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Original Article

A Study of the Antioxidant and Antibacterial Effect of Dichloromethane Roots Extract of *Salvia larifolia* and *Salvia atropatana* Medicinal Plants

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ABSTRACT

Regarding to the importance of bacterial resistance to many antibiotics and due to the adverse nutritional and carcinogenic effects of many synthetic antioxidants, the discovery and development of antibacterial and antioxidant ag ents derived from plants have been of interest in recent years. This study was conducted on two species of *Salvia* to determine their antibacterial and antioxidant properties. The antibacterial effect of dichloromethane extract of the roots of *Salvia larifolia* and *Salvia atropatana* was studied using disk diffusion method. Additionally, three methods DPPH, FRAP, and BCB were used to evaluate the antioxidant power of the extracts. The results of this study showed that the extracts at concentrations of 40 and 80 μg/mL had the highest antibacterial and antioxidant properties, respectively. The effect of the extracts on gram-positive bacteria was greater than on gram-negative bacteria. The antioxidant power of *Salvia larifolia* was higher than that of *Salvia atropatana* in the DPPH and FRAP methods, but lower at some concentrations in the BCB method, indicating a difference in the amount of compounds present in these two species. The results of this study suggest that the studied species can be used as effective natural antibacterial and antioxidant agents in various industries.

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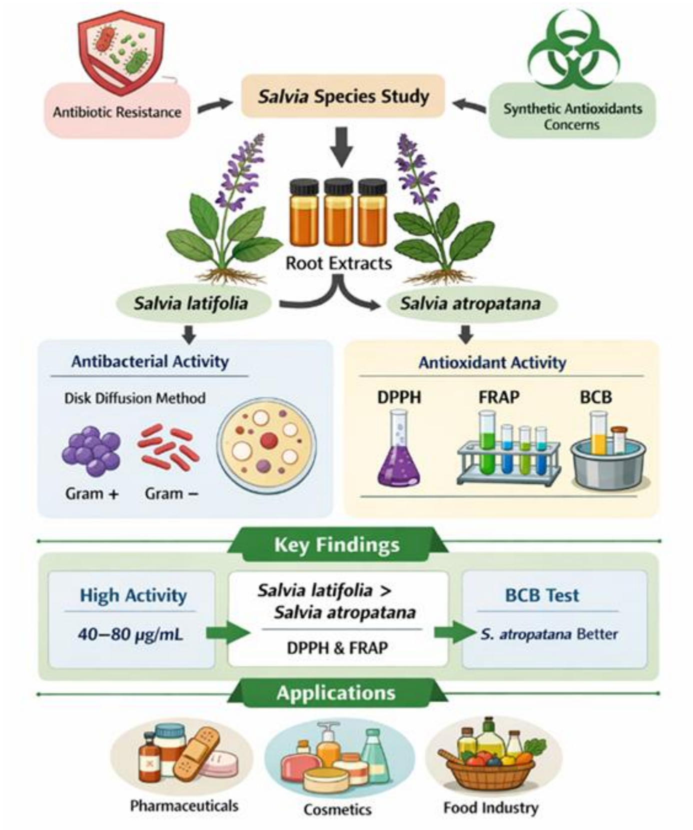
GRAPHICALABSTRACT



Introduction

For centuries, humans have used various plants with different medicinal effects for their therapeutic applications. Today, it is estimated that at least one third of existing drugs are composed of plant compounds and their derivatives [1]. The rare side effects of medicinal plants compared to chemical drugs are one of the main reasons for the endorsement of these products by the medical community [2]. In recent years, many studies have been conducted on the antimicrobial activity of plant extracts. The increase in resistance of pathogenic microorganisms to common antibiotics, which is due to the excessive use of these antibiotics, has led to the warnings from the World Health Organization (WHO) [3], motivating the researchers to discover new natural substances with antimicrobial effects. Compounds such as alkaloids, polyphenols, coumarins and terpenes are among the main compounds found in plant extracts that affect the growth of microorganisms [4]. Another disadvantage of chemical drugs is the formation of peroxides and free radicals due to undesirable metabolic reactions. Meanwhile, herbal medicines are rich in bioactive substances that prevent the formation of oxidants [5]. Antioxidants are substances that have the ability to slow down, delay, and even stop oxidation processes, and as a result, prevent changes in the taste and color of foods caused by these processes. Antioxidants are considered as the first line of defense against free radicals and play an important role in maintaining celluar health and improving body health [6]. In recent years, the use of synthetic antioxidants such as TBHQ, BHT, and BHA has been limited due to their carcinogenic effects, and scientists are focusing on the use of new and less dangerous antioxidants with herbal origin [7].

