

Essential Elixir: Harnessing Antioxidant Power to Combat Staphyloxanthin-Producing *Staphylococcus Aureus*

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ABSTRACT

In the battle against *S. aureus* infections and the growing crisis of antibiotic resistance, health departments are facing challenges and exploring alternative treatments such as antivirulence drugs. Staphyloxanthin (STX), protects *S. aureus* against neutrophil-mediated eradication by providing antioxidant properties, has emerged as a novel target for antivirulence therapy. Stripping off this pivotal pigment from STX-producing *S. aureus* (SPSA) strains is a promising approach. Plant-derived products, specifically Essential Oils (EOs) natural antioxidants are complex mixtures of secondary metabolites derived from aromatic plants and offer a rational strategy to tackle the complexities associated with SPSA infections presenting an intriguing avenue for therapy. This study correlates antioxidant activity with sensitivity patterns of 16 EOs employed in the battle against SPSA. Clove EO (98.9%) exhibited exceptional antioxidant activity, closely followed by cinnamon (98.06%), thyme (92.79%), peppermint (80.43%), and tea tree (77.22%) EOs. Tulsi, eucalyptus, and ginger EO had moderate activity, while lemon, orange, lemongrass, linseed, castor, mustard, sunflower, and sesame exhibited poor antioxidant activity. Statistical analysis was used to assess their sensitivity patterns. This study demonstrates a significant linear correlation ($p < 0.05$) between the antioxidant activity and sensitivity patterns of EOs, suggesting that EOs with strong antioxidant properties may effectively inhibit SPSA isolates. These results highlight the potential of EOs as effective agents in combatting SPSA infections and aiding targeted therapies to counter *S. aureus* infections. By harnessing the natural antioxidant properties of EOs, it may develop innovative approaches to address the challenges posed by *S. aureus* infections and the antibiotic resistance crisis.

Keywords: *S. aureus* infection, Antivirulence, Staphyloxanthin, Essential Oils, Therapeutic activity

1. INTRODUCTION

It is a major challenge to protect against various clinical manifestations of the etiological agent, *S. aureus* that has a large repertoire of virulence factors (VFs) (Turner *et al.*, 2019). It has been reported that the inappropriate use of antibiotics has led to the spread of multidrug-resistant (MDR) strains, which spread outside the health care environment and influence people who may belong to diverse environments (McNeil *et al.*, 2019). It is noteworthy that hospital-associated and community-associated cases of *S. aureus* have complex virulence and resistance genes, which lead to increased morbidity and mortality rates. Its consequences are extensive and include issues like drug hypersensitivity and immunosuppression, which affect the healthcare framework of the whole world (De Carvalho *et al.*, 2019). As a result, there is a pressing necessity to pursue the potential options of fighting the staphylococcal infections, to reduce the negative effects of the existing methods of treatment.

A viable option appears to be antivirulence drug and the emergent drug targeting antivirulence therapy is the staphyloxanthin (STX). A golden carotenoid pigment is produced by more than 90% of *S. aureus* clinical isolates (Jorgensen *et al.*, 2015). STX concentrates within the functional membrane micro-domain having membrane integrity and a high quality of antioxidant. The deprivation of this crucial pigment in clinical isolates of STX pigment-producing *S. aureus* (SPSA) has become a new treatment method since Nizet and co-workers explained the protective role of STX the VF that can shield *S. aureus* against neutrophil-based killing (Escajadillo and Nizet, 2018).

Both the pharmaceutical and food industry have a lot to gain in the botanical world due to the rich number of different compounds that such a world can provide. Among these classes of compounds, there are the volatile members of

essential oils (EOs) (Rao *et al.*, 2019). Complex mixtures of aromatic plant-derived secondary metabolites known as EOs have natural antioxidant properties and offer a rational way to meet the challenges presented by the SPSA infections (Mittal *et al.*, 2018). Besides antioxidant effect, EOs have also drawn interest due to their antimicrobial action. They are powerful agents that can inhibit different multidrug-resistant (MDR) strains and increase the effectiveness of the antibiotics that work in multiple bacterial areas such as cell membranes, protein production, efflux pumps, and virulence-associated pathways such as biofilm formation and toxin generation (Langeveld *et al.*, 2014).

The main aim of this research paper is to develop correlations between the antioxidant and antimicrobial action of EOs used in the war against SPSA. The study of these correlations can help find valuable information concerning the potential of EOs as multitarget agents in managing *S. aureus* infections. The proposed investigation is aimed at supporting the creation of new therapeutic approaches, using the antioxidant and antimicrobial properties of EOs that are inherent to those compounds, which will open the way to managing the SPSA infection successfully.

