds (Moshiri-Roshan et al., 2020). In the study of Ghorbanipour et al. (2023), the extract was subjected to different extraction conditions at different temperatures and concentrations. The results showed that by increasing the extraction time, the amount of phenolic compounds increased, whereas the extracted phenolic compounds decreased upon increasing the solubility.

The solvent polarity plays a crucial role in the appropriate recovery of phenolic compounds. It is well known that phenolics are often most soluble in organic solvents less polar than water. Solvent systems with a lower (EtOH) or excessively high ($H_2O$) polarity index were not appropriate for the higher response of natural antioxidants recovery from wild fruits. Results of Sik et al (2022) showed that a certain degree of increase in the solvent polarity enhances the solubility of antioxidant compounds from fruits.

In the present study, the results of analysis of variance of data obtained from measurement of total phenolic compounds for ethanolic extract were significant at 5% level with a second-order statistical model (Table 2). According to the results of analysis of variance (Table 2) and regression coefficients (Table 3), the second-order model was obtained for calculating the total phenolic compounds of ethan-

**Table 2.** The analysis, variance of the regression coefficients of predicted linear and quadratic polynomial models for predicting antioxidant properties of *Rosmarinus officinalis* extract under different extraction conditions.

| Response | Source | SS | DF | MS | F-value | p-value | significance |
|---|---|---|---|---|---|---|---|
| Total phenolic compounds | Model | 1.556E+005 | 5 | 31118.62 | 3.98 | 0.0498 | significant |
| | A-Concentration | 11454.29 | 1 | 11454.29 | 1.46 | 0.2654 | |
| | B-Extractiontime | 73944.21 | 1 | 73944.21 | 9.46 | 0.0179 | |
| | AB | 3232.38 | 1 | 3232.38 | 0.41 | 0.5407 | |
| | $A^2$ | 14725.95 | 1 | 14725.95 | 1.88 | 0.2123 | |
| | $B^2$ | 66330.58 | 1 | 66330.58 | 8.48 | 0.0226 | |
| | Residual | 54737.74 | 7 | 7819.68 | | | |
| | Lack of Fit | 54737.67 | 3 | 18245.89 | 1.033E+006 | < 0.0001 | significant |
| | Pure Error | 0.071 | 4 | 0.018 | | | |
| | Cor Total | 2.103E+005 | 12 | | | | |
| Total antioxidant capacity | Model | 59137.42 | 5 | 11827.48 | 29.79 | 0.0001 | significant |
| | A-Concentration | 5523.46 | 1 | 5523.46 | 13.91 | 0.0074 | |
| | B-Extractiontime | 30273.26 | 1 | 30273.26 | 76.26 | < 0.0001 | |
| | AB | 436.77 | 1 | 436.77 | 1.10 | 0.3291 | |
| | $A^2$ | 1540.56 | 1 | 1540.56 | 3.88 | 0.0895 | |
| | $B^2$ | 22527.95 | 1 | 22527.95 | 56.75 | 0.0001 | |
| | Residual | 2778.78 | 7 | 396.97 | | | |
| | Lack of Fit | 2778.75 | 3 | 926.25 | 1.116E+005 | < 0.0001 | significant |
| | Pure Error | 0.033 | 4 | 8.298E-003 | | | |
| | Cor Total | 61916.21 | 12 | | | | |
| FRAP | Model | 19044.25 | 5 | 3808.85 | 23.89 | 0.0003 | significant |
| | A-Concentration | 2685.08 | 1 | 2685.08 | 16.84 | 0.0046 | |
| | B-Extractiontime | 9631.95 | 1 | 9631.95 | 60.40 | 0.0001 | |
| | AB | 221.56 | 1 | 221.56 | 1.39 | 0.2770 | |
| | $A^2$ | 323.02 | 1 | 323.02 | 2.03 | 0.1977 | |
| | $B^2$ | 6327.80 | 1 | 6327.80 | 39.68 | 0.0004 | |
| | Residual | 1116.23 | 7 | 159.46 | | | |
| | Lack of Fit | 1116.16 | 3 | 372.05 | 23880.29 | < 0.0001 | significant |
| | Pure Error | 0.062 | 4 | 0.016 | | | |
| | Cor Total | 20160.48 | 12 | | | | |
| DPPH | Model | 418.73 | 2 | 209.37 | 1.03 | 0.3910 | not significant |
| | A-Concentration | 160.27 | 1 | 160.27 | 0.79 | 0.3947 | |
| | B-Extractiontime | 258.46 | 1 | 258.46 | 1.28 | 0.2851 | |
| | Residual | 2026.68 | 10 | 202.67 | | | |
| | Lack of Fit | 2026.62 | 6 | 337.77 | 21084.24 | < 0.0001 | significant |
| | Pure Error | 0.064 | 4 | 0.016 | | | |
| | Cor Total | 2445.42 | 12 | | | | |

