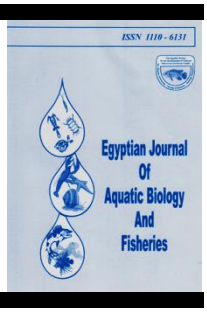
Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(5): 207 – 218 (2022)
ww.ejabf.journals.ekb.eg



Antibacterial, antibiofilm, antioxidants and phytochemical profiling of *Syzygium aromaticum* extract

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ARTICLE INFO
Article History:
Received: July 25, 2022
Accepted: Sept. 2, 2022
Online: Sept. 18, 2022

Keywords:
S. aromaticum extract, antibacterial, antibiofilm. antioxidant, GC-MS analysis.

ABSTRACT

Syzygium aromaticum (*S. aromaticum*) is a clove used traditionally for the treatment of several diseases. This work aimed to evaluate the biological activity and phytochemical profile of *S. aromaticum* extract. The *S. aromaticum* was extracted by petroleum ether and chemically characterized by gas chromatography-mass spectrometry (CG-MS). The antibacterial action of the *S. aromaticum* was tested against standard strain and three clinical isolates of viridans group streptococci previously isolated from dental caries. The MIC of *S. aromaticum* extract was ringing from 5. 53 to 12.5 mg\ml. In addition, *S. aromaticum* extract showed antibiofilm activity at 1 and l/2 MIC against streptococci species and has strong antioxidant properties. The major chemical constituent of *S. aromaticum* extract were eugenol (21.72%), phenol, 2-methoxy-4-(2-propenyl)-acetate (16.75%), espintanol (10.41%) and caryophyllene oxide (9.55%). *S. aromaticum* extract showed antibacterial action against viridans group streptococci strains, showing a promising natural alternative in clinical therapy

INTRODUCTION

Syzygium aromaticum, commonly called clove is from the family *Myrtaceae* have been reported to possess biological activities (**Pandey and Singh, 2011**). *S. aromaticum* is a rich source of bioactive compounds and has many therapeutic uses including control of vomiting. nausea, cough, dyspepsia, flatulence, diarrhea, stomach distension, and gastro-intestinal spasm relieve pain, stimulate the nerves and cause uterine contractions **(Tanko *et al.*, 2008).** The plant extracts have long been used in traditional medicine as antimicrobial agents, as they are composed of biologically active compounds with important health effects. Numerous medicinal plant products have been explored for their antibacterial properties several of these approaches have targeted cariogenic bacteria **(Isabelade *et al*., 2020; Sharaf *et al*., 2021; El-Sherbiny and Mahmoud, 2022, Abdullah *et al*., 2022).** The common uses of *S. aromaticum* have been attributed to some of its biological activities such as antioxidant properties. antibacterial,

antifungal, and insecticidal **(Oluwasina *et al*., 2019)**. The ethanolic of *S. aromaticum* extract showed high antioxidant efficacy in addition to its hepatoprotective on liver damage caused by paracetamol treatment **(Nassar *et al*., 2007)**. Its extract exhibited antibacterial activities against the growth of *S. aureus* and *E. coli* in a concentration range from 50 to 100µg/ml **(Abo El-Maati *et al*., 2016)**. Previously reported, eugenol compound extract from *S. aromaticum* cause lysis to bacterial membranes and inhibits β-lactamase production in *E. coli* **(Ginting *et al*., 2021). Dhamodhar *et el*., (2012)** reported the antibacterial activity of *S. aromaticum* extract against multidrug resistance *S. mutans*. Several studies have been focusing on the use of plant extract against both pathogen bacteria, often responsible for antibiotic-resistant infections in a healthcare setting, and their biofilm formation **(Condò *et al*., 2018).** The methanolic extract of *S. aromaticum* was found to contain β-caryophyllene, caryophyllene oxide, humulene, and oleanolic acid **(Merr and Perry, 2019)**. The major constituent of the *S. aromaticum* is eugenol (83.58%), phenol, 2-methoxy-4-(2-propenyl)-, acetate (12.07%) caryophyllene (4.35%) **(Oluwasina *et al*., 2019)**. The present study aimed to identify the major chemical constituent of *S. aromaticum* showing antibacterial activity against cariogenic bacteria and investigated other biological activity.