2. MATERIALS AND METHODS

2.1 Collection of Essential Oils (EOs):

A comprehensive collection of 16 distinct EOs was assembled based on their availability and documented antimicrobial properties. Only EOs sourced from natural herbs were included in the study to ensure their purity and authenticity.

2.2 Procurement of Commercial Chemicals and Media:

All necessary commercial chemicals and media were procured from Hi-Media Company, Mumbai, India. These high-quality materials were utilized to maintain consistency and accuracy in the experimental procedures.

2.3 Bacteria Strains and Growth Conditions:

The 135 clinical samples (pus, blood and urine) were taken at the hospitals and pathology labs in Chandrapur region and delivered using bacterial transport swabs. All samples were transported to the microbiology laboratory within the 24 h in order to preserve viability. Swabs were aseptically inoculated into Trypton Soya Broth and incubated at 37°C during a period of 18 h to enrich bacteria. Sub-cultures were then transferred onto Baird Parker Agar and Mannitol Salt Agar which selectively isolated *S. aureus*. Pure colonies were acquired further screened against staphyloxanthin (STX) production by inoculating them onto Nutrient Agar plates which were further incubated at 37 °C overnight (24 h) and then further incubated at room temperature overnight (24 h) to see the formation of typical pigmented colonies.

2.4 Antioxidant Activity Determination:

The EOs antioxidant activity was assessed through the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging microassay as proposed in the method by Sharififar *et al.* (2007) with few alterations. DPPH solution of 0.004% in methanol was prepared and 100 uL of all EO concentrations were dissolved in 3.9 mL of DPPH solution of 0.1 mM in methanol. The mixtures were thoroughly mixed and allowed to incubate at room temperature in the dark over 30 minutes to ensure free-radical scavenging occurs. A UV-visible spectrophotometer was used to measure the absorbance at 520 nm. The positive control was the BHT (butylated hydroxytoluene). Reduced absorbance values referred to greater antioxidant activity, which means that the EOs scavenged more DPPH radicals.

$$\% \text{ Free radical scavenging} = \frac{(\text{Blank} - \text{Sample}) \times 100}{\text{Blank}}$$

2.5 Antistaphylococcal Activity Determination:

The antimicrobial property of 16 EOs were assessed against *S. aureus* standard strains (ATCC 6538, ATCC 25923, and ATCC 25913) and 44 clinical SPSA isolates using a modified disc diffusion technique (Muthiyan *et al.*, 2012). Overnight cultures were transferred to TSB and allowed to grow until they reached a 0.5 McFarland standard. Swabbing of MHA plates with standardized inoculum was done, and 10 µL of EOs were applied to the agar surface using sterile discs (6 mm). Plates were incubated at 37°C, 24 h and inhibition zones were determined in mm. Inhibition zones were categorized as extremely sensitive (20 mm and above), very sensitive (15 to 19 mm), sensitive (9 to 14 mm), and not sensitive (8 mm and below). This technique enabled it to establish the relative antistaphylococcal action of the EOs.

2.6 Statistical Analysis

For this study, all assays were conducted in triplicate, and at least two independent experiments were performed to ensure reliable results. The collected data were subjected to statistical analysis using Minitab software. The study was able to draw statistically supported conclusions and provide valuable insights into the antioxidant and antimicrobial activities of the EOs against SPSA isolates.

3. RESULTS

3.1 Isolation and Identification of SPSA

From the 135 clinical samples (pus, urine, and blood) collected, a total of 80 *S. aureus* isolates were obtained. Among these isolates, 44 were identified as SPSA isolates based on their ability to produce the STX pigment.

3.2 Antioxidant Assay

The 16 collected EOs were evaluated for their antioxidant activity using the DPPH scavenging method. The results revealed varying degrees of antioxidant activity among the tested EOs.

Clove EO exhibited excellent antioxidant activity, with a scavenging inhibition percentage of 98.9%. This was followed by cinnamon EO (98.06%), thyme (92.79%), peppermint (80.43%), and tea tree EO (77.22%). These EOs demonstrated high levels of antioxidant potential.

On the other hand, tulsi, eucalyptus, and ginger EO exhibited moderate antioxidant activity, with scavenging inhibition percentages ranging between 50 to 45%. Lemon, orange, lemongrass, linseed, castor, mustard, sunflower, and sesame EOs displayed poor antioxidant activity, with scavenging inhibition percentages ranging from 15 to 20% compared to the positive control BHT.