Among the various aromatic plants with medicinal properties, plants of the mint family have been investigated more extensively [8]. Salvia is one of the largest genera of the mint family, belonging to the subfamily Stachys and the genus Salvia, and is an important ornamental, industrial, and medicinal plant that is widespread in warm and subtropical regions of the world. This genus grows in different regions of Iran as annual, perennial, shrub, and semi-shrub species [9]. Salvia has more than 1,000 species in the world, of which 55 species are found in Iran [10]. In various studies, many medicinal properties of different species of this genus have been reported. Monoterpenes with disinfectant properties have been found in several Salvia species [11]. Antitumor, antimicrobial, antibacterial, antioxidant, and antiviral properties have been observed in species of this genus [12].

Salvia larifolia is native to Khorasan Province and part of Semnan Province. Many medicinal properties have been reported for this species. Antimicrobial and antioxidant effects [13], lowering blood sugar, reducing dependence on morphine, analgesic, hypnotic and sedative [14], preventing the formation and development of gastric ulcers [14], anticancer [15], and neuroprotection against local anemia [16] are some of the medicinal effects reported for this species. *Salvia atropatana* is considered as one of the most variable Salvia species and is widespread in the northeastern and northwestern regions of Iran [17]. Biological properties such as antioxidant [18] and antitumor have also been reported for this species. Since the antioxidant and antimicrobial properties of plant extracts depend on the compounds present in these extracts [19,20] and the amount of extracted compounds from plant extracts is affected by many factors, including the extraction method [21], the extraction method used affects the antimicrobial and antioxidant properties of the extracts. Seasonal and climatic changes (changes in the plant harvest season) are other factors affecting the secondary metabolites present in plants [22]. It has also been shown that the morphology of the genus *Salvia* varies with changes in the place of growth [23]. Therefore, in this study, an attempt has been made to investigate the antimicrobial and antioxidant properties of two species, *Salvia larifolia* and *Salvia atropatana*, collected from Sabzevar City in the growing season of 2018.

Materials and Methods

Preparation of Plant Extracts

The species *Salvia larifolia* and *Salvia atropatana* were collected from Sabzevar City in the growing season of 2018 and identified by the Biology Department of the Faculty of Science, Ferdowsi University of Mashhad. The roots of the plants were separated and after washing with distilled water, they were dried in the air and in the shade. After complete drying, they were ground using an electric grinder and the resulting powder was used for extraction. Extraction was performed by soaking. For this purpose, the powders of each plant were weighed and soaked in dichloromethane (1:10 plant powder to dichloromethane ratio), and then shaken. In the next step, to separate the excess parts of the plant from its extract, the samples were passed through 20 μm Whatman paper. The extraction process was repeated three times until all the plant extract was removed. After that, the extracts were concentrated using a rotary device. Finally, the concentrated extracts were completely desolvated using a freeze dryer. Finally, dilutions of 80, 40, 20, 10, 5, and 2.5 μg/mL of dry extracts were prepared in dichloromethane solvent.

