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Antimicrobial activity of *Syzygium aromaticum* L. essential oil on extended-spectrum beta-lactamases-producing *Escherichia coli*

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Abstract

Background: To test the antimicrobial potential of clove essential oil that has been less investigated on antimicrobial-resistant organisms (extended-spectrum β-lactamase-ESBL-producing *Escherichia coli),* we collected 135 ESBL-producing *Escherichia coli* strains given that *E. coli* is the major organism increasingly isolated as a cause of complicated urinary and gastrointestinal tract infections, which remains an important cause of therapy failure with antibiotics for the medical sector.Then, in this study, we evaluated the relationship between the antibacterial potential activity of *Syzygium aromaticum* essential oil (EOSA) and the expression of antibiotic-resistant genes (SHV-2, TEM-20) in plasmidic DNA on ESBL-producing *E. coli* using RT-PCR technique.

Results: EOSA was obtained by hydrodistillation. Using Kirby-Baüer method, we found that EOSA presented a smaller media (mean = 15.59 mm) in comparison with chloramphenicol (mean = 17.73 mm). Thus, there were significant differences ($p < 0.0001$). Furthermore, EOSA had an antibacterial activity, particularly on ECB132 (MIC: 10.0 mg/mL and MBC: 80.0 mg/mL), and a bacteriostatic effect by bactericidal kinetic. We found that the expression of antibiotic-resistant gene blaTEM-20 was 23.52% (4/17 strains) and no expression of blaSHV-2.EOSA presented such as majority compounds (eugenol, caryophyllene) using the GC–MS technique.

Conclusions: Plant essential oils and their active ingredients have potentially high bioactivity against a different target (membranes, cytoplasm, genetic material). In this research, EOSA might become an important adjuvant against urinary and gastrointestinal diseases caused by ESBL-producing *E. coli.*

Keywords: Antimicrobial activity, Essential oil, Herbal medicine, Multidrug resistance, Urinary and gastrointestinal diseases

Background

The importance of extended-spectrum β-lactamase-producing Enterobacteriaceae (especially, *E. coli* and *K. pneumoniae*) lies in the increased human antimicrobial-resistant organisms (AROs). Additionally, they arise as main causes of urinary tract and bloodstream infections in both developed and developing countries (Pitout 2012; Domarackỳ et al. 2007; Pana and Zaoutis 2018).

The extended-spectrum beta-lactamases (ESBLs) are classified as a group of enzymes that cause resistance to the oxyiminocephalosporins (i.e., cefotaxime, ceftazidime, cefepime) and the monobactams (i.e., aztreonam). However, they do not produce resistance to the cephamycins (i.e., cefoxitin, cefotetan) nor to the carbapenems (i.e., imipenem, meropenem, doripenem, ertapenem) (Pitout and Laupland 2008). The majority of ESBLs are classified by Ambler, where the class

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A includes the SHV or TEM types. These types have evolved from parent enzymes such as TEM-1, TEM-2 and SHV-1 (Sawa et al. 2020).

Additionally, a higher prevalence of AROs is troublesome for the medical sector (due to complications such as chemotherapy, surgery) because infections increase patient mortality and morbidity. These issues remain as an important cause of therapy failure with antibiotics and have serious infection-control-related consequences according to the World Health Organization (WHO) (Seale et al. 2017).

The latter suggests other therapeutic options, mainly of natural origin (i.e., mineral, vegetable and animal) that were the main sources of active compounds; however, with the development of organic chemistry, the preference for synthetic products were above those of natural origin (Wright 2019). In this instance, out of the 252 substances that are considered basic and essential, only 11% are of plant origin (World Health Organisation 2001, 2013).

Clove has gained much attention among other spices, due to its antimicrobial and antioxidant activities (Batiha et al. 2020; Shan et al. 2005; Shrivastav et al. 2019) to help alleviating toothache, inflammation and gastrointestinal disorders (El-Shouny et al. 2020; Santin et al. 2011; Oluwasina et al. 2019). Some studies mentioned the antibacterial potential activity of clove. The first study refers that clove's phenolic extract (*Syzygium aromaticum*) has antibacterial activities against the growth of *S. aureus* and *E. coli* in a concentration range of 50–100 µg/mL (El-Maati et al. 2016). The second study mentions that *Syzygium aromaticum* seeds contain eugenol (component with antimicrobial activity) with a minimum inhibitory concentration (MIC) of 0.06 mg/mL, which affect the membrane permeability, and increase oxidative stress enzymes such as catalase and superoxide dismutase (Ajiboye et al. 2016).

The synergistic activity of essential oils (EOs) in combination with antibiotics has beneficial potential that allows to minimize the side effects (Gündüz et al. 2009; Langeveld et al. 2014). This interaction has the potential pro to reduce the concentration of EOs needed to obtain an effective antimicrobial activity and minimize the negative sensory impact (Nazer et al. 2005; Vuuren et al. 2009).