**Table 3.** The regression coefficients of predicted quadratic polynomial and liner models for predicting different antioxidant activity of *Rosmarinus officinalis* extract under different extraction conditions.

| Response | Factor | coefficients | $R^2$ | $R^2$ adjusted |
|---|---|---|---|---|
| Total phenolic compound | Intercept | 117.96 | 0.8601 | 0.7602 |
| | A-Concentration | 43.69 | | |
| | B-Extraction time | 111.01 | | |
| | AB | 28.43 | | |
| | $A^2$ | -73.02 | | |
| | $B^2$ | 154.97 | | |
| Total antioxidant capacity | Intercept | 106.93 | 0.9628 | 0.9362 |
| | A-Concentration | 30.34 | | |
| | B-Extractiontime | 71.03 | | |
| | AB | 10.45 | | |
| | $A^2$ | -23.62 | | |
| | $B^2$ | 90.31 | | |
| FRAP | Intercept | 58.99 | 0.7154 | 0.5121 |
| | A-Concentration | 21.15 | | |
| | B-Extractiontime | 40.07 | | |
| | AB | 7.44 | | |
| | $A^2$ | -10.81 | | |
| | $B^2$ | 47.87 | | |
| DPPH | Intercept | 26.69 | 0.3352 | 0.2022 |
| | A-Concentration | -5.17 | | |
| | B-Extractiontime | 6.56 | | |

olic extract that was presented, in equation 2, where A is the concentration of solvent (v/v) and B is the extraction time (hours). According to the results of regression analysis, if the equation is 2, the amount of phenolic compounds of the extracts can be predicted with 86.01% accuracy.

$$\text{Total phenolic compounds} = +12.73 - 9.01\,A + 109.37\,B - 52.07\,AB + 121.39\,A^2 + 108.50\,B^2 \quad (2)$$

Where A and B are the solvent type (ethanol concentration) and extraction time, respectively.

**Ferric Reducing Power Assay of Extracts**

Figures 3 and 6 show the three- and two-dimensional curves of the ferric reducing power of the ethanolic extracts of rosemary plant and propolis, respectively, under different extraction conditions (extraction time and concentrations of ethanolic solvent). As evidenced, with increasing the extraction time, ferric reducing ability of extracts increased and by increasing the concentration of ethanolic solution, the ferric reducing power of extracts increased, the increase in the ferric reducing power along with the extraction time elevation can be due to the increase in total phenolic compounds of extracts under prolonged extraction conditions (Rezvannejad et al., 2017; Shabanian et al., 2021).