It is worth noting that the absorbance and the percentage of scavenging inhibition showed an inverse relationship, indicating that higher absorbance values corresponded to lower scavenging inhibition percentages (Fig. 3.1). These results suggest that certain EOs possess significant antioxidant properties, which could be attributed to the presence of bioactive compounds within the oils.

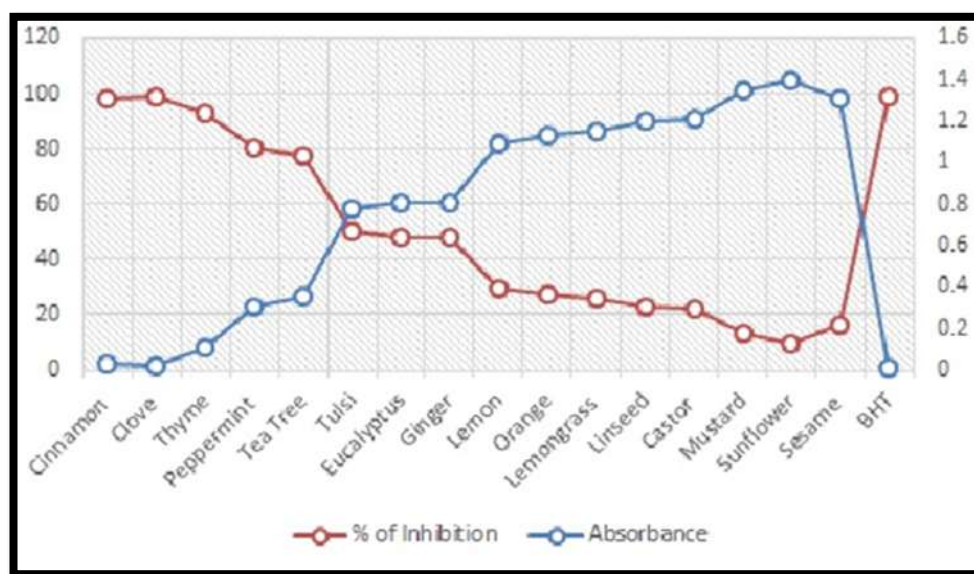


Fig. 3.1: Correlation between absorbance and % of scavenging inhibition of EO

3.3 Effect of EOs on SPSA

The antimicrobial efficacy against 16 EOs of 44 SPSA isolates was assessed and an extremely uneven distribution of antimicrobial susceptibility was observed. Cinnamon, clove, thyme, tea tree, tulsi, peppermint, ginger and eucalyptus EOs showed great inhibitory effect, far much to the growth of SPSA isolates. Lemon, lemongrass, sesame, linseed, and castor EOs were moderate in their action and had observable but relatively weak inhibitors. Contrarily, EOs of sunflower, mustard, and orange did not show any significant inhibitory effect meaning they had no significant activity against SPSA.

The difference in the EO effectiveness indicates that their specific chemical compositions, the concentration and synergy of the bioactive compounds, determine their antimicrobial efficacy. Such findings highlight the potential of cinnamon, clove, thyme, tea tree, tulsi, peppermint, ginger and eucalyptus as promising antibiotics in the formulation of alternative measures to deal with SPSA infections.

Moreover, these findings were proved by the results of the analysis of the mean and standard deviation (Fig. 3.2). The variability of cinnamon and clove was the greatest, and most of the isolates were extremely sensitive with the only minor part of them belonging to other groups. The relative uniformity of the activity of thyme, tulsi, tea tree, and ginger, as expressed by their smaller standard deviations, indicated a similar antimicrobial effect of these four ingredients on the different isolates. On the other hand, the standard deviations of eucalyptus, lemon grass, sesame, linseed, lemon, castor, sunflower, orange and mustard were higher because the isolates were mostly non-sensitive showing weak and erratic activity. In general, cinnamon, clove, thyme, tulsi, tea tree and ginger were the most promising and dependable essential oils to stop the growth of SPSA.