MATERIALS AND METHODS

1. Plant material

S. aromaticum was purchased from the local market of El-Minia governorate, Egypt. Buds of *S. aromaticum* were washed with distilled water, allowed to dry at room temperature, and ground to powder using an electrical blender.

2. Extracts of *S. aromaticum*.

Preparation of extract of *S. aromaticum* was carried out by mixing 10 g of *S. aromaticum* powder with 100 ml of petroleum ether (Sigma-Aldrich, St. Louis, USA), and soaking for 24 h. The extract was then filtered using Whatman No.1, and the filtrate was then evaporated in a vacuum evaporator at 50∘ C. The extracts were stored in sterile bottles and kept frozen at −10 ^{o}C until further studies.

3. Antibacterial activity of *S. aromaticum* extract

3. 1. Inoculum of *Streptococcus* species preparation.

Previously isolated *Streptococcus* species and standard strain *Streptococcus mutans* ATCC 25175 were cultivated in (TSB) at 37 ^{o}C to exponential phase. The bacterial growth was evaluated as turbidity using a spectrophotometer at 620 nm and checked every 30 minutes to determine exponential phase. Then, the inoculum density of each bacterial suspension was adjusted to a final density equivalent to 0.5 McFarland Standard (1.5×10^6 CFU/ml) in sterile saline (0.84% NaCl).

3. 2. Antibacterial activity of *S. aromaticum* extract using disc diffusion methods

Antibacterial activity of *S. aromaticum* extract was determined against *Streptococcus* isolates as well as *Streptococcus mutans* ATCC 25175 **(Parekh and Chanda, 2007)**. Briefly, Muller Hinton Agar (MHA) plates were inoculated with 100µl of bacterial suspension (10^6 CFU/ml). Dried paper discs (8 mm) were loaded with 50µl of crude extracts at a concentration of

(10 mg of each one/ml of DMSO). The loaded paper discs were plated on the surface of the inoculated agar plate. penicillin paper disc 10Uwas used as a control antibiotic on the same plates. The loaded plates were incubated at 35 °C for 18 h and the inhibition zone diameter was measured in millimeters (mm). The experiment was performed in three replicates.

3. 3. Determination of minimum inhibitory concentrations (MIC) of *S. aromaticum*.

The MIC values of *S. aromaticum* extract and penicillin (benzylpenicillin potassium salt, HiMedia Laboratories Pvt. Ltd. India.) were determined using microdilution method in a 96-well plate. Muller Hinton broth medium was inoculated with a *Streptococcus* isolates suspension and *Streptococcus mutans* ATCC 25175 (10^6 CFU/ml) and 200 µl of the inoculated medium was distributed in each well. Tested compounds (*S. aromaticum* extract and penicillin) were tested in a twofold serial dilution. *S. aromaticum* extracts was tested at concentrations ranged from 100, 50, 25, 12.5, 6.25, 3.13and 1.56 mg/ml and penicillin started with 20, 10, 5, 2.5, 1.25 and 0.613µg/ml. To experiment was performed according to the criteria of M7-A7 **(CLSI, 2018).** Wells containing negative control (medium + *S. aromaticum* extracts or penicillin at the tested concentrations) were performed to determine the differences in optical density. To plates were incubated for 18 h at 37 °C and the absorbance was measured at 630 nm. MIC was defined as the lowest concentration of the *S. aromaticum* extracts or penicillin which is able to inhibit the visible growth of bacteria.