Sik et al. (2022) studied the antioxidant properties of the six fruits using the FRAP and DPPH assays. Their study showed that EtOH–$H_2O$ mixtures were significantly ($P < 0.05$) more effective than the water or pure ethanol solvent system used for extraction. In general, the antioxidant activity of fruit extracts increased with the increasing concentration of ethanol. For the FRAP assay, the highest antioxidant activity levels were measured when using an 80:20 (v/v) EtOH–$H_2O$ mixture for European crab apple (27.0 ± 1.25 mg GAE/100g) and red-skinned greengage (49.4 ± 1.46 mg GAE/100g). Additionally, the yellow-skinned greengage extracts obtained by 50:50 (v/v) and 80:20 (v/v) EtOH–$H_2O$ mixtures did not show a significant difference ($P < 0.05$). At the same time, the antioxidant properties of bilberry and quince decreased when the ethanol concentration was higher than 50%. Similar tendencies were also

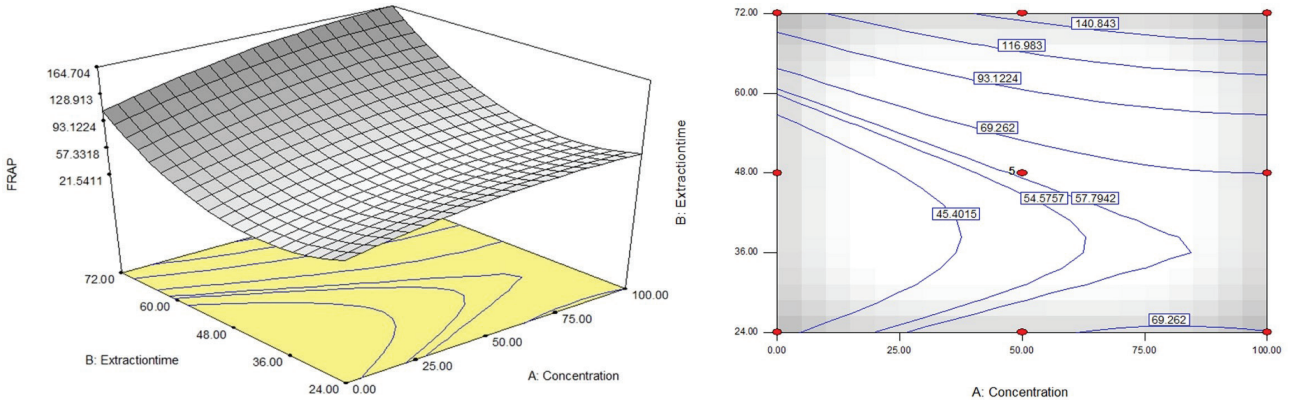


**Figure 3.** 3D and 2D diagram of changes in reducing power of ethanolic extracts of a *Rosmarinus officinalis* under the influence of solvent concentration and extraction time (extraction temperature of 40°C).

observed for the DPPH assay. Insang et al. (2022) reported that when the ethanol concentration was increased above 60%, the antioxidant activity of mulberry tended to decrease.

The second-order model is used to calculate antioxidant activity in terms of the ferric reducing power of the purified extracts in equation (Yakhchali, 2017), where A is the concentration of solvent (v/v) and B is the extraction time (hours). According to the results of regression analysis, if equation 3 is used, the antioxidant activity can be predicted with 71.54% accuracy in terms of the ferric reducing ability of extracts.

$$FRAP = +28.97 - 3.13\,A + 0.1697\,B - 13.89\,AB + 36.53\,A^2 + 8.19\,B^2 \quad (3)$$

Where A and B are the solvent type (ethanol concentration) and extraction time, respectively.

## Antioxidant properties of active radical inhibition of DPPH extracts

The 2D and 3D curves of the changes in antioxidant properties of DPPH active radical scavenging of ethanolic extracts of rosemary under different extraction conditions (extraction time and concentrations of ethanolic solvent) are presented in Figure 4. On this basis, with increasing the concentration of ethanol, the antioxidant activity (DPPH active radical scavenging) decreases in the resulting ethanolic extracts. Also, by increasing the extraction time, the DPPH radical scavenging activity increased.

