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Original Article

Evaluation of Phenolic and Flavonoid Content, Alkaloids, Antioxidant Capacity and Antibacterial Properties of Methanolic Extract of Zahak Native Medicinal Plants Against Seven Pathogens

Bahman Fazeli-Nasab[1*], Mehrangiz Ghafari[2], Mehdi Jahantigh[3], Zahra Beigomi[4] and Saeide Saeidi[5]

[1]*Department of Agronomy and Plant Breeding, Agriculture Institute, Research Institute of Zabol, Zabol, Iran*
[2]*Department of Pathology, School of Medicine, Zabol University of medical sciences, Zabol, Iran*
[3]*Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran*
[4]*Zahedan University of Medical Sciences, Zahedan, Iran*
[5]*Agricultural Biotechnology Research Institute, University of Zabol, Zabol, Iran*

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***Corresponding author**
bfazeli@uoz.ac.ir

ABSTRACT

In general, the resistance of microorganisms to antibiotics has become one of the major concerns of human society and the health care system. The aim of this study was to evaluate total phenol, flavonoids content, alkaloids, antioxidant capacity and antibacterial activity of 7 medicinal plants extracts in Sistan climatic conditions against pathogens. Plant samples were collected from the collection of the Agricultural Research Institute of medicinal plants, University of Zabol and dried in normal shade and room temperature. A Methanolic extract of the leaves of snake grass, eucalyptus, tatura, Musquit bean, Watercress, rosemary and olive was prepared by cold maceration method. Total phenol content by using folin-ciocalteu reagent method, total flavonoid content by aluminum chloride colorimetric method, antioxidant capacity by DPPH free radical scavenging assay and antibacterial activity of extracts by agar diffusion method and measurement of growth inhibition zone or Disk diffusion was measured. The analysis of variance showed that there was a statistically significant difference between different medicinal plants at a probability level of 5%. In the present experiment, the highest phenolic content (110.78 mg/q D.W.) in the Rosemary methanolic extract, the highest total flavonoid content (4.55 mg GA/g D.W.) in Eucalyptus extract and the highest antioxidant activity (93.1%) in the Olive extract were observed. Also, the largest diameter of Disk diffusion (25 mm) was observed in the medium containing Rosemary extract and against *Streptococcus pyogenes*.

INTRODUCTION

Antibiotic resistance is rising to dangerous high levels in Human society and the health system [1-3]. The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, and this case is becoming multidrug-resistant with the attendant increased risk of failure of standard therapies [4-7]. In addition, the widespread use of industrial drugs and the misuse of these drugs cause many side effects that sometimes have toxic effects that are more serious than the diseases themselves [8-11].

Due to the increasing incidence of antibiotic resistance, there is an urgent need to new antibacterial drugs. Among the potential sources, medicinal plants are very important [12].

There have been an increasing number of tendentious to discover new antimicrobial compounds from plant origin; Plants form compounds with complex molecular structures, some of which are associated with plant antimicrobial properties. Alkaloids, flavonoids, isophalonoids, tannins, glycosides, terpenes, and phenolic compounds are secondary metabolites that can exert antimicrobial properties [13-17].

Recently, some bacterial infections have re-emerged due to increased antibiotic resistance. They are able to survive and even multiply in the presence of an antibiotic. The outbreak of antibiotic-resistant

pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* is a global threat to public health[18]. The antimicrobial activities of some plant species have been widely researched. For example, raw extracts of cinnamon, garlic, basil, curry, ginger, sage, mustard, etc. were tested antimicrobial properties against a wide range of gram-positive and gram-negative bacteria [19]. In addition, it has been reported that the extracts from Chinese chives and cassia can effectively reduce the growth of Escherichia coli and other bacteria during storage of meat, juices, and milk [20]. It was investigated [21] the effect of some plant extracts on the growth of Candida albicans, the results indicated that the alcoholic extract of curry leaves effectively inhibit the growth of C. albicans with 24.05 ± 0.07 after 48 h. it was reported [22] that thyme oil extract could decrease the growth of *C. albicans* and *P. aeruginosa*.

