

Original article

Antimicrobial and *in vitro* antioxidant activity of *Salvia officinalis* L. against various re-emergent multidrug resistance microbial pathogens

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Abstract

Routine antimicrobial testing was evaluated by using agar disk-diffusion testing method. The total phenolic, total flavonoid content, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) antioxidant activity were also performed on the extract/fractions. The results revealed fraction II possess maximum total phenolic and flavonoids content (330 ± 9.81 mg GAE/g and 106 ± 6.23 mg NE/g, respectively). *Salvia officinalis* L. extract and its all fractions (I and II) exhibited antioxidant potential, but fraction II displayed significant DPPH and ABTS antioxidant potential, having an IC_{50} value of 49.43 ± 6.21 μ g/ml and 76.88 ± 7.42 μ g/ml, respectively. The fraction II displayed substantial activity against clinical isolates for both Gram-positive bacteria (93 A, P 15 and P 9 *Staphylococcus aureus*), but against Gram-negative bacteria (715 *Escherichia coli* and ETT 10 and P 34 *Klebsiella* sp.). There were no inhibition in the growth of the microbes. *S. officinalis* extract and fractions would be useful to reduce the infectious diseases, caused by various re-emergent multidrug resistance microbial pathogens or slow the advancement of different oxidative stress related ailments. However, further study is needed to isolation and characterization of bioactive molecules, which may provide potential benefits source of natural antioxidants and antimicrobial properties.

Key words: *Salvia officinalis* L., leaves, antimicrobial, antioxidant, pathogens

1. Introduction

Antimicrobial resistance is one of the utmost threat to health related issues, associated with a rise in morbidity and mortality where more than 25% of the worldwide yearly mortality is mainly due to infectious diseases (Mahady, 2005). Antimicrobial resistance among the pathogens is one of the most predominant obstacle in the management/treatment of various infectious diseases. There are several reasons/factors which are associated with the development of antimicrobial resistance. One of the important causes of the antibiotic resistance is the misuse of antibiotics that causes progress and proliferation of resistance among the microbes. A number of infectious diseases are being difficult to treat for a number of antibiotics, used in the management/treatment have developed resistance. The investigation on the chemotherapeutic tenacity of

microbial biofilms, whose antimicrobial resistant is multiple times higher contrasted with planktonic bacterial cells, has been increased. The viability of natural antimicrobial competitors can be credited to their synergistic effect and wide pharmaceutical range coming about because of secondary metabolic reactions (Karygianni *et al.*, 2019).

Natural products have been recognized for centuries as the most acknowledged for therapeutic purposes; various research has been done on plant extract/isolates as antimicrobial agents. Plant base products are considered as the most promising antimicrobial activity due to the presence of polyphenolic molecules (Dorman *et al.*, 2000). Plants can manufacture diverse bioactive metabolites, and those metabolites have the potential for huge pharmacological action. In conventional drug, plants are utilized from numerous points of view to treat different diseases/disorders. The folklore claims of the plants can be evaluated scientifically to explore the promising bioactive compounds which can be utilized in the management of various ailments (Eruygur *et al.*, 2019).

Sage (*Salvia officinalis* L.) is native to the Mediterranean regions. This pungent herb is a member of the Lamiaceae, or mint family.

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The specific name officinalis indicates that sage was included in the official lists of medicinal herbs. *S. officinalis* (Lamiaceae) is a wide spread perennial shrub mostly located in the Middle East and Mediterranean regions (Ahmad *et al.*, 2017). It is commonly known as sage or culinary sage and has been used by Egyptians, Greeks, and Romans from centuries in the treatment of various diseases and disorders (Demet *et al.*, 2016).

Some studies revealed that the pharmacological activities of *S. officinalis* is due to the presence of polyphenolic and essential oils content (Martins *et al.*, 2015; Abu-Darwish *et al.*, 2013).

In this study, an effort has been made to investigate the phytochemical screening, radical scavenging and antimicrobial potential of diffraction fraction collected from flash chromatography.