The results showed that solvent concentration had a significant effect on the DPPH radical scavenging activity. Also, the ability of the extracts to scavenge DPPH free radicals depends on the solvent concentration; hence, by increasing the solvent con-

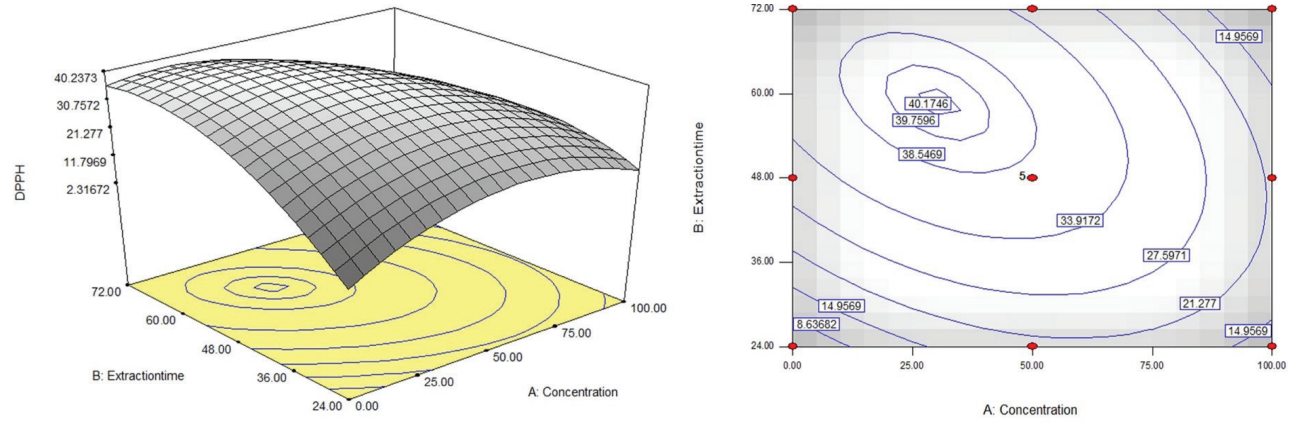


**Figure 4.** 3D and 2D diagram of changes in antioxidant properties of DPPH activated radical inhibition of ethanolic extracts of a *Rosmarinus officinalis* under the influence of ethanol solvent concentration and extraction time (extraction temperature of 40°C).

centration, the inhibitory activity of free radicals increases. Since there is a direct association between the scavenging activity of radical and the amount of phenolic compounds in fruits (Ghorbanipour et al., 2023; Shabanian et al., 2021), therefore, upon increase in total phenolic content in the purified extracts, it is expected that the DPPH scavenging percentage of the extracts will increase; nevertheless, in the present study, despite increasing the amount of phenolic compounds due to the the increase in extraction time, the inhibitory effect of DPPH active radical of the prepared extracts decreased. This finding is in contrast to that of previous studies.

Equation 4 shows the antioxidant activity of the extract in terms of the DPPH active radical scavenging model. As demonstrated in Tables 2 and 3, the DPPH active radical scavenging activity of the extracts showed a linear correlation that was not accurate enough for prediction, and in the case of using this equation, only 33.52% accuracy can be expected.

DPPH scavenging ability = +15.57 +1.70 A -5.18 B (4)

Where A and B are the solvent type (ethanol concentration) and extraction time, respectively.

**Total antioxidant activity of extracted extracts**

The 2D and 3D curves of the total antioxidant properties of rosemary and propolis under different extraction conditions (extraction times and concentrations of ethanolic solvent) showed that an increase in the extraction time led to an increase in the total antioxidant activity of ethanolic extracts (Figures 5 and 6). Also, by increasing the concentration of ethanol solvent, the total antioxidant activity of the extracts increased. Long-term extraction increased the mass transfer rate in the extraction process, hence the effective compounds in antioxidant activity could be better removed from the cells. In the case of propolis, the antioxidant activity increased upon an increase in extraction time, while the antioxidant activity remained constant with an increase in the concentration of ethanol solvent, which is in agreement with the result of Shabanian et al. (2021). They showed that by increasing the extraction time, the antioxidant activity of the obtained extract increased, which may be due to the more phenolic compounds extracted from the green walnut peel by increasing the extraction time.