Recent studies demonstrate the antibacterial activity of clove on multidrug-resistant strains. However, multidrug-resistant strains are mostly confirmed with phenotypic screening (Shrivastav et al. 2019; Faujdar et al. 2020) but barely using RT-PCR technique. In this study, we evaluate the relationship between the antibacterial potential activity of EOSA and the expression of antibiotic-resistant genes (SHV-2, TEM-20) in plasmidic DNA on ESBL-producing *E. coli* using RT-PCR technique.

Methods

Plant material

Syzygium aromaticum L. seeds were bought from a grocery store located in the municipality of El Oro de Hidalgo, State of Mexico, with geographical coordinates 19° 48′03 "N 100° 07′53" W in November–December 2017. The botanical authentication of the specimen was performed by M.D. Maria E. Lopez-Villafranco, curator at the IZTA Herbarium, in FES Iztacala of the Autonomous University of Mexico. The specimen was properly labeled with its voucher IZTA 2818.

Preparation of essential oil of *Syzygium aromaticum* L. (EOSA)

Dried flower buds of *S. aromaticum* (236.2 g) were ground with mortar and stood in distilled water approximately for 12 h. The obtention of essential oil was performed using hydrodistillation method (Dominguez 1985). Furthermore, EOSA were dissolved in Müeller Hinton Broth (BD Bioxon) to evaluate antibacterial activity.

Microorganism culture and maintenance

Collection and maintenance

In total, 135 ESBL-producing *E. coli* strains were collected from clinical isolates and identified by culture media, biochemical tests and antibiograms. These strains were identified as ESBL by the automated equipment VITEK ® 2 Compact (BioMerieux, Germany) in the Bacteriology Laboratory of a Health Center in Mexico. ESBL-producing *E. coli* strains were sampled from July to December 2017, and they were kept in Nutritious Agar (Bioxon, State of Mexico, Mexico) and refrigeration under 4° C for their conservation.

Criteria selection of strains

During the first selection, the total of samples ($n=135$) were evaluated using Kirby-Baüer method (i.e., inhibition halos); the samples were analyzed using the statistically significant difference between strains with an inhibition halo greater or equal than 21 mm and control (chloramphenicol), were selected by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluations and using antibiotic-resistant genes by RT-PCR technique. The last selection considered the strains with expression of antibiotic-resistant genes, and the higher concentration of EOSA by CMB was selected by bactericidal kinetic.

Pre-treatment for antibacterial activity

The bacterial inoculum of ESBL-producing *E. coli* strains was incubated in 10 mL of Müeller Hinton Broth overnight at 37 °C. The colonies were adjusted to turbidity comparable to the McFarland standard (1.5×10^8 CFU/mL) using disk diffusion agar method by the Kirby-Baüer (Vanden Berghe et al. 1991; CLSI 2015).

Evaluation of the antibacterial activity of EOSA

To evaluate the antibacterial potential of EOSA, disks of 5 mm diameter (Whatman No. 5) were impregnated with 10 µL of EOSA and 25 µg of chloramphenicol. This component was used as positive control above the microbial pre-treatment suspensions on plates of Müeller Hinton Agar plates (BD Bioxon), which were previously striated using Kirby-Baüer method and methods of Martinez-Elizalde et al. (Vanden Berghe et al. 1991; CLSI 2015; Martinez-Elizalde et al. 2015).

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were estimated using the broth dilution method. The assay tubes of pre-treatment microbial suspension were transferred to fresh tubes in concentration of 10^5 CFU/mL on Müeller Hinton Broth with the concentrations of EOSA at levels between 0.875 and 80 mg/mL. The MIC values were defined as the lowest concentration that prevented visible bacterial growth overnight at 37 °C.

The bactericidal kinetic assay was performed using the appropriate concentrations of EOSA (corresponding to MIC_{50}, MIC and MBC) (Lennette et al. 1981).

Bacteria DNA extraction

The DNA extraction was performed by the alkaline lysis method (Birnboim 1983). The bacteria grew previously in Luria broth overnight, and then 3 mL of bacteria was centrifuged in 2 fractions at 9500 rad/s for 5 min. For this method, three solutions were used: 1) solution I (50 mM glucose, 25 mM Tris–Cl (pH 8.0), 10 mM EDTA (pH 8.0); 2) solution II (0.2 N NaOH, 1% SDS prepared on the same day and added from concentrated solution); and 3) solution III (60 mL of 5 M sodium acetate, 11.5 mL of glacial acetic acid, 28.5 mL of H_2O).