4. Effect of *S. aromaticum* extracts on biofilm formation.

S. aromaticum extracts will be tested for their potential to inhibit biofilm formation. Two-fold dilutions of *S. aromaticum* extract will be prepared in 96-well microtiter plates containing TSB with glucose (TSBG) under MIC values. Transferred 100µl of bacterial suspensions (5×10^6 CFU/ml, final concentration) into the plate. TSBG containing 0.2% DMSO was used as a negative control. TSBG without the extract is employed as the non-treated well and the medium with extract is used as the blank control. After incubation at 37◦C for 24 h, the effect of the extract on the growth of bacterial species will estimate using the microplate reader at an optical density of 620nm (OD 620 nm) **(Chusri *et al*., 2012).**

5. Antioxidant activity of *S. aromaticum* extract

5. 1. DPPH method

Antioxidant activity of *S. aromaticum* extract was carried out using DPPH (2, 2-diphenyl-1-picrylhydrazyl) method according to **Sharaf *et al*., (2022)** with minor modifications. Various concentrations of *S. aromaticum* extract (1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml, and 7.81 µg/ml) were used to estimate the scavenging of DPPH radicals. Antioxidant activity of standard (ascorbic acid) and extracts was estimated as DPPH scavenging activity (%): [control absorbance - *S. aromaticum* extract absorbance/control absorbance x 100].

5. 2. ABTS method

Estimation of the antioxidant activity of *S. aromaticum* extract with ABTS method (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) was performed according to method used by **(Nicoletta, 1999).**

6. Identification chemicals constitute of *S. persica* extract by GC-MS.

Major compounds present in *S. aromaticum* extract were analyzed, counted, and identified using GC-MS, as described by (**Zothanpuia *et al*., 2017).**

7. Statistical analysis

The data were expressed as the mean ±SE value, which was calculated by using Minitab 18 software extended with a statistical package and Microsoft Excel 365.

RESULTS AND DISCUSSION

1. Antibacterial activity of *S. aromaticum* and MIC.

The crude extract of *S. aromaticum* was screened for its antibacterial potential against cariogenic *Streptococcus* isolates and *Streptococcus mutans* ATCC 25175. Results obtained revealed that *S. aromaticum* extract was active against cariogenic *Streptococcus* isolates and *Streptococcus mutans* ATCC 25175 showed the mean ± SD of growth inhibition zone which ranged from 21±0.66 to 24±0.23mm. Penicillin control antibiotic had no inhibition activity on cariogenic *Streptococcus* isolates, but it showed mean ± SD of growth inhibition zone (24±0.62 mm) against *Streptococcus mutans* ATCC 25175 as shown in (**Table. 1**). Several studies reported the antibacterial activity of *S. aromaticum* extract against different standard bacterial species and bacterial species isolated from clinical samples **(Condò *et al*., 2018; Oluwasina *et al*., 2019; Haro-González *et al*., 2021)**. The MIC values of *S. aromaticum* varied depending on the tested bacteria. The highest MIC was observed with *Streptococcus oralis* and *Streptococcus mutans* (12.50 µg/ml), while the lowest MIC was observed with *Streptococcus parasanguinis,* and standard strain *Streptococcus mutans* ATCC 25175 with MIC values of 6.25 and 5.53 µg/ml respectively as listed in (**Table.2**). A previous study reported MIC values of *S. aromaticum* essential oil against *Streptococcus* spp., *Staphylococcus* spp., *Candida albicans,* and *Rhodotorula* spp with MIC ranging from 0.16 mg/ml and 0.29 mg/ml **(Ebani *et al*., 2021)**. **Mostafa *et al*., (2018)** reported that ethanolic extract of *S. aromaticum* exhibits potential antibacterial activity against food-borne bacteria *S. aureus* and *P. aeruginosa* with MIC ranging from 2.5 to 5.0 mg/ml. Clove oil is listed as a ‘‘generally regarded as safe’’ substance by the FDA when administered at levels not exceeding 1500 ppm in food categories. In addition, the World Health Organization (WHO) Expert Committee on Food Additives has established the acceptable daily human intake of clove oil at 2.5 mg/kg body weight for humans **(Hassanien *et al*., 2014).**