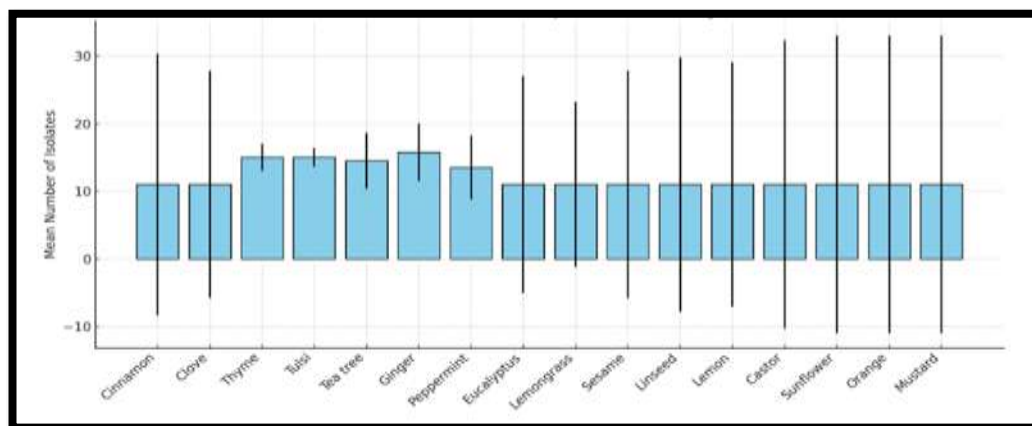


Fig.3.2 Mean $\pm$ Standard Deviation of Sensitivity Pattern of SPSA against EOs

3.4Comparative Study between Antioxidant and Antistaphyloxanthin Activity of EOs

A comparative study was done to examine the correlation between the antioxidant activity and sensitivity pattern of the EOs to SPSA isolates. The statistical analysis was conducted at the significance level $\alpha=0.05$.

The findings indicated that there was a notable linear relationship between the sensitivity pattern of the EOs and the antioxidant activity ($p<0.05$). This means that EOs that had high antioxidant qualities also had higher antimicrobial effects on the SPSA isolates.

The findings of this comparative study are summarized in Table 3.1, which shows the antioxidant activity and the pattern of sensitivity of the EOs. The EOs that had high antioxidant capabilities like clove, cinnamon, thyme, peppermint, and tea tree also exhibited effective inhibitory act on the SPSA isolates.

These findings indicate that the antioxidant properties of the EOs may also be a contributing factor to the antimicrobial efficacy of the EOs against SPSA. EOs with great antioxidant potential can potentially contain compounds that do not just scavenge free radicals, but also show an anti-proliferation effect on the growth and virulence of SPSA.

Table No.3.1: Correlation between antioxidant activity and sensitivity pattern of EOs against SPSA (n=44)				
Test	Non-sensitive	Sensitive	Very-sensitive	Extremely Sensitive
Correlation	-0.367	0.055	0.274	0.618
p-Value	$p<0.05^*$			

4. DISCUSSION

The DPPH assay was selected in the present study as in earlier experiments to determine the antioxidants of the 16 selected EOs. Assessment of the antioxidant potential of plant extracts and compounds is a common technique, which is known as the DPPH assay. It is a quick and easy test and can be used to screen large number of samples within a fairly short time (Sanchez-Moreno, 2002).

The outcome of the antioxidant activity determination of the study revealed that there were significant differences between the tested EOs. Clove EO had the highest percentage of antioxidant effect, which is 98.9 percent of the EOs under analysis. This also indicates that clove EO possesses good antioxidant properties, which is agreeable with other past researches (Rahimi *et al.*, 2010; Hasani-Ranjbar *et al.*, 2009) that have also indicated that clove EO is a powerful antioxidant.

After clove EO, cinnamon EO had a high antioxidant activity, having a percentage of 98.06. Thyme EO had a comparatively lower and yet, noteworthy antioxidant activity percentage of 92.79. Peppermint EO and tea tree EO exhibited an antioxidant activity of 80.43% and 77.22 respectively. These results point towards the fact that these EOs have significant antioxidant characteristics.

These findings are in accordance with the past studies which have also indicated that these particular EOs have a high antioxidant activity. This uniformity implies that the antioxidant effects reported in this study are not single incidences but rather are consistent with the available scientific understanding of the same. The above studies of Edris, (2007) who presented corroborative evidences to the high and moderated antioxidant activities of tested EOs were made. Such a direct correlation between the antioxidant activity of extracts and the concentration of polyphenols and flavonoids in these extracts was proven in the previous studies (Thusoo *et al.*, 2014; Shahidi and Wanasundara, 1992; Kulisic *et al.*, 2004).