The process of DNA extraction is described below: The first step consisted to resuspend the bacterial bottom in 100 µL of solution I. Secondly, 200 µL of solution II was added to the mixing of previous step by inversion and incubated on ice for 3 min. In the last step, 150 µL of solution III was added, followed by an incubation on ice for 3 min, and a centrifugation at 9500 rad/s for 5 min. The supernatant was removed in a fresh tube, and 420 µL of chloroform-phenol-isoamyl alcohol was added, mixing both by inversion. Then, the mix was centrifuged at 9500 rad/s for 5 min. The aqueous phase (upper) was separated in a new tube, and 1 mL of absolute ethanol was added. The supernatant was discarded, and the pellet was washed with cold 70% ethanol, allowing to dry at room temperature for 5 min and resuspending in 50 µL of sterile deionized H_2O.

Detection of bacteria resistance genes

A RT-PCR was done to detect antibiotic-resistant genes, and 5 µL of the supernatant obtained in the extraction of DNA was used with two pairs of oligonucleotides (IDT): blaTEM-20 (F: GAT AAC ACT GCG GCC AAC TT and R: TTG CCG GGA AGC TAG AGT AA) and blaSHV-2 (F: CTT TCC CAT GAT GAG CAC CT and R: CGC TGT TAT CGC TCA TGG TA) in a concentration of 12.5 uM. The RT-PCRs (Techne-TC-512) were evaluated using the kit of Master Mix PCR M7502 (Promega) (Araujo Díaz and Salas Asencios 2008).

For the TEM-20 gene, the PCR steps are described next: a denaturation of 5 min at 97 °C, 30 cycles of 1 min at 94 °C, followed by a banding during 1 min at 56 °C (except for the blaSHV gene that requires 54 °C for banding). After that, an extension for 1 min at 72 °C is followed by 10 min at 72 °C of final extension. The PCR products were visualized on 1.5% agarose gels (Merck, Germany), in 1X TEB with ethidium bromide staining. Through this process, a 5X colorless molecular weight marker buffer of 100–2000 bp DNA was used (Bioline, USA) (Araujo Díaz and Salas Asencios 2008).

Analysis of the Chemical Composition by GC–MS

Then, 1µL of EOSA was injected into the equipment. A 6850 Network GC System chromatograph from Agilent Technologies coupled to a 5975C VL MSD mass spectrometer with a triple-axis detector, of the same brand, equipped with an RTX capillary column 30 m long, 0.25 mm internal diameter and 0.25 µm thick (dimethylpolysiloxane (95%) and phenyl polysiloxane (5%)).

The injection mode used was a Split, with radius 80:1 and running flow 37 cm/seg. The temperature of the oven was programmed as follows: initial temperature of 70 °C for 2 min, after which, it was increased at a rate of 8 °C/min until reaching 270 °C and then at 10 °C/min during 6 min. The total time was 75 min, and the higher temperature used was 290 °C. The mass spectra were obtained by electronic impact with ionization energy of 70 electron volts (eV) and detected in a mass range from 35 to 600 m/z. Helium was used as the entraining gas, while the identification of individual components in EOSA was made by GC–MS.

Separated compounds were identified using the chemical library and the database of the NIST library and the PubMed Compound of the National Center of Biotechnology Information (NCBI) (NIST 2016; US National Library of Medicine 2017).

Statistical analysis

All experiments were done three times; however, inhibition halos experiments were done under a completely randomized design, and they were expressed as the mean ± standard deviation (mm). Inhibition halos data were studied using the factorial analysis of variance (ANOVA) with a Tukey–Kramer multiple comparison test ($P < 0.05$) using the R-project Core-Team (2017).

Results

EOSA had a yield of 6.35% and a density of 1.16 g / mL.

Evaluation of the antibacterial activity of EOSA

Using Kirby-Baüer method, we found that EOSA presented a smaller media (mean = 15.59 mm), in comparison with chloramphenicol (mean = 17.73 mm). Thus, there were significant differences ($F_{0.05,1,134} = 371.02$, $p < 0.0001$). Furthermore, we found 18 samples with statistically significant differences. They showed an inhibition halo similar to control (chloramphenicol) that were selected by MIC, MBC evaluation and expression of antibiotic-resistant genes by RT-PCR technique (Table 1).

By dilution method, we found that strain 132 had the lowest MIC and the higher MBC (10.0 mg/mL and 80.0 mg/mL), respectively. Then, this strain was selected by bactericidal kinetic, and we found that EOSA had bacteriostatic effect (Fig. 1).

The concentrations were: 1/2 MIC: 5 mg/mL; MIC: 10 mg/mL; MBC: 80 mg/mL. CFU: colony-forming unit; hours (h); control (chloramphenicol); MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration. For kinetic curve the times measured were 0, 2, 4, 6, 8, 12 h with a decrease of bacteria except for MBC at 6 h until 24 h an increasing of bacteria survival in comparison with control.

Table 1 Antibacterial activity of EOSA

Bacteria ESBL-*E. coli*	Inhibition Halos ± S.D *S. aromaticum*	MIC	MBC	Inhibition Halos