2. Antibiofilm activity of *S. aromaticum* extract.

Biofilm inhibition studies were carried out using petroleum ether extract of *S. aromaticum*. In the present study, it was found that the extract from *S. aromaticum* showed strong activity and prevents the formation of biofilm by all streptococci clinical isolates. The maximum suppression of biofilm formation by *S. aromaticum* extract on bacterial isolates was observed at MIC with a percentage of 78.183%, 74.88, and 72.87 against *Streptococcus parasanguinis, Streptococcus*

oralis, and *Streptococcus mutans*, respectively as shown on (**Fig.1**). Significant biofilm reduction by clove extract at various concentrations has also been reported by **(Chavan *et al*., 2015; Liaqat *et al*., 2016).** These results show that the petroleum ether extract of *S. aromaticum* is effective against biofilm-producing MDR isolates. **Fuqua *et al*., (1994)** reported that phytochemicals of medicinal plants suppress the expression of genes involved in bacterial pathogenicity by interfering with the formation of biofilm. However, plant extracts comprise a large number of components, and it is expected that their mechanisms of action involve several targets in the *S. pyogenes* cells rather than a single mechanism **(Burt, 2004).** Several previous studies have reported that plant extract prevents the various steps of biofilm in different genera of Gram-positive bacteria **(Darsini *et al*., 2015; Mutalib *et al*., 2015).**

Table 1. Antibacterial activity of *S. aromaticum* extracted with petroleum ether

Isolates	**Mean of inhibition zone diameter (mm) ± standard error**	
	***S. aromaticum* extract**	**Penicillin**
Streptococcus oralis	22±0.36	0.0
Streptococcus parasanguinis	24±0.62	0.0
Streptococcus mutans	21±0.66	0.0
Streptococcus mutans ATCC 25175	24±0.23	24±0.62

Table 2. MIC of *S. aromaticum* extracted against *Streptococcus* species

Isolates	**Minimum inhibitory concentration (µg/ml)**	
	***S. aromaticum* extract**	**Penicillin**
Streptococcus oralis	12.50	9
Streptococcus parasanguinis	6.25	8
Streptococcus mutans	12.50	10
Streptococcus mutans ATCC 25175	5.53	0.11

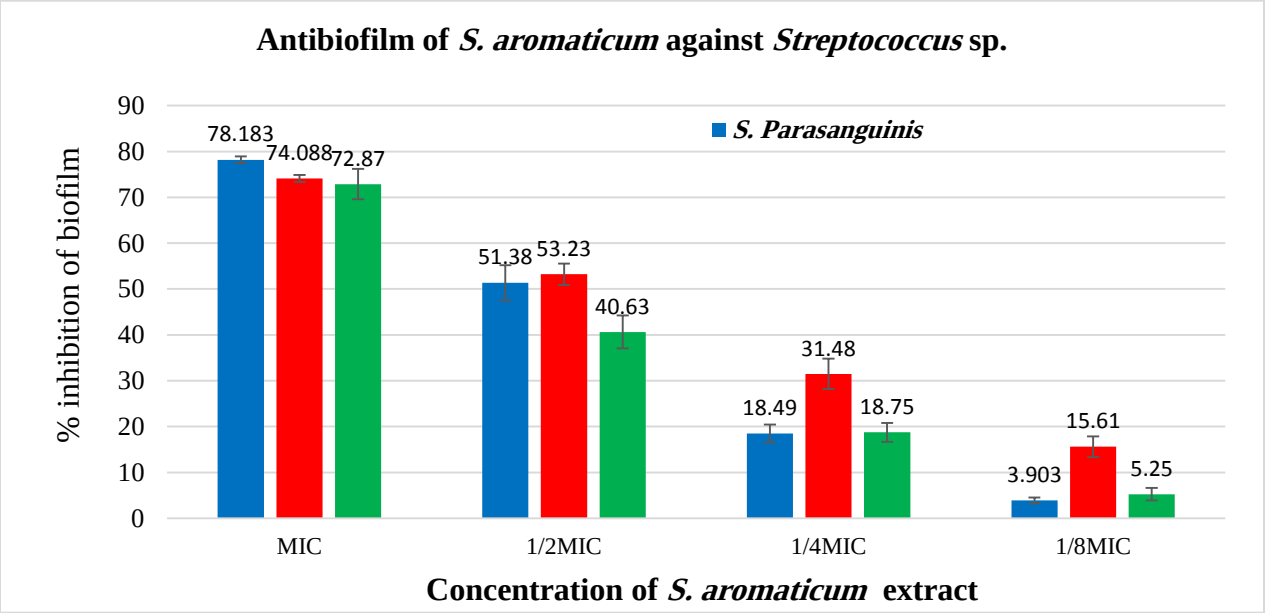

Fig. 1. Antibiofilm activity of *S. aromaticum* extract against *Streptococcus* sp.