According to the CDC, each year in the United States, at least 2 million people develop serious infections with bacteria that are resistant to one or more antibiotics. The total crude cost burden of antibiotic resistance was estimated to be at $ 20 billion in direct health care and $ 35 billion in reduced productivity in one year. In low-income countries, financial constraints become more complex due to the lack of effective monitoring systems, weak laboratory capacity, and lack of access to appropriate antimicrobial drugs. If there were no successful efforts to intervene in terms of looking for new drugs, the number of deaths will rise to ten million and costs the world up to 100 trillion dollars by the year 2050. To this effect, the search for an innovative antibiotic from natural products is ultimately an important segment of modern medicine to overcome the socio-economic and health impact caused by multidrug-resistant microbes [23].

MATERIALS AND METHODS

The leaves of snake grass, eucalyptus, tatura, Musquit bean, Watercress, rosemary and olive (Figure 1) were collected from a farm of Agricultural Research Institute (located in Zahak city (Coordinates: 30°53′38″N 61°40′49″E)). The plants were identified by botanical laboratory at University of Zabol (Dr. A. Sirousmehr).

The samples were then dried and grounded under normal shading and room temperature conditions. Using cold maceration method, 20g of powdered plant leaves was soaked separately in methanol solvent and kept on a shaker for 24 hours.

Fig. 1 snake grass (A), eucalyptus (B), tatura (C), Musquit bean (D), Watercress (E), rosemary (F)

After 24 hours, the material was passed through Whatman No. 2 filter paper. The solvents were then removed from the filtered material by a vacuum rotary apparatus. In order to reach the pure extract and complete removal of the solvent, the concentrated extract was kept in an oven at 40 °C for 48 hours. Finally, the extracts obtained after weighing were stored in the refrigerator at 4 °C until the experiment.

Bacterial strains were obtained from the standard laboratory of the veterinary department of University of Zabol, Zabol, Iran. Bacterial strains include: *S. pyogenes* ATCC® 19615 S., *S. saprophyticus* ATCC®15305, *Streptococcus pneumoniae* ATCC 49619, *Hafnia alvei* ATCC 51873, *S. aureus* ATCC® 25923, *Serratia marcescens* EnterCC ATCC 35, *Serratia marcescens* ATCCC *Acinetobacter baumannii* ATCC 19606 was propagated on nutrient agar medium and stored in a refrigerator at 4 °C until use.

In order to prepare the bacterial suspension from fresh and young bacterial cultures, several colonies were transferred to Mueller-Hinton Broth culture medium. To even out the turbidity of the microbial

suspension prepared according to the McFarland Standard Tube No. 0.5 (turbidity equivalent to 1.5 $\times 10^8$ bacteria per milliliter), the light absorption was adjusted at a wavelength of 630 nm in the range of 0.08 to 0.1. To reach a concentration of 1.5 $\times 10^7$ bacteria per milliliter, the bacterial suspension with turbidity 0.5 McFarland and the ratio 0.1 was diluted [24]. The antimicrobial effects of the all extracts were investigated at a concentration of 250 mg/ml by agar diffusion method. Using a sterile swab of turbidity equivalent to 1.5× 10^7 bacteria per ml was cultured uniformly on Mueller-Hinton Broth culture medium. Then, at appropriate time intervals, a number of wells with a diameter 6 mm and a depth 5 mm were created. One hundred microliters of the extracts were poured into its own well. The antibiotic ciprofloxacin was used as a positive control. After 24 hours of incubation at 37 °C, the growth inhibition zone or Disk diffusion of bacterial specimens was measured in millimeters. To confirm the results, the experiment was repeated three times.

Extraction for Total phenol, Flavonoids and Antioxidants

Methanolic extract was prepared by the cold maceration method with a ratio of 1:20 of plant dry matter and 80% methanol solvent. The samples were soaked in solvent for 48 hours on an orbital shaker at 120 rpm. It was then filtered with Whatman *No. 1* filter paper and transferred to a rotary evaporator at 45 °C for concentration. 1 hour after concentration, the extract was transferred under a laminar hood so that the rest of the solvent was gradually evaporated to give a dry extract. Dry extract was used to prepare methanolic extract for other assays at a concentration of 1mg/ml [25,26].