Antibacterial Properties Study

Bacterial strains of two types of gram-negative bacteria, *Escherichia coli* (ATTC 10536) and *Pseudomonas aeruginosa* (ATCC 15442), and two types of gram-positive bacteria, *Bacillus cereus* (PTCC 1247) and *Staphylococcus aureus* (ATCC 29737), were used. These strains were purchased from the Microbiology Laboratory of the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad. Fresh (24-h) cultures of microorganisms were used to prepare microbial suspensions. For this purpose, microorganisms were transferred from the stock culture medium to Mueller Hinton agar culture medium 24 h before the experiments. After this period, a portion of each microorganism was removed with a sterilized loop and transferred separately to sterile test tubes containing sterile normal saline. Sterile normal saline solution was added to the test tubes until the turbidity of the solutions equaled the turbidity of 0.5 McFarland standard solution. The number of bacteria present in each milliliter of the suspensions produced was 1.5 × 108 (CFU/mL).The sensitivity of bacterial strains to extracts of *Salvia atropatana* and *Salvia laurifolia* was determined by the disk diffusion method. For this purpose, Mueller Hinton agar was poured onto sterile plates and incubated at 37 °C. After this, a mixture of 10 ml of Mueller Hinton agar and 1 ml of microbial suspension was poured on the medium layer. Finally, Whatman filter paper discs with a diameter of 6 mm containing different dilutions of plant extracts were placed on the plates using sterile forceps at a distance of 2.5 cm from each other and from the edges of the plates. The plates were kept in a greenhouse at 37 °C for 24 h [24]. After this period, the diameter of the zones of no growth was measured using a caliper. Clear halos around the discs mean no bacterial growth and consequently antibacterial activity of the extract. Discs containing standard antibiotics gentamicin and cephalexin were used as positive controls for gram-negative and gram-positive bacteria, respectively. For each microorganism, one disc was also prepared with solvent alone (negative control) to determine that the antimicrobial effect observed was not caused by the solvent. Experiments were performed for all dilutions of the extracts with three replicates. The effect of the extracts on inhibiting the growth of microorganisms was divided into 4 categories according to the diameter of the halo of bacterial growth inhibition: negative sign (-) which was correspondent to diameter less than 5 mm which indicated inactivity of the extract), positive sign (+) illustrate the diameter between 5 and 10 mm which confirm the weak effect), double positive sign (++) which shows the diameter between 10 and 15 mm demonstrate the moderate effect) and triple positive sign (+++) indicate the diameter more than 15 mm which confirm the strong effect.

Antioxidant Property Investigation

DPPH Method

The high sensitivity and fidelity of DPPH radical assay (1,1-diphenyl-2-picrylhydrazyl) have made its use one of the most widely used methods for investigating the antioxidant power of natural compounds. The highest absorption of this radical is seen at a wavelength of 517-515 nm. By absorbing a hydrogen from an antioxidant, the DPPH radical is converted to a non-radical state, which causes its color to change from dark purple to yellow and a decrease in its absorption at a wavelength of 517-515 nm. To investigate the antioxidant property by DPPH method, the method described by Yamashoji [25] was used. For this, 1 ml of different dilutions of the extracts in methanol was added to 3 ml of a 1 mM methanolic solution of DPPH and mixed vigorously. The prepared solutions were kept in a dark place for 30 minutes at room temperature. After this time, the absorbance of the solutions was read at a wavelength of 517 nm and the percentage of antioxidant activity was calculated with the following Equation 1:

$$\%RSA = \frac{A_C - A_S}{A_C} \times 100 \quad (1)$$

Where, %RSA, A_S and A_C are the percentage of DPPH free radical inhibition, the absorbance of the sample containing the extract and the absorbance of the control sample (without extract), respectively. All experiments were repeated 3 times and the average results were reported. After that, the antioxidant activity of the extract was also obtained as the IC_{50} value (the amount of antioxidant required to reduce the DPPH concentration to 50% of the initial value). This value was determined by linear correlation analysis of the RSA values for different sample concentrations [26]. Obviously, the smaller this number, the greater the antioxidant power or inhibition of free radicals. The results were compared with the IC_{50} value of the antioxidant ascorbic acid as a positive control.

FRAP Method

The basis of this method is the reduction of ferric ions (Fe^{+3}) to ferrous ions (Fe^{+2}) in the presence of antioxidants. This operation is carried out in the presence of 6,4,2-triprydylstriazine (TPTZ) as a chromogenic ligand and in an acidic medium. After reduction of the complex, Fe-TPTZ is formed, which is blue in color and its absorption is visible at a wavelength of 593 nm [27]. In this study, the FRAP test was performed using the method described by Benzie *et al.* [28]. First, the FRAP reagent was prepared by mixing TPTZ (10 mM) in HCl (10 mM), acetate buffer (300 mM, pH = 3.6), and $FeCl_3$ solution (20 mM) in the ratios of 1: 1: 10. To 1.5 mL of this reagent, 1.5 µL of different concentrations of the sample was added and after thorough mixing, the absorbance of the samples was read at 593 nm. To draw the standard curve, ferrous sulfate ($FeSO_4.7H_2O$) was used in typical concentrations of 100 to 2,000 Mm, and finally the antioxidant power of the extracts was reported based on micromoles of Fe II per gram of sample.