3. Antioxidant activity of *S. aromaticum* extract

The antioxidant activity of *S. aromaticum* extract was studied by inhibiting the stable free radical DPPH. In our study, the antioxidant activity of *S. aromaticum* extracts at different concentrations from 1000 to 7.81 µg/ml was performed using ABTS and DPPH assays, as shown in (**Fig. 2A, 2B**). The *S. aromaticum* extract showed DPPH scavenging activity at a higher IC_{50} value of 42µg/ml as compared to standard ascorbic acid (8.2 µg/ml). Standard ascorbic acid was able to scavenge ABTS radical at IC_{50} values of 7.5µg/ml while *S. aromaticum* extract exhibited moderate free radical scavenging activity by ABTS method with IC_{50} values of 46µg/ ml. In the present study, antioxidant activity levels were found to be relatively high in an extract of *S. aromaticum* estimated by ABTS from DPPH methods. Antioxidants are compounds that overcome reactive oxygen species (ROS) which resulted from biological activities as a by-product **(Kurutas, 2016).** In addition, antioxidants are capable of holding and balancing the free radicals which cause several diseases**(Pham-Huy *et al.*, 2008)**. Antioxidants have been considered therapeutic agents where that possess anti-atherosclerotic, anti-inflammatory, antitumor, anticarcinogenic, antimutagenic, and antimicrobial properties. Previous studies confirmed the potential antioxidant activity of *S. aromaticum* **(Gülçin *et al.*, 2012; Chatterjee and Bhattacharjee, 2013; Teles *et al.*, 2021)**. Moreover, **Gülçin *et al.*, (2012)** measured the scavenging of the DPPH radical of clove oil in comparison to some artificial antioxidant agents, alfa-tocopherol, BHT, Trolox, and butylated hydroxyanisole, and they demonstrated that the clove oil antioxidant activity declined as follows: clove oil > BHT > alfa-tocopherol > butylated hydroxyanisole > Trolox. Different in vitro methods including DPPH, oxygen radical absorbance capacity, ferric reducing antioxidant power, 2-deoxyguanosine, 2, 20 -azino-bis (3-

ethylbenzothiazoline-6-sulphonic acid) (ABTS), and xanthine oxidase used to examine the antioxidant activity of aqueous *S. aromaticum* extract. They documented that the potent antioxidant efficacy of aqueous *S. aromaticum* extract may be due to the strong hydrogen donating ability, scavenging of hydrogen peroxide, free radicals, and superoxide and metal chelating ability **(Gülçin *et al.*, 2004).** Antioxidant agents like clove extracts and essential oils play a significant role in treating memory deficits resulting from oxidative stress **(Mehta *et al.*, 2010).**