The analysis of variance of data obtained from total antioxidant properties measurement for ethanolic extract was statistically significant at 5% level and can be expressed by using a second-order statistical model (Tables 2 and 4). According to the analysis variance results (Table 2 and 4) and regression coefficients (Table 3 and 5), the second order (quadratic) equation of the statistical model for calculating the total antioxidant properties of ethanolic extract was given in equation (5), where A is the concentration of solvent (v/v) and B is the extraction time (hours). According to the results of regression analysis, if using equation 5, antioxidant properties of extracts can be predicted with 96.28% accuracy.

Total antioxidant capacity = +19.14 +30.25 A +63.57 B -35.97 AB +92.39 $A^2$ +50.56 $B^2$ (5)

Where A and B are the solvent type (ethanol concentration) and extraction time, respectively.

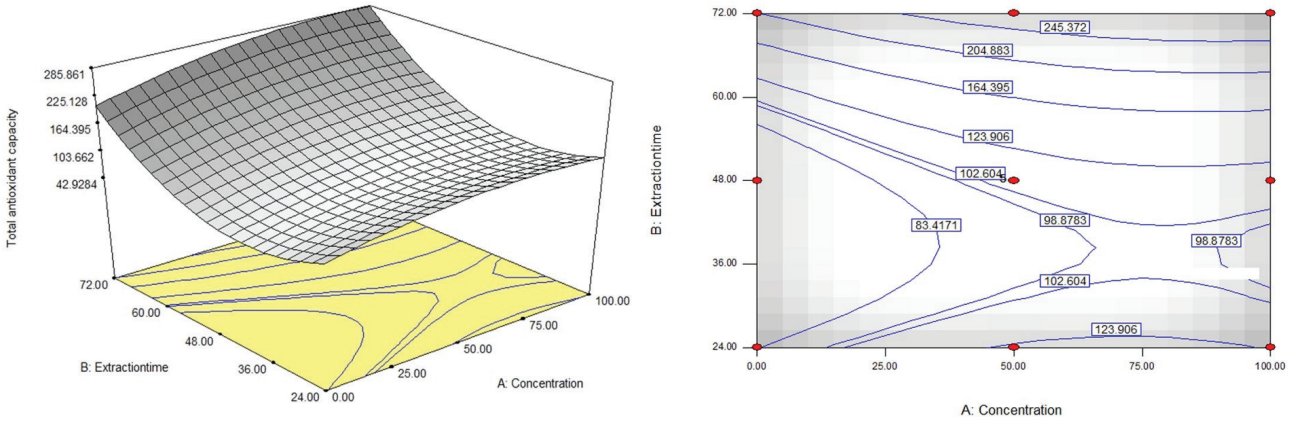


**Figure 5.** 3D and 2D diagram of changes in total antioxidant capacity of ethanolic extracts of a *Rosmarinus officinalis* under the influence of ethanol solvent concentration and extraction time (extraction temperature of 40°C).