Assessment of Total Phenol Content

The amount of phenolic compounds was measured in the plant methanolic extract [27]. The total phenolic contents are expressed as mg of Gallic acid equivalent (GAE) per gm of extract. According to this method, 200 µl of extracts (at a concentration of 1 mg/ml) were poured into test tubes. 400 µl of Folin Ciocalteu reagent (diluted 1: 10 with distilled water) and 400 µl of 7% sodium carbonate were added to the mixture. After 30 minutes of storage at ambient temperature, its light absorption was read by spectrophotometer at a wavelength of 765 nm. Finally, by placing the amount of adsorption in the linear standard curve equation of Gallic acid (10, 50, 100, 150, 200 and 250 mg/ml), the total phenolic content was calculated. Data expressed as milligrams of Gallic acid equivalent (GAE) (mg GAE/g). All experiments were performed at three replications.

$Y=0.004 X +0.1$

Y absorption number recorded in the spectrophotometer

X Total phenol content

Total Flavonoid Assay

The total flavonoid content was estimated using aluminum chloride colorimetric assay. In this method, 100 µl of aluminum chloride solution (10%), 100 µl of 1 M potassium acetate solution and 2.8 ml of distilled water were added to 500 µl of methanolic extract. Samples were incubated at room temperature for 40 minutes and absorbance of the resultant solution mixture was then measured at 415 nm. Standard curve drawn based on solution with different concentrations (550-450-350-250-150-50 mg/ml) Quercetin and the amount of flavonoid equivalent to mg of quercetin per gram of dry plant powder (mgQUEg-1) was calculated. The solution blank was prepared in the same way without extract (Chang *et al*., 2002). All assays were performed at three replications.

$Y=0.004 X +0.1$

Y absorption number recorded in the spectrophotometer

X Total flavonoid content

Measurement of Antioxidant Activity

The measurement of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity was carried out according to the method of Barros *et al*. [28]. This method is based on the color change of purple methanolic solution 2 and 2- diphenyl-1-picryl- hydrazyl to yellow solution of diphenyl-picryl hydrazine. 250 µl of the extract was mixed with 750 µl of DPPH solution (2 mg DPPH dissolved in 50 ml methanol). The samples were incubated for 30 minutes in the dark condition at room temperature. Then its absorbance at 517 nm was read by spectrophotometer. Percentage of DPPH radical scavenging activity was calculated by the following equation

$(Ac-As)/Ac\times100$ = Percentage of free radical scavenging

AC: Absorption rate for control sample

AS: Absorption rate of plant samples

Statistical Data Analysis

Three replications were measured for each treatment. All statistical analysis is performed using SAS statistical software version 9.1.

RESULTS

The results of analysis of variance showed that the amount of total phenol content of the different medicinal plant extracts was significant at a level of 5% ($P \leq 0.05$) (Table 1). In this experiment, the highest amount of phenolic compounds (110.78 mg quercetin/g dry matter) was observed in rosemary and then in olives (78.05 mg/g dry matter), respectively. However, the lowest amount of total phenol content in the leaves of the tatura plant (8.01 mg quercetin / g dry matter) was evaluated (Figure 2).

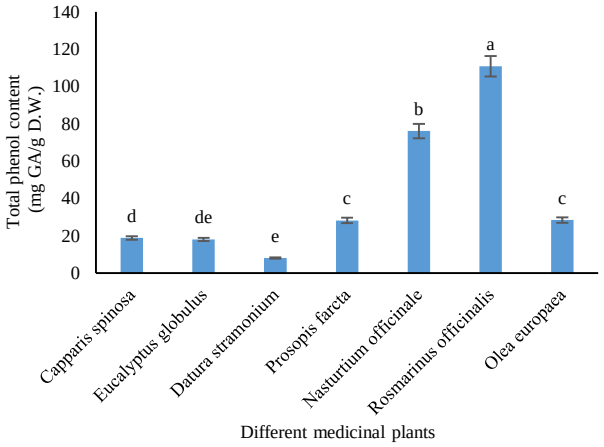

Fig. 2 Investigation of total phenol content in the different medicinal plant methanolic extracts