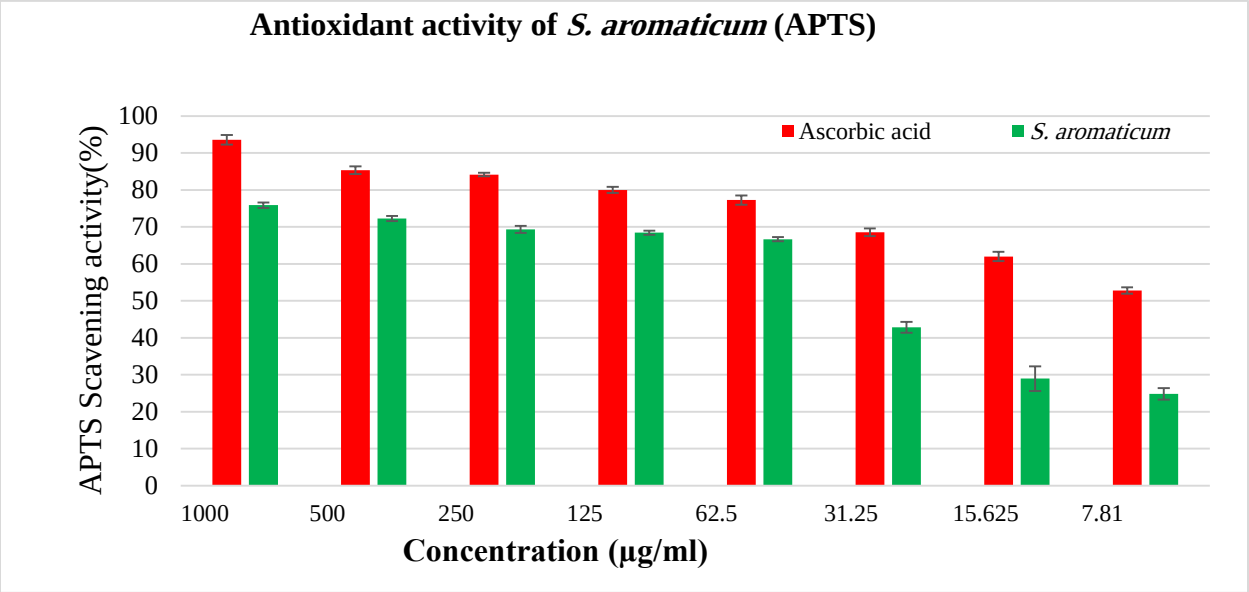

Fig. 2A. Antioxidant activity of *S. aromaticum* extract with (DPPH) methods

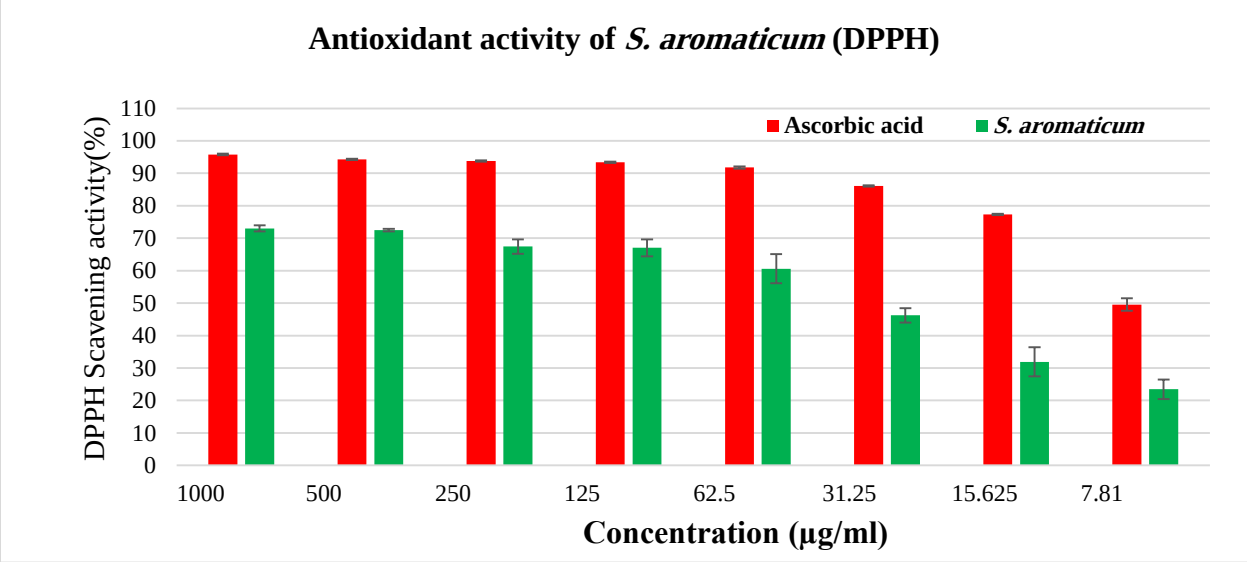

Fig. 2B. Antioxidant activity of *S. aromaticum* extract with (APTS) methods

4. Chemical Composition of *S. aromaticum* extract

The quantitative phytochemical screening of *S. aromaticum* extract using GC-MS revealed that extract contained 28 compounds, highest peak area at 16.22 RT with the presence of the major chemical constituents include on eugenol (21.72%), phenol, 2-methoxy-4-(2-propenyl)-acetate (16.75%), espintanol (10.41%), caryophyllene oxide (9.55%), n-hexadecanoic acid (6.80%), 6-octadecenoic acid (4.56%), isopropyl myristate (3.62%) and oleic acid (2.74%) as shown in (**Table 3**). Eugenol (49.71%) and caryophyllene (18.94%), were detected in the buds oil *S. aromaticum* (**Elekwa *et al*., 2011; Bhuiyan *et al*., 2010)**. Caryophyllene, eugenol, 9,12-octadecadienoic acid (Z, Z)- and octadecanoic acid among other compounds are found in the ethanolic extract of *S. aromaticum* **(Hema *et al*., 2012).**

Table 3. Chemical profile of *S. aromaticum* extract by GC-MS.

P.N	RT	Compound name	Molecular Formula	Contents %
1	16.22	Eugenol	$C_{10}H_{12}O_2$	21.72
2	18.13	Naphthalene	$C_{15}H_{24}$	2.73
3	19.33	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	$C_{12}H_{14}O_3$	16.75
4	19.72	Caryophyllene oxide	$C_{15}H_{24}O$	9.55
5	20.58	Bicyclo[5.1.0]octan-2-one, 4,6-diisopropylidene-8,8-dimethyl-	$C_{16}H_{24}O$	0.99
6	21.23	10,12-Tricosadiynoic acid, methyl ester	$C_{24}H_{40}O_2$	1.74
7	22.15	Espintanol	$C_{12}H_{18}O_3$	10.41
8	22.57	Benzene, (1-pentylheptyl)-	$C_{18}H_{30}$	0.88