2. Methods

2.1 Collection of plant material and extraction

Salvia officinalis L. leaves were collected from Ras Al Khaimah (Coordinates: 25°47'N 55°57'E) UAE. The collected leaves were chopped and ground, using a grinding mill. Then, the leaves were transferred into a beaker and extracted with deionized water at room temperature by using the ultrasonic homogenizer extraction technique for 1 h (BioLogics, Inc. 300 VT). The extract was filtered and recovered under reduced pressure using Buchi Rotavapor® R-210. The aqueous extract of *S. officinalis* (ASO) was then kept in a desiccator for further use.

2.2 Flash chromatographic analysis of aqueous extract (ASO)

Flash chromatography (Reveleris® X2) was used to classify the presence of different secondary metabolites in fractions with an increase in the polarity ratio of different organic solvents. ASO, ethyl acetate and methanol were filtered through 0.05 µm membrane filter. 40 mg of ASO was loaded in Reveleris® 12 g silica normal phase cartridge at the flow rate of 10 ml/min using both ultra-violet and evaporative light scattering detector. Fractionation was carried out with 1% acetic acid in methanol "A" and methanol "B" gradient elution.

2.3 Preliminary phytochemical investigation

The phytochemical screening of ASO, F1 and F2 of *S. officinalis* leaves were evaluated by several chemical tests for the detection of different secondary metabolites like alkaloids, carbohydrate, glycosides, terpenoids, phenols, flavonoids and saponins. ASO, F1 and F2 dried extracts were reconstituted in suitable solvent and all the chemical tests were performed as described by Ali *et al.* (2015) and Trease (1989).

2.4 Total phenolic content (TPC)

The total phenolic acid content of ASO, F1 and F2 dried extract/fractions was evaluated by Folin-Ciocalteu method (Singleton *et al.*, 1965). A 1000 µg/ml solution of ASO, F1 and F2 was prepared from extract, fractions and gallic acid (reference standard), pipette out 500 µl of dilute solution of extract, fractions and gallic acid were mixed with 2.5 ml of Folin Ciocalteu reagent (Sisco Research Laboratories Pvt. Ltd.) and kept for 5-6 min at room temperature. Then 4 ml of aqueous sodium carbonate (1M) (HiMedia

Laboratories) and the mixture was made up to 10 ml with millipore water. All the mixtures were allowed to stand in a dark place for 10-15 min and the absorbance of the extract, fractions and standard were measured by UV-Vis spectrophotometer (Shimadzu Corp., Japan Model UV-1800) at 760 nm. Gallic acid (Sigma Aldrich) was used as a standard for constructing the calibration curve (50 to 150 mg/ml). Gallic acid equivalent (GAE) was used as the reference standard for constructing calibration curve (50 to 150 mg/ml) and the total phenolic content was expressed as mg of GAE equivalents per gram of each extract on dry basis.

2.5 Total flavonoid content (TFC)

The total flavonoid content of ASO, F1 and F2 dried extracts was evaluated by aluminum chloride colorimetry (Wali *et al.*, 2016). All the samples were diluted with methanol (SD Fine-chem Limited) up to a concentration of 500 µg/ml. All the extract, fractions and reference standard 2.0 ml were mixed with 100 µl of freshly prepared aluminum chloride solution (10% (w/v) and 100 µl of potassium acetate (SD Fine-chem Limited) (0.1 mM). All the samples and reference standard were kept at room temperature for 30 min and the absorbance was measured by UV-Vis spectrophotometer (Shimadzu Corp., Japan Model UV-1800) at 415 nm. The calibration curve was constructed by using naringenin as reference standard (Sigma Aldrich) (50 to 150 mg/ml). Total flavonoid content was expressed as milligram of naringenin equivalent per gram (mg NE/g).

2.6 In vitro antioxidant capacity

2.6.1 Evaluation of 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging assay of ASO, F1 and F2 extract and fractions were investigated by a modified DPPH method (Kallel *et al.*, 2014). 300 µl of all extracts and reference standard (ascorbic acid) of different concentrations (50-250 µg/ml) were mixed with a solution of 0.2 mmol/l DPPH in 2.7 ml of methanol (SD Fine-chem Limited). The absorbance values were documented at 517 nm using UV-Spectrophotometer (Shimadzu Corp. Model UV-1800). The percentage of free radical scavenging activity was compared with that of ascorbic acid and is evaluated by using the following formula: Free radical scavenging activity % = $[A_x - A_z / A_x * 100]$

where A_x is the absorbance of DPPH without sample and A_z DPPH with sample. All the samples were investigated in triplicate.