Conversely, lemon, orange, lemongrass, linseed, castor, mustard, sunflower and sesame EOs exhibited rather low antioxidant activity of between 15-20% against the positive control, which was probably the antioxidant BHT. The antioxidant activities of these EOs were lower than those of the aforementioned EOs. It is necessary to mention that these EOs can have lower antioxidant activity, but still, it may be recommended that they have other positive qualities or uses.

The correlation of the antioxidant activity with the sensitivity pattern of the EOs against SPSA isolates using correlation analysis carried out in the study produced significant results. These two parameters showed a linear relationship where the p-value was below 0.05. This observation indicates that the antioxidant effects of the EOs and their inhibitory effect against the SPSA isolates are related.

These findings show that EOs exhibiting better antioxidant activity also had better inhibitory ability against SPSA isolates. This finding indicates that the antioxidant properties of the EOs may be one of the reasons why they are antimicrobial against SPSA. That is, the high antioxidant potential of the EOs could contribute to their effect to prevent the growth or viability of the SPSA. The results obtained are in line with the earlier studies which have examined the antimicrobial properties of EOs. Antimicrobial properties against different microorganisms, such as bacteria have been discussed to be exhibited by antioxidants (Kuroda *et al.*, 2007; Musthafa and Voravuthikunchai 2015; Martuccia *et al.*, 2015). The antimicrobial properties of EOs are also considered to be the result of EOs chemical compounds which can affect the microbial cells and interfere with their essential functions (Sikkema *et al.*, 1995; Ahmad, *et al.*, 2011).

With the example of SPSA isolates, the antioxidant activity and inhibitory effect had a positive correlation indicating that the antioxidant compounds found in the EOs might tamper with the STX pigment production or other metabolic pathways within the SPSA resulting in inhibition. This could be an added evidence of the possible application of these EOs in the prevention or treatment of SPSA infections.

One should also mention that antimicrobial action of EOs is not only connected with antioxidant activity. The EOs have been reported to have multiple ways of action on bacteria such as disrupting bacterial membrane, inhibiting protein synthesis, rupturing efflux pumps, and disrupting virulence-related pathways (Langeveld *et al.*, 2014). The other antimicrobial processes can be synergistic to the overall effect of EOs on SPSA isolates.

5. CONCLUSION

The general observation indicates that EOs can be utilized as therapeutic agents in the treatment of *S. aureus* infections especially SPSA-causing strains on the basis of their antioxidant property, they suppress the formation of STX, stimulate immune response and promote clinical outcome. The insights can be useful in considering the application of EO as alternative treatment of multidrug-resistant strains, as such approach is natural and may be rather effective. Nevertheless, additional *in vivo* and clinical studies are required to confirm the effectiveness, safety, and the potential of EOs as

supplementary treatment to conventional antibiotics. Also, in future studies, emphasis must be made on the identification of particular constituents and action mechanisms of synergistic interactions between the various constituents in EOs that are involved in their antimicrobial activity against SPSA isolates.