BCB Method

This method uses a beta-carotene/linoleic acid emulsion. Beta-carotene has the ability to react with radicals and eliminate them due to its eleven double bond pairs in its structure and thus acts as an antioxidant [29]. The highest absorption of beta-carotene occurs at a wavelength of 470 nm. In this test, beta-carotene reacts with linoleic acid radicals and becomes a radical, and its yellow color progresses toward colorlessness. Adding another antioxidant to the reaction medium causes competition between the two antioxidants and results in less destruction of beta-carotene. In this study, the beta-carotene discoloration test was performed according to the method described by Zhang *et al.* [30], with slight modifications. For this purpose, 0.5 mg of beta-carotene was first dissolved in 1 mL of chloroform. 600 µL of this solution was added to a round-bottom flask containing 40 mg of linoleic acid and 400 mg of Tween 40. The flask was connected to a rotary evaporator and the chloroform was evaporated under reduced pressure. After that, 100 mL of oxygen-saturated distilled water was added to the flask and the mixture was stirred vigorously to form an emulsion. 5 mL of the formed emulsion was added to test tubes containing 200 µL of different concentrations of extracts. The absorbance of the contents of each test tube was recorded immediately after the addition of the emulsion (at time zero) at a wavelength of 470 nm. Then, the tubes were closed and placed at room temperature for 48 h, and after this time, the absorbance of the samples was read. The antioxidant activity of the samples was calculated using the following Equation 2:

$$\%I = \frac{A_{S(48)} - A_{C(48)}}{A_{C(0)} - A_{C(48)}} \quad (2)$$

Where, I% is the percentage of inhibition, $A_{S(48)}$ is the absorbance of the sample after 48 h, and $A_{C(0)}$ and $A_{C(48)}$ are the absorbance of the control sample (without extract) at time zero and after 48 h, respectively. All experiments were repeated 3 times and the average results were reported.

Statistical Analysis

For data analysis, SPSS version 18 software was used using two-way analysis of variance for homogeneity of variance and normality of the data. Comparison of data averages was performed with Duncan's test at the 5% level. Graphs were also drawn in Excel software.

Results and Discussion

Antibacterial Properties

The results of the antibacterial test are summarized in Tables 1 and 2. The results showed that the extracts were inactive at a concentration of 2.5 µg/mL against the strains of evaluated microorganisms and showed weak effectiveness at a concentration of 5 µg/mL. The samples at a concentration of 10 µg/mL also had weak effectiveness on gram-negative bacteria but showed moderate inhibitory effect on gram-positive bacteria. In general, both plant species had a better effect on gram-positive bacteria than gram-negative bacteria and showed a strong effect on all microorganisms from a concentration of 40 µg/mL. According to the data obtained and as it is clear from the results in the table, the *Lorifolia* species performed better than the *Atropatana* species, especially at a concentration of 20 µg/mL.

Table 1: Growth inhibition of samples prepared from Salvia Lari folia species

Sample concentration (μg/mL)	***Staphylococcus aureus***	***Bacillus cereus***	***Pseudomonas aeruginosa***	***Escherichia coli***
Negative control	–	–	–	–
2.5	–	–	–	–
5	+	+	+	+
10	++	++	+	+
20	+++	+++	+++	++
40	+++	+++	+++	+++
80	+++	+++	+++	+++
Positive control	+++	+++	+++	+++

Table 2: Growth inhibition of samples prepared from Salvia Atropa tana species

Sample concentration (μg/mL)	***Staphylococcus aureus***	***Bacillus cereus***	***Pseudomonas aeruginosa***	***Escherichia coli***
Negative control	–	–	–	–
2.5	–	–	–	–
5	+	+	+	+
10	++	++	+	+
20	+++	+++	++	++
40	+++	+++	+++	+++
80	+++	+++	+++	+++
Positive control	+++	+++	+++	+++