2.6.2 Evaluation of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay

The ABTS radical scavenging activity of ASO, F1 and F2 extracts and fractions were investigated by ABTS method (Shen *et al.*, 2009), in which the ABTS radical cation (ABTS^{•+}) was generated by reacting 7 mM ABTS (Solution 'A') and 2.4 mM potassium persulfate (Solution 'B'). The working solution was prepared by mixing solution "A" and "B" in a 1:1 ratio and allowing them to react for 24 h at 37°C in a dark place. After 24 h, 1000 µl solution was pipette out and diluted with 60 ml methanol to acquire an absorbance of 0.706 ± 0.01 units at 734 nm using UV-visible Spectrophotometer (Shimadzu Corp. Model UV-1800). The percentage of ABTS radical scavenging activity was compared with that of naringenin and is evaluated by using the following formula:

Free radical scavenging activity % = $[(Ax - Az)/Ax * 100]$

where Ax is the absorbance of ABTS radical solution without ASO, F1, F2 and ascorbic acid and Az with ASO, F1, F2 and ascorbic acid. All the samples were investigated in triplicate.

Microbiological Screening

Six clinical isolates (93A, P15 and P9 *Staphylococcus aureus*; 715 *Escherichia coli*; ETT10 and P34 *Klebsiella*) were isolated from the Department of Medical Microbiology and Immunology, RAK Medical and Health Sciences University (Ras al Khaimah), United Arab Emirates. The experiment was performed using Agar Well Diffusion Method according to Clinical and Laboratory Standards Institute (CLSI) (Adil *et al.*, 2015). Standardized inoculums were accustomed to 0.5 Mcfarl and standard (1-2 X 10⁸ cfu/ml) with measured using a UV-visible Spectrophotometer (Shimadzu Corp. Model UV-1800). Bacterial strains were cultured overnight at 37°C in Mueller Hinton plates and all the extracts ASO, FI and FII were dissolved in 5% DMSO and diluted to the concentration 250 µg/ml, were then carefully placed onto the agar. The petri plates were incubated aerobically at 37°C and examined for zone of inhibition after 24 h. Each zone of inhibition was measured. The experiment was performed in triplicates and mean values were taken.

3. Statistical analysis

All the results from the experiments were expressed as mean ± Standard error of the mean of obtained measurements. Linear regression analysis was used to calculate the IC₅₀ values for the DPPH and ABTS antioxidant activity.

4. Results

4.1 Preliminary phytochemical evaluation

Preliminary phytochemical evaluation of aqueous extract and its fraction collected were performed by using various chemical tests in order to categorize the presence or absence of secondary metabolites. Table 1 summarizes the phytochemical screening results of extract and fractions. The results obtained from the preliminary phytochemical screening of leaves of *S. officinalis* were revealed that FII contains terpenoids and flavonoids in high amount along with other phytoconstituents. FII and ASO extract revealed the presence of higher concentration of terpenoids, flavonoids and phenolic compounds than FI. Whereas, in all fraction and extract confirmed the presence of glycosides, carbohydrate and alkaloids in moderate concentration and absence of saponins.

4.2 Flash chromatography

The aqueous extract of sage was loaded in the flash chromatography C18 column at 5 ml/min flow for 3 min. Then, the column was leached with methanol at 2 ml/min for 20 min, followed by elution with 1% acetic acid in methanol: methanol (8/2, v/v) at 5 ml/min for 10 min (Figure 1) using evaporative light scattering detector.

4.3 Total phenolic content

The total phenolic content of extract and fractions is given in Table 2. The concentration of phenolic components in extract and fractions was expressed in mg GAE/g. The results revealed that ASO was found to contain 270 mg GAE/g, followed by F I with 330 mg GAE/g and F II with 186 mg GAE/g.

Table 1: Phytochemical screening of *S. officinalis*

S.No	Test(s)	ASO	FI	FII
1.	Detection of Alkaloids	++	+	++
	Dragendroff's	+	++	+
2.	Detection of Carbohydrate	++	+	+
	Benedict's	++	+	+
	Fehling's	+	+	+
3.	Detection of Glycosides	+++	+	++
4.	Detection of Terpenoids	+++	+++	++
	Liebermann Burchard's	++	++	++
5.	Detection of Phenols	+++	+++	++
	Ferric Chloride	+++	+++	++
6.	Detection of Flavonoids	++	++	++
	Alkaline Reagent	++	++	++
7.	Detection of Saponins	-	-	-

(-) Absent; (+) present in a negligible quantity; (++) Present in moderate quantity; (+++): Present in a considerable quantity.