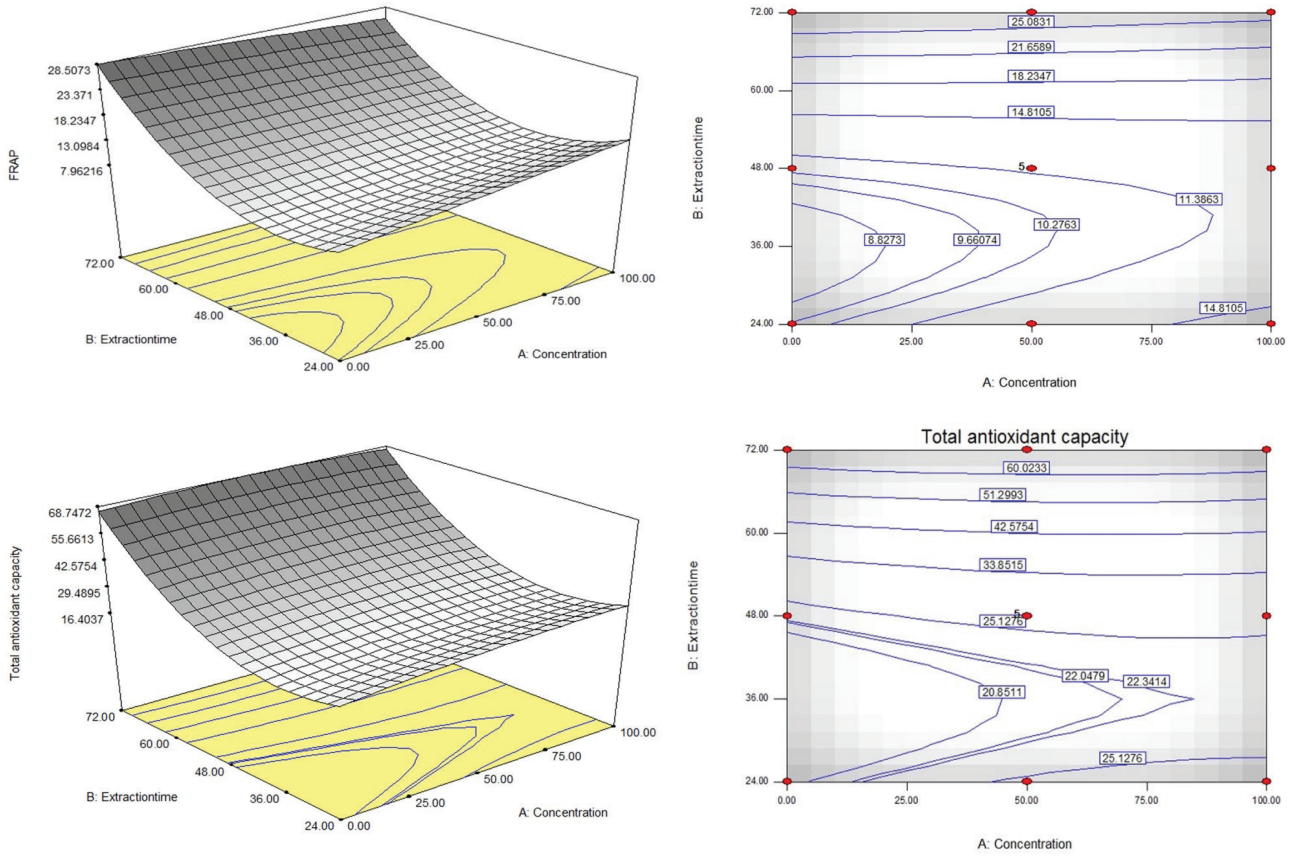


**Figure 6.** 3D and 2D diagram of changes in reducing power and total antioxidant capacity of ethanolic extracts of propolis under the influence of ethanol solvent concentration and extraction time (extraction temperature 40°C).

### Optimization of the extraction process of rosemary and propolis under different extraction conditions

Since the aim of the extraction processes is to achieve the highest antioxidant activity and maintain active compounds, therefor for minimizing thermal damage in long extraction times, independent variables of solvent concentration were chosen in the applied range (0-100%) and extraction time were considered minimum to reduce the adverse effect of heat on phenolic and antioxidant compounds, as observed in Table 6. Also, dependent variables such as total phenolic compounds, total antioxidant capacity, and DPPH scavenging activity of extracts were considered as maximum. During the optimization process, all independent parameters were given the same weight and importance. Considering the desired conditions, the best and most preferred solution involved the extraction time of 24 hours, and the concentration of ethanolic solvent 100% was to achieve optimum conditions. When applying the optimum conditions (Figure 7), the antioxidant properties and phenolic compounds of rosemary plant and propolis extracts can be maintained optimally (Shabanian et al., 2021).