Antioxidant Properties

Different compounds can be responsible for the antioxidant properties of a plant extract, and different antioxidants also act in different ways *in vitro*, so the effect of all the features involved in antioxidant properties cannot be determined using only one method [31]. In this study, DPPH, FRAP, and BCB methods were used to investigate the antioxidant properties of the extracts. As can be seen from Figure 1, the antioxidant properties raised with increasing extract concentrations in all three methods. In the DPPH and FRAP methods, the antioxidant power of the *Lorifolia* species was higher (Figure 1a,b), while in the linoleic acid inhibition method (BCB method), the *Atropatana* species has shown greater antioxidant ability at some concentrations (Figure 1c). The IC_{50} value, which is equivalent to the concentration of the extract that provides 50% radical inhibition, was also obtained as 25.96 and 30.49 for the species *Lorifolia* and *Atropatana*, respectively. The IC_{50} value, which is obtained from the interpolation of linear regression analysis, has an inverse relationship with the antioxidant capacity and the higher value showed lower antioxidant power of the extract. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$.

Research has shown that bacteria are able to develop resistance to antibiotics. In addition, adverse nutritional and carcinogenic effects of synthetic antioxidants have been observed. Therefore, the discovery and development of new antibacterial and antioxidant agents from plants have received much attention.

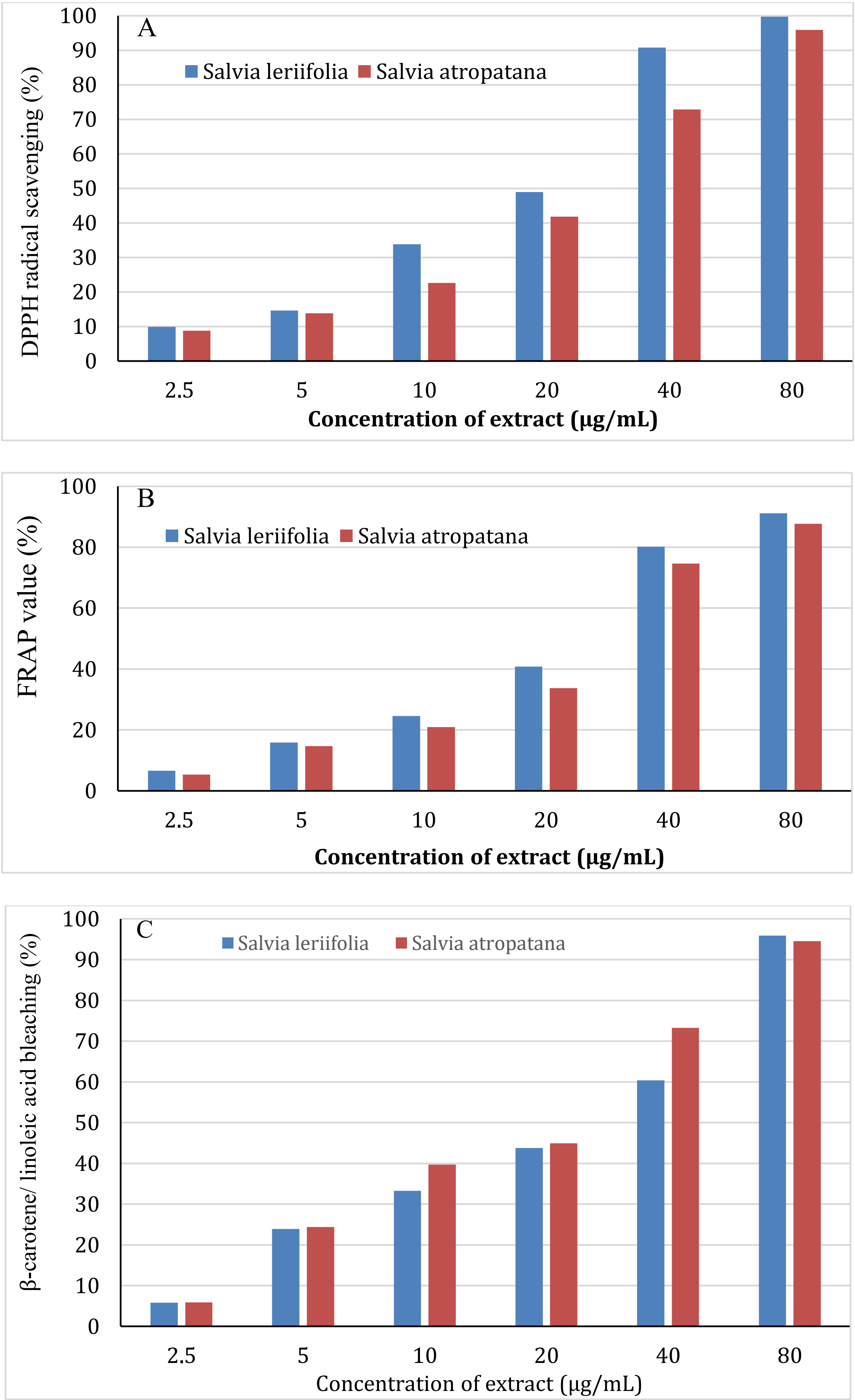


Figure 1: Antioxidant activity of different concentrations of dichloromethane extracts of roots of Salvia Lari folia and Salvia Atropa tana species by A) DPPH, B) FRAP, and C) BCB methods

In this regard, this study was conducted to investigate the antibacterial and antioxidant properties of different concentrations of dichloromethane extracts of two species, *Salvia* *larifolia* and *Salvia atropatana*. It has been found that antibacterial compounds in plant extracts cause destruction of the phospholipid bilayer membrane of bacteria and cause the release of intracellular fluids and consequently their death [32]. In the present study, plant extracts with concentrations of 20 μg/mL and above showed moderate to strong antibacterial effects on all bacterial strains. The growth inhibition zones diameters are measured as criteria of microbicidal reaction and diameter of structured clear zones in disk diffusion method were 7.16 mm for *B. subtilis* ag gram-positive bacteria and 3.67 mm for *E. coli* as gram-negative bacteria. However, the results showed that both extracts were more effective against Gram-positive bacteria than Gram-negative bacteria. This result can be justified by examining the outer membrane of Gram-negative and Gram-positive organisms. In general, a phospholipid membrane is present in the outer layer of Gram-negative bacteria containing lipopolysaccharide structural components, making the cell wall strong against lipophilic solutes. In contrast, Gram-positive organisms are weaker because they have only an outer peptidoglycan layer that cannot effectively prevent the penetration of destructive compounds [33].

The antioxidant properties of plants depend on various factors, including climatic conditions, harvesting and storage methods, the plant parts used, and different stages of plant growth [34]. These factors influence both the type and concentration of secondary metabolites produced in plants. Previous studies have demonstrated a significant relationship between antioxidant activity measured by the DPPH assay [19,20] and the FRAP assay [35] with total phenolic and total flavonoid contents. Accordingly, the higher antioxidant activity observed for *Salvia laurifolia* using the DPPH and FRAP methods can be attributed to its higher content of phenolic compounds. In contrast, the greater antioxidant activity of *Salvia atropatana* in the β-carotene bleaching (BCB) assay indicates a stronger ability to inhibit lipid peroxidation. Several studies have reported that carotenoids play an important role in preventing lipid oxidation [36]. The presence of both phenolic and carotenoid compounds in *Salvia* species has been well documented [37,38].

Since antibacterial activity is also closely associated with the presence of phenolic compounds in plants [39], the higher antibacterial activity observed for *Salvia laurifolia* compared to *Salvia atropatana* may likewise be attributed to its higher phenolic content.

Conclusion

The obtained results showed that the species *Salvia erifolia* and *Salvia atropatana* at concentrations of 40 and 80 μg/mL exhibited strong antibacterial power against various bacterial strains of both gram-negative and gram-positive types. Additionally, high antioxidant properties of both these species were observed at these two concentrations. Therefore, the extracts obtained from these two species can be used as an effective natural antibacterial and antioxidant agent in various industries.

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