Table 2: Total phenolics and flavonoids content of *S. officinalis*

Extract/Fractions	mg GAE/g ^a	mg NE /g ^b
ASO	270 ± 20.00	75 ± 5.00
FI	330 ± 20.00	106 ± 6.63
FII	180 ± 10.00	45 ± 5.00

^aTotal phenolics content is expressed in terms of gallic acid equivalent (µg of GA/g).

^bTotal flavonoids content is expressed in terms of naringenin equivalent (µg of NE/g).

4.4 Total flavonoids content

The total flavonoids content in extract and fractions are shown in Table 2. Among all, the highest amount of flavonoid content was found in FI 106.00 mg NE/g dry wt. (Naringenin acid equivalent) 45.00 NE/g dry weight in FII followed by 75.00 g/NE/g dry wt. in ASO.

4.5 In vitro antioxidant evaluation

The free radical scavenging potential of the extract and fractions of *S. officinalis* leaves were examined by their capacity to scavenge the stable free radicals and shield against damage brought about by oxidizing agents. The outcomes were contrasted were compared with the scavenging capability of reference samples of vitamin C.

In DPPH assay the free radical scavenging activities are shown in Table 3, IC₅₀ values obtained for ASO, FI and FII were 86.15 ± 7.95 g/ml (R²: 0.987), 49.43 ± 6.21 g/ml (R²: 0.970) and 68.23 ± 8.15 g/ml (R²: 0.955) respectively. The results were compared with the reference sample of Vit. C which is showed an IC₅₀ value of 47.44 ± 4.76 µg/ml (R²:0.976).

The relative antioxidant capacity to scavenge the radical ABTS+ has been related with the standard Naringenin Table 3. The ABTS+ scavenging activity IC₅₀ values was found lowest for ASO 124.94 ± 5.51 µg/ml (R²: 0.997) followed by FII and FI were 91.39 ± 9.34 µg/ml (R²:0.948) and 76.88 ± 7.41 µg/ml (R²:0.973), respectively. The results were compared with the reference sample of naringenin which is showed an IC₅₀ value of 54.08 ± 2.53 µg/ml (R²: 0.978).