### Lethal effect of optimum extract on *Echinococcus granulosus* protoscoleces

In the present study, rosemary extract obtained by ethanol 77.01% had a high scolicidal activity, so that during 5, 15, and 30 min, it eliminated 86.66%, 100% and 100% of protoscoleces, respectively. In addition, the scolicidal properties of the propolis extract obtained by ethanol 43.58% at different exposure times (5, 15, and 30 min) were 94.16%, 100% and 100%, respectively. In a study, hydroalcoholic extract of rosemary was suggested as one of the treatment options against giardiasis *in vivo* (Vazini et al., 2017). Also, the inhibitory effect of the essential oil of this plant has been proven on *Trichomonas* growth. In another study, the inhibitory effect of propolis on toxoplasmosis infection in mice has been studied *in vivo* (Feyzi et al., 2015). The antibacterial effects on the secondary cause of

**Table 4.** The analysis, variance of the regression coefficients of predicted linear and quadratic polynomial models for predicting antioxidant properties of Propolis extract under different extraction conditions.

| Response | Source | Sum of Squares | DF | Mean of Squares | F-value | p-value | significance |
|---|---|---|---|---|---|---|---|
| Total antioxidant capacity | Model | 2796.51 | 2 | 1398.25 | 10.87 | 0.0031 | significant |
| | A-Concentration | 27.28 | 1 | 27.28 | 0.21 | 0.6549 | |
| | B-Extractiontime | 2769.23 | 1 | 2769.23 | 21.54 | 0.0009 | |
| | Residual | 1285.76 | 10 | 128.58 | | | |
| | Lack of Fit | 1285.61 | 6 | 214.27 | 5569.73 | < 0.0001 | significant |
| | Pure Error | 0.15 | 4 | 0.038 | | | |
| | Cor Total | 4082.27 | 12 | | | | |
| FRAP | Model | 575.19 | 5 | 115.04 | 37.95 | < 0.0001 | significant |
| | A-Concentration | 5.90 | 1 | 5.90 | 1.95 | 0.2056 | |
| | B-Extractiontime | 311.98 | 1 | 311.98 | 102.91 | < 0.0001 | |
| | AB | 18.88 | 1 | 18.88 | 6.23 | 0.0413 | |
| | $A^2$ | 0.025 | 1 | 0.025 | 8.342E-003 | 0.9298 | |
| | $B^2$ | 205.55 | 1 | 205.55 | 67.80 | < 0.0001 | |
| | Residual | 21.22 | 7 | 3.03 | | | |
| | Lack of Fit | 20.99 | 3 | 7.00 | 122.01 | 0.0002 | significant |
| | Pure Error | 0.23 | 4 | 0.057 | | | |
| | Cor Total | 596.41 | 12 | 596.41 | | | |

**Table 5.** The regression coefficients of predicted quadratic polynomial and liner models for predicting different antioxidant activity of propolis extrac under different extraction conditions.

| Response | Factor | coefficients | $R^2$ | $R^2$ adjusted |
|---|---|---|---|---|
| Total antioxidant capacity | Intercept | 35.41 | 0.9628 | 0.9362 |
| | A-Concentration | 2.13 | | |
| | B-Extractiontime | 21.48 | | |
| FRAP | Intercept | 11.58 | 0.7154 | 0.5121 |
| | A-Concentration | 0.99 | | |
| | B-Extractiontime | 7.21 | | |
| | AB | -2.17 | | |
| | $A^2$ | -0.096 | | |
| | $B^2$ | 8.63 | | |

European foulbrood disease, as well as on the *Nocardia* pathogenic species in honey bees, have been reported (Rezvannejad et al., 2017). In another study, 10 minutes of exposure to propolis extract with protoscoleces caused the elimination of daughter cysts (Özçelik et al., 2015).

As a limitation of this study, we could not perform an *in vivo* study to provide a concrete conclusion on the applicability of the obtained *Rosmarinus officinalis* and propolis extracts because of the budget and the pandemic situation. Additionally, we did not fractionate the extracts to explicitly find the efficient material(s) that is/are acting the best scolicidal activity.