9	22.66	Benzene, (1-butyloctyl)-	$C_{18}H_{30}$	0.83
10	22.89	Benzene, (1-propylnonyl)-	$C_{18}H_{30}$	0.74
11	23.30	Benzene, (1-ethyldecyl)-	$C_{18}H_{30}$	0.86
12	24.08	Benzene, (1-methylundecyl)-	$C_{18}H_{30}$	1.34
13	24.59	Isopropyl myristate	$C_{17}H_{34}O_2$	3.62
14	24.91	Methyl octadec-6,9-dien-12-ynoate	$C_{19}H_{30}O_2$	0.92
15	25.34	Benzene, (1-ethylundecyl)-	$C_{19}H_{32}$	0.91
16	26.10	Benzene, (1-methyldodecyl)-	$C_{19}H_{32}$	1.25
17	26.51	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	1.20
18	28.06	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	6.80
19	29.71	methyloctahydro-3a,6-ethanoinden-5 -ol	$C_{15}H_{26}O$	1.19
20	29.84	Phenol, 2-methoxy-5-(1-propenyl)-, (E)-	$C_{10}H_{12}O_2$	0.53
21	30.21	Cyclopropanebutanoic acid,	$C_{25}H_{42}O_2$	0.70
22	30.99	Oleic acid	$C_{18}H_{34}O_2$	2.74
23	31.11	6-Octadecenoic acid	$C_{18}H_{34}O_2$	4.56
24	32.67	Tributyl acetylcitrate	$C_{20}H_{34}O_8$	0.82
25	36.84	Methanone	$C_{19}H_{22}N_2O_3$	0.64
26	37.08	Benzene-1,4-diamine	$C_{16}H_{17}N_5O_6$	0.66
27	37.43	Tofisopam	$C_{22}H_{26}N_2O_4$	0.70
28	40.02	3-Allyl-6-methoxyphenol	$C_{10}H_{12}O_2$	0.90

CONCLUSION

Based on the presented results, we conclude that *S. aromaticum* possesses a powerful antibacterial and antibiofilm activity against resistance viridans group streptococci and has antioxidant activity. It utilizes as a mouth rinse and could therefore be recommended as a prophylactic to preserve from tooth decay and treat sore throats. Future studies are needed to know the mechanism of *S. aromaticum* extract action on bacterial resistance antibiotics.

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