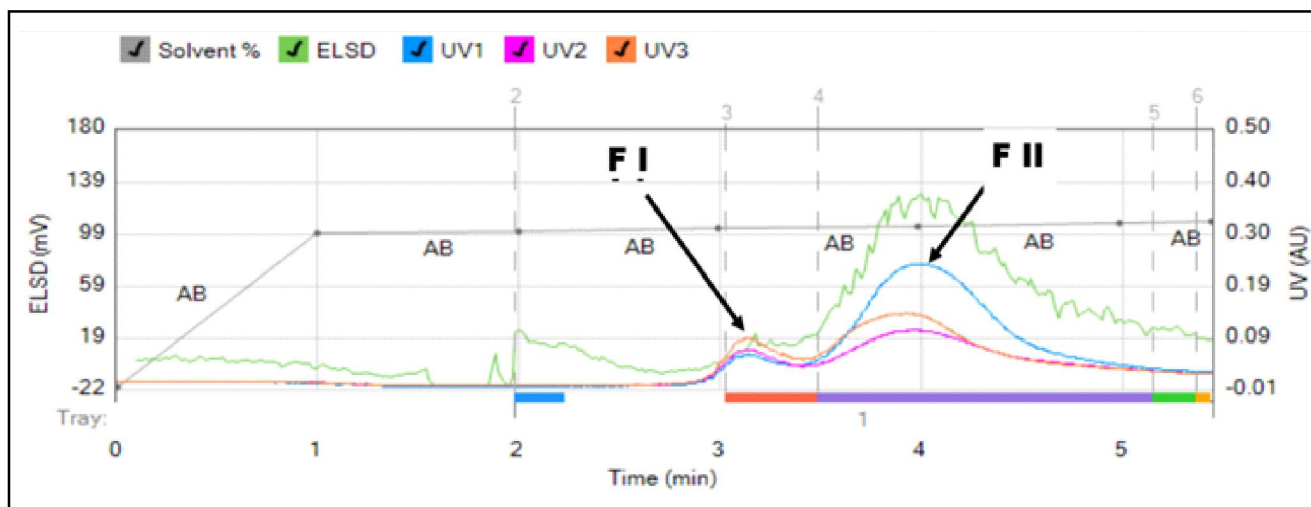


Figure 1: Flash chromatogram of two fractions collected from aqueous extract of *Salvia officinalis*.

Table 3: Antibacterial screening of *S. officinalis* against clinical isolated microbes (Zone of inhibition mm)

	93 A * <i>S. aureus</i>	P 15 * <i>S. aureus</i>	P 9 * <i>S. aureus</i>	715 # <i>E. coli</i>	ETT 10 # <i>Klebsiella</i>	P 34 # <i>Klebsiella</i> sp
ASO	11	16	14	-	-	-
F I	9	16	12	-	-	-
F II	13	15	11	-	-	-
Streptomycin	27	23	29	20	16	11

All values are expressed as means \pm standard deviation; samples were analyzed in triplicate.

4.6 Antimicrobial activity screening

Results of the antimicrobial screening of extract and fractions were evaluated by the disc diffusion method along with the reference drug streptomycin which are presented in Table 3. It has been observed that all fractions of *S. officinalis* leaves inhibited the growth of the microbes against clinical isolates for both Gram-positive

cocci (93 A, P 15 and P 9 *Staphylococcus aureus*), but against Gram-negative bacteria (715 *Escherichia coli* and ETT 10 and P 34 *Klebsiella*), there were no inhibitions in the growth of the microbes. When equated to the reference standard used, all the extract and fractions indicated potential antimicrobial activity against the respective clinical isolated microbes used in the current study and their zones of inhibitions were also distinguished Figure 2.

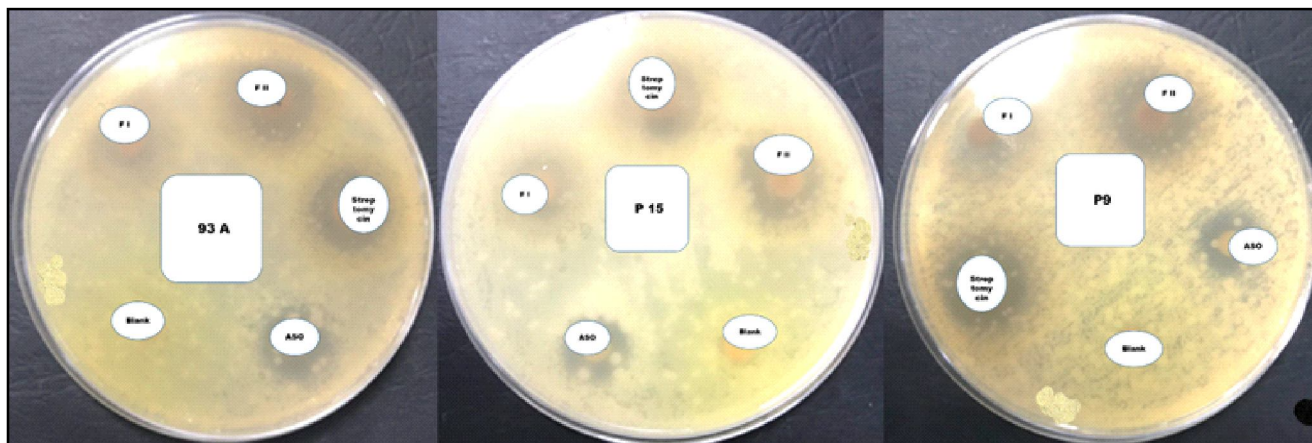


Figure 2: Plate showing zone of inhibition of *S. officinalis* fractions against multidrug resistant (MDR) pathogenic clinical isolates.

5. Discussion

The preponderance of current medicines that have been explored from flora and fauna have been examined due to human understanding and then clinical trials.