**Table 6**. The constrain used to optimize the antioxidant properties of ethanolic extracts of *Rosmarinus officinalis* and propolis under the influence of different extraction conditions

| name | Goal | Lower limit | Uper limit |
|---|---|---|---|
| A:Concentration | is in range | 0 | 100 |
| B:Extractiontime | minimize | 24 | 72 |
| Total phenolic compound | maximize | 37.47 | 675.00 |
| Total antioxidant capacity | maximize | 23.57 | 665.74 |
| FRAP | maximize | 21.52 | 320.62 |
| DPPH | maximize | 16.80 | 38.87 |

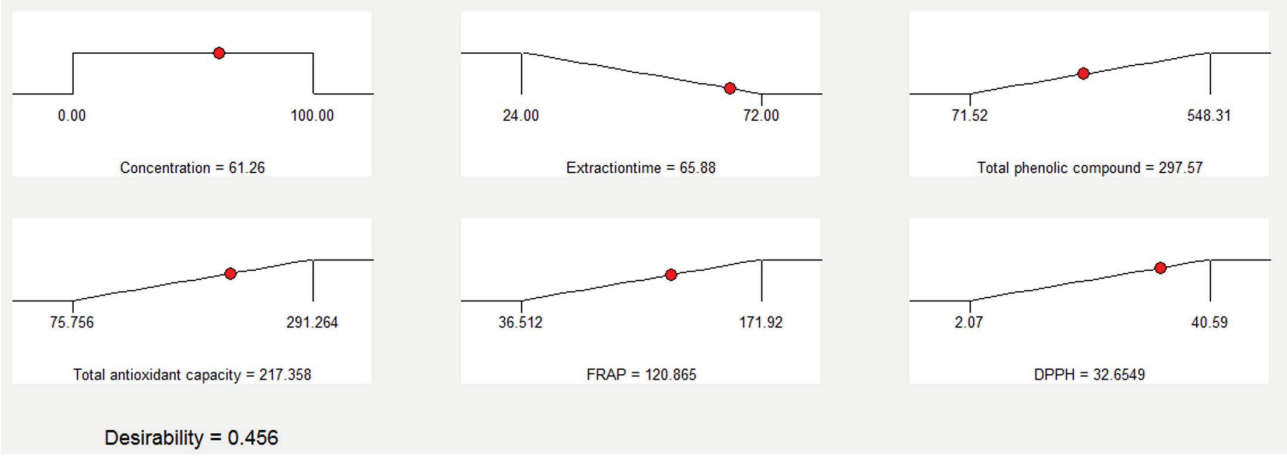


**Figure 6.** Results of optimizing the extraction of ethanolic extracts of *Rosmarinus officinaliss*.

## CONCLUSION

Data presented herein shows for the first time that the addition of water to the ethanol solvents of *Rosmarinus officinalis* and propolis increases the efficiency of phenolic compounds and antioxidant properties of the resulting extracts due to the increasing polarity of the solvent. In addition, *in vitro* experiments indicated that rosemary and propolis extracts can be used as suitable candidates for scolicidal agents. Evaluation of the efficacy of these extracts in an experimental *in vivo* study is recommended to better understand their protoscolicidal effects.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## ACKNOWLEDGEMENTS

We are very grateful to Dr. Alireza Sazmand (Bu-Ali Sina University, Hamedan, Iran). Although he critically read the manuscript, he did not follow our invitation to become a co-author. Nevertheless, many thanks for his help.

## FUNDING

This work was supported by Bu-Ali Sina University, Hamedan, Iran, under the framework of the Master's thesis project of Milad Ghorbanipoor.

### Author's contribution

ZSD designed the study, conducted laboratory indicators, drafted the manuscript, and was responsible for the financial support of the study. MG collected samples and carried out the experiments. ADG performed data analysis. FM provided the herbal plants and modified the test, conceived and designed the study, and provided final supervision. ASari performed antioxidant tests. All authors have approved the final version of the manuscript.

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