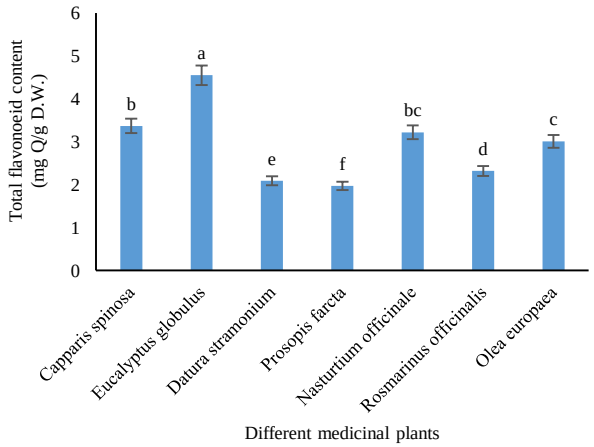

Fig. 3 Investigation of total flavonoids in methanolic extracts of different medicinal plants

The results of Table 1 showed that there is statistically significant difference in the level of 5% probability between different medicinal plants in terms of flavonoid compound (Table 1). As shown in Figure 2, the highest number of flavonoid compounds (4.55 mg Gallic acid / g dry weight) is present in eucalyptus leaves; The lowest number of flavonoid compounds (1.97 mg Gallic acid / g dry weight) was found in Musquit bean leaves (Fig. 3). Our results were consistent with previous result [29] which confirm the high polyphenolic content in rosemary, especially flavonoid compounds. Martin and colleagues Identified 20 bioactive compounds in E. oilda essential oil. The most important compounds are piperitone, alpha-flanders, p-semen and terpene-4-L. The results of analysis of variance showed that there was a statistically significant difference ($P \leq 0.05$) between the studied medicinal plants in terms of antioxidant activity (Table 1). The highest antioxidant activity (93.1%) was observed in methanolic extract of olive leaf and rosemary (84.1%), respectively. However, the lowest antioxidant activity (29.25%) was present in the methanolic extract of tatura leaves (Figure 4).

Pearson correlation coefficient between phenolic, flavonoid and antioxidant content showed that there is a positive and high correlation (P = 0.87) between phenolic content and DPPH free radical scavenging power (Table 2). Plant samples with higher total phenol content (rosemary and olive) also have strong antioxidant properties.

Table 3 shows the diameter of the growth inhibition zone of different bacteria in the vicinity of medicinal plant extracts in this experiment (Fig. 5). The antibiotic ciprofloxacin showed an inhibition zone about 36 mm. One-way analysis of variance (ANOVA) was used to compare the mean diameter of growth inhibition zone in extracts of different medicinal plants. One-way analysis of variance (ANOVA) was used to compare the mean diameter of growth inhibition zone of different medicinal plant extracts. It is noteworthy that the mean diameter of growth inhibition zone in all treatments was lower than antibiotics. According to Table 2, the highest diameter of growth inhibition zone (25.2 ± 4.32 mm) was observed in *Streptococcus mutans* and rosemary extract treatment. The most resistant bacterium was *Hafnia alvi* (Gram-negative), which grew 100% in treatment with tatura leaf extract. it was shown that gram-positive bacteria were more sensitive to the eucalyptus essential oil than gram-negative bacteria. *S. aureus* was the most sensitive strain to eucalyptus essential oil: *P. aeruginosa* was the most resistant strain [29].

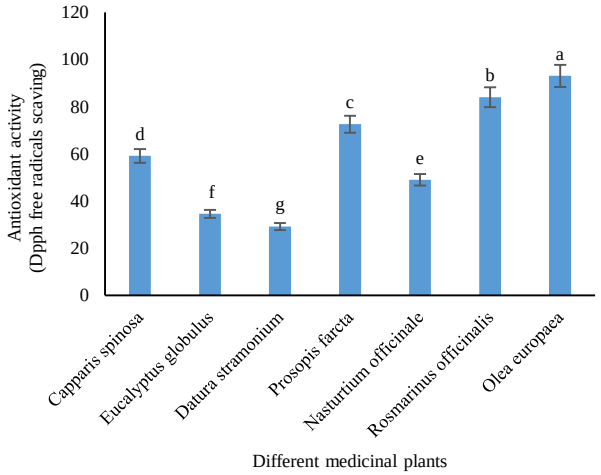

Fig. 4 Investigation of Antioxidant activity in methanolic extracts of different medicinal plants