Phytochemical components present in the natural products are identified to be pharmacologically active compounds and they are responsible for different activities, for example, cancer prevention agent, antimicrobial, antifungal, and anticancer (Lee *et al.*, 2010).

All constituents present in natural products play a pivotal role in preventing or delaying humans from various chronic diseases/disorder due to free radical scavenging property (Hossain *et al.*, 2011).

This current study provides information on the phytochemical investigation, total phenolic and flavonoid contents of *S. officinalis* in relation to the *in vitro* antioxidant and antimicrobial activities.

The results acquired from the phytochemical investigation of *S. officinalis* (Table 1), displayed the presence of chemical compounds such as terpenoids, phenol, glycosides, carbohydrate, flavonoids and alkaloids. The maximum effective chemical components present such as terpenoids and flavonoids were found in F1 fraction as compared to FII and ASO. Saponins were absent in all fractions and extracts. Therefore, the presence of various chemical components in various fraction and extract might be responsible for the *in vitro* antioxidant and antibacterial activities (Chiang *et al.*, 2015).

Phenolic moiety is one of the major category that are aromatic in nature. The plants biosynthesized the phenolic moiety in defense of UV radiation and to guard against foreign threat (Anyasor *et al.*, 2010). Phenols are an essential chemical constituent present in the natural products, which show antioxidant capacity due to scavenging ability of free radicals of hydroxyl group present in the nuclei/skeleton of the chemical structure (Stevenson *et al.*, 2007). The antioxidant activity of *S. officinalis* was observed by DPPH and ABTS assays to investigate the free radical scavenging potential and antioxidant potential of the extract/fractions.

In the DPPH assay, the stable DPPH^o radical has been generally used in the evaluation of the antioxidant potential of various natural products through the capacity of compounds to act as free radical scavengers or hydrogen donors. There will be discoloration of purple color of the DPPH solution to yellow due to reaction of antioxidants present in the extract/fractions react which will reduce a number of DPPH compounds equal to the present number of the hydroxyl groups. The interaction between the antioxidant moieties and DPPH will cause decrease in absorbance at 517 nm (Rice-Evans *et al.*, 1996).

The current study indicates that the *S. officinalis* leaves were effectively active and extract/fractions contain phenolic moieties that are capable of donating hydrogen to a free radical in order to remove an odd electron which is responsible for radical's reactivity.

In the ABTS⁺ radical scavenging assay, scavenging takes place through electron donation. The antiradical activity of extract/fractions has been compared with the reference standard ascorbic acid. The ABTS radical cation was formed in the stable state by using potassium persulfate. The absorbance of the extract/fractions were taken at 734 nm; the antiradical capacity was determined by change of the blue color ABTS radical cation to its colorless form (Jao *et al.*, 2001; Xu *et al.*, 2005). The antiradical activity of antiradical was found to be in order of: FII > ASO > FI. The highest radical scavenging ability of F II may be due to extraction of more electron donating/hydrogen donating antioxidant.

As results revealed that the FI, FII and ASO inhibited the growth of the microbes against clinical isolates for both Gram positive cocci (93 A, P 15 and P 9 *Staphylococcus aureus*) but against Gram-negative bacteria (715 *Escherichia coli* and ETT 10 and P 34 *Klebsiella*) there was no inhibition in the growth of the microbes. Literature review

revealed that the antimicrobial chemical components of the natural product extract extracts such as terpenoid, alkaloids flavonoid and glycoside compounds interact with enzymes and proteins of the microbial. Considering the fact that extracts/fractions are a mixture of different bioactive compounds, therefore it is impossible to realm their antimicrobial potential to a specific constituent. It has been reported that the antimicrobial potential of different extracts/fractions is because of the synergistic effect presence of different bioactive compounds content (Clinical and Laboratory Standards Institute, 2017; Gill *et al.*, 2006; Moreira *et al.*, 2016)]

6. Conclusion

The results obtained in this work showed comprehension of *S. officinalis* leaves which contains a good amount of phenolics and flavonoids content which might be responsible for antiradical and antimicrobial activities against Gram-positive microorganisms. Consequently, it might be utilized as an alternative medicine in the administration of the human pathosis originated because of oxidative stress and organisms. Our team is further investigating the bioactive compounds/s isolated from *S. officinalis* which is/are responsible for antiradical, antimicrobial and other pharmacological activities.

Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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