6. REFERENCES

- Ahmad, A., Khan, A., Akhtar, F., Yousuf, S., Xess, I., Khan, L., and Manzoor, N., 2011. Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against *Candida*. *Eur. J. Clin. Microbiol. Infect. Dis.* 30, 41–50. <https://doi.org/10.1007/s10096-010-1050-8>
- De Carvalho, S., De Almeida, J., De Freitas, L., Guimaraes, A., Do Nascimento, N., Dos Santos, A., Campos, G., Messick, J., Timenetsky, J., and Marques, L. M., 2019. Genomic profile of Brazilian methicillin-resistant *Staphylococcus aureus* resembles clones dispersed worldwide. *Journal of Medical Microbiology*, 68, 693–702. <https://doi.org/10.1099/jmm.0.000956>
- Edris, A.E., 2007. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. *Phytotherapy Research*, 4, 308–323. <https://doi.org/10.1002/ptr.2072>
- Escajadillo, T., and Nizet, V., 2018. Pharmacological targeting of pore-forming toxins as adjunctive therapy for invasive bacterial infection. *Toxins* 10, 542. <https://doi.org/10.3390/toxins10120542>
- Hasani-Ranjbar, S., Nayebi, N., Larijani, B., and Abdollahi, M., 2009. A systematic review of the efficacy and safety of herbal medicines used in the treatment of obesity. *World J Gastroenterol*, 15(25), 3073–3085. <https://doi.org/10.3748/wjg.15.3073>
- Jorgensen, J. H., Pfaller, M. A., Carroll, K. C., Funke, G., Landry, M. L., Richter, S.S., Warnock, D. W., 2015. *Manual of Clinical Microbiology*, 11th ed., American Society of Microbiology, Washington, DC.
- Kulicic, T., Radonic, A., Katalinic, V., Milos, M., 2004. Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chemistry*, 85 (4), 633–640. <https://doi.org/10.1016/j.foodchem.2003.07.024>
- Kuroda, M., Nagasaki, S., Ito, R., and Ohta, T., 2007. Sesquiterpene farnesol inhibits recycling of the C55 lipid carrier of the murein monomer precursor contributing to increased susceptibility to β -lactams in methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 59, 425–432. <https://doi.org/10.1093/jac/dkl519>
- Langeveld, W. T., Veldhuizen, E. J., and Burt, S. A., 2014. Synergy between essential oil components and antibiotics: a review. *Critical Reviews in Microbiology*, 40 (1), 76–94. <https://doi.org/10.3109/1040841X.2013.763219>
- Martuccia, J.F., Gendeb, L.B., Neiraa, L.M., and Ruseckaite, R.A., 2015. Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. *Ind. Crops Prod.*, 71, 205–213. <https://doi.org/10.1016/j.indcrop.2015.03.079>
- Mcneil, J. C., and Fritz, S. A., 2019. Prevention strategies for recurrent community-associated *Staphylococcus aureus* skin and soft tissue infections. *Curr. Infect. Dis. Rep.* 21, 12. <https://doi.org/10.1007/s11908-019-0670-0>
- Mittal, R. P., Rana, A., and Jaitak, V., 2018. Essential oils: an impending substitute of synthetic antimicrobial agents to overcome antimicrobial resistance. *Current Drug Targets*, 20 (6), 605–624. <https://doi.org/10.2174/1389450119666181031122917>
- Musthafa, K. S., and Voravuthikunchai, S. P., 2015. Anti-virulence potential of eugenyl acetate against pathogenic bacteria of medical importance. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 107(3), 703–710. <https://doi.org/10.1007/s10482-014-0364-4>
- Muthaiyan, A., Martin, E. M., Natesan, S., Crandall, P.G., Wilkinson, B.J., and Ricke S. C., 2012. Antimicrobial effect and mode of action of terpeneless cold pressed valencia orange essential oil on methicillin-resistant *Staphylococcus aureus*. *Applied Microbiology*, 112(5), 1020–1033. <https://doi.org/10.1111/j.1365-2672.2012.05270.x>
- Rahimi, R., Shams-Ardekani, M. R., and Abdollahi, M., 2010. A review of the efficacy of traditional Iranian medicine for inflammatory bowel disease. *World J Gastroenterol*, 16(36), 4504–4514. <https://doi.org/10.3748/wjg.v16.i36.4504>
- Rao, J., Chen, B., and McClements, D. J., 2019. Improving the efficacy of essential oils as antimicrobials in foods: mechanisms of action. *Annual Review of Food Science and Technology*, 10 (1), 365–387. <https://doi.org/10.1146/annurev-food-032818-121727>
- Sanchez-Moreno, C., 2002. Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Science and Technology International*, 8 (3), 121–137. <https://doi.org/10.1106/108201302026770>
- Shahidi, D., and Wanasundara, P. K., 1992. Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition*, 32 (1), 67–103. <https://doi.org/10.1080/10408399209527581>

- Sharififar, F., Moshafi, M.H., Mansouri, S.H., Khodashenas, M., and Khoshnoodi, M., 2007. In vitro evaluation of antibacterial and antioxidant activities of the essential oil and methanol extract of endemic *Zataria multiflora* Boiss. *Food Control*, 18, 800–805. <https://doi.org/10.1016/j.foodcont.2006.04.002>
- Sikkema, J., De Bont, J.A.M., and Poolman, B., 1955. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.*, 59, 201–222. <https://doi.org/10.1128/mr.59.2.201-222.1995>
- Thusoo, K., Gupta, S., Sudan, R., Kour, J., Bhagat, S., Hussain, R., and Bhagat, M., 2014. Antioxidant Activity of Essential Oil and Extracts of *Valeriana jatamansi* Roots. *BioMed Research International*, 1, Article ID 614187, 1-4. <https://doi.org/10.1155/2014/614187>
- Turner, N. A., Sharma-Kuinkel, B. K., Maskarinec, S. A., Eichenberger, E. M., Shah, P. P., Carugati, M., et al. 2019. Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nat. Rev. Microbiol.* 17, 203–218. <https://doi.org/10.1038/s41579-018-0147-4>