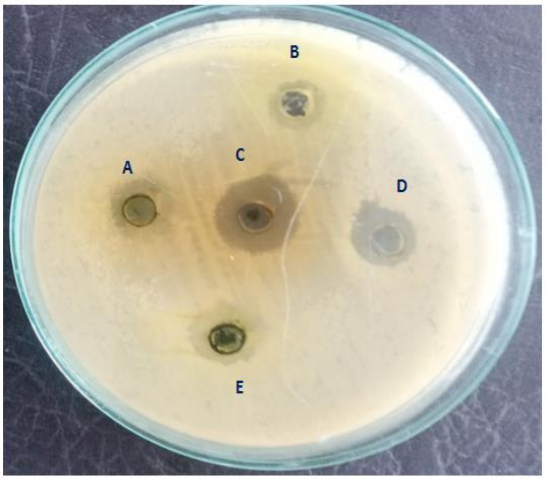

Fig. 5 The inhibition zone diameter of Musquit bean (A), Eucalyptus (B), Rosemary (C), tatura (D) andsnakegrass (E) on *S. pyogenes*

Table 1 Analysis of variance of total phenol, total flavonoids and antioxidant activity of different medicinal plant extracts

Variable	Antioxidants	Flavonoids	Phenol
Mean square	2494.32	8.015 *	3980.3 *
Test Error	5.24	28.39	11.30
coefficient of variation (c.v.)	6.97	0.44	3.96

Table 2 Evaluation of Pearson correlation coefficient between total phenol, flavonoids and antioxidant activity

Variable	Total phenol	Total flavonoids	Antioxidants
Total phenol	1.00	0.4 ns	0.87 *
Total flavonoids	0.4 ns	1.00	0.502 **
Antioxidants	0.87 *	0.502 **	1.00

* Significance at 5% probability level, ** Significance at 1% probability level, ns Non significance

Table 3 Evaluation of the antibacterial effect of different medicinal plants methanolic extract based on diameter of growth inhibition zone (mm)

	Musquit bean	Eucalyptus	Rosemary	tatura	Snake grass	Olive	watercress
S. aureus	2.17±0.0	1.37±0.005	2.1±0.0	2.7±0.0	2.64±0.04	1.31±0.0	3.87±0.004
S. pyogenes	1.14±0.0	2±0.0	10.36±1.96	8.97±1.33	15±47±3.25	20±3.4	10.12±1.26
S. pneumoniae	15±3.04	25±4.06	5.31±2.1	20±4.62	13±2.86	1.41±0.03	25.2±4.32
H. Alvi	1.34±0.0	1.86±0.0	1.69±0.05	0±0.0	2.3±0.25	2.78±0.16	2±0.05
E. faecalis	6±0.0	2±0.03	1.1±0.0	2.36±0.05	2.1±0.0	1.9±0.002	2.39±0.005
P. Mirabilis	20.3±1.1	18.7±3.64	10±1.86	20.1±4.83	15±2.36	4.32±0.11	20±3.4
S. mutans	8±2.46	2±0.12	6.98±1.74	10.99±2.78	10±1.64	18±3.15	15.3±2.46

Data are reported based on the standard deviation of the mean.

DISCUSSION

The results of the present study showed that there is a positive relationship between phenolic content and antioxidant activity of different extracts. The higher phenolic and flavonoid content showed stronger antibacterial activity. In this experiment, the highest amount of phenolic compounds was observed in rosemary, followed by olives.

Studies on citrus fruits showed that the amount of total phenol and total flavonoids in the fruits of citrus cultivars was significantly different and these compounds were also affected by climatic conditions [30]. Therefore, based on the findings, it can be concluded that the degree of effectiveness of

qualitative characteristics depends on the cultivar from one cultivar to another and their physiological conditions[31]. A study also reported that in addition to the fact that all genotypes differed in terms of phenol and flavonoids, citrus cultivars alone were examined and it was found that the best genotypes were red grapefruit and March grapefruit genotypes. In contrast, the environment did not affect the amount of flavonoids and phenol in citrus, i.e. the citrus genotypes of Jiroft and Rudan did not differ significantly [31].

In a study [32] , the comparison of the content of phenolic, flavonoid compounds and antioxidant activity of shoots of two spike plate populations in northern Iran and concluded that the differences between the mean phenolic and flavonoid compounds of the whole extract. Under the influence of three factors: population, extraction method and solvent type, were significant and had the highest antioxidant activity in methanol extract and the lowest in aqueous extract. In the present study, it was found that some of the changes between plants must be found for the reasons mentioned.

Measurement of free radical scavenging based on DPPH is a reliable, accurate, easy and cost-effective method with high reproducibility that is used to evaluate the antioxidant activity of plant extracts in vitro [33]. It has been reported that antioxidant activity has a positive correlation with antimicrobial properties [34,35]. Basically, the antioxidant properties of the extracts increase with increasing concentration of total phenol compounds [36-39] and this ability depends on the number of aromatic rings and the nature of the hydroxyl-displaced groups in the higher concentration. Phenolic compounds increase the probability of hydrogen transfer to free radicals due to the increase in the number of hydroxyl groups present in the reaction medium [38-41]. In a study, it was reported that snake leaf extract with the highest flavonoid and phenolic content among fruit and stem extracts had the highest percentage of antioxidant activity [42]. In a study, it was reported that yarrow plant in Ilam region had the highest amount of phenol in aqueous extract (15.33), hydroalcoholic extract (20/180), Estonian extract (31.59) and methanolic extract (51.47) and the highest amount of flavonoids. In aqueous extract (87/60), hydroalcoholic extract (1447/48), Estonian extract (171.98) and methanolic extract (277.48) and had the highest antioxidant properties [43]. In the present study, it was found that rosemary and olive plants, which had the most technical substances, also had the highest antioxidant activity.

The researchers acknowledged that rosemary essential oil (*R. officinalis*) grown in Sistan contains 19 biologically active compounds that make up 97.5% of the plant essential oil content. The most important bioactive compounds are: alpha-cement, 1 and 8-cineole, borneol, geraniol and camphor. Rosemary essential oil has been shown to inhibit the strong growth of *S. aureus* (MBC = 312.5 µg / ml), *P. aeruginosa* (MBC = 625 µg / ml) and *Salmonella typhi* (MBC = 1250 µg / ml). The researchers acknowledged that the oxygen monoterpenes in rosemary essential oil is a major factor in inhibiting the growth of pathogenic bacteria [44]. In another study, the phenolic, flavonoid and antioxidant content of olive leaves and fruits grow in Gorgan and Zabol ecosystems were evaluated. The results showed that the leaves and fruits of olives grown in Zabol climate have stronger antioxidant properties than Gorgan sample [45]. It was showed [46] that the increased concentration of total phenol and especially flavonoids for many plant species is probably due to the leaves being exposed to increased levels of UV radiation. These cases confirm the positive function of the leaf as a defense mechanism against UV damage. Secondary metabolites and related phenolic compounds are likely to be the main source of UV-B uptake into the leaf epidermis. Herbal leaves (especially olive leaves) with high phenolic and antioxidant content can be used as a tea drink. Plant antioxidants play a vital role in maintaining optimum health care, protecting against coronary heart disease, cancer, etc. The results of this research can encourage researchers and consumers to consume these plant resources as functional foods.

Plants containing high flavonoid compounds have been reported to have high antioxidant activity [42, 47]. In a study, the amount of phenolic compounds in the leaf extracts of plants of the squash and jujube families is high, so it is expected that they have high antioxidant activity [31]. In the present study, it was found that rosemary and olives, which have the highest amount of phenol, also have the highest antioxidant activity. Also, due to the carcinogenic risks of synthetic and synthesized antioxidants, it is suggested that the antioxidants of these plants be used as a suitable alternative to preservatives and can also

be used as rich and available sources in the food and pharmaceutical industries.

CONCLUSION

In general, given that alkaloids, phenols, and polyphenolic compounds such as flavonoids are widely found in food and pharmaceutical products and have been shown to have significant antioxidant activity [48, 49] and on the other hand increasing the level of flavonoids in the diet leads to Reduction of some diseases in humans leads [38,39] and considering the negative effects of synthetic antioxidants and also according to the results of this study, olives and rosemary can be suggested as a substitute for synthetic antioxidants.
Conclusion

Conflict of Interest

All authors declare no conflict of interest.

Ethics Approval and Consent to Participate

No human or animals were used in the present research.

Consent for Publications

All authors read and approved the final manuscript for publication.

Availability of Data and Material

All the data are embedded in the manuscript.

Authors' Contributions

All authors had equal role in study design, work, statistical analysis and manuscript writing.

Informed Consent

The authors declare not used any patients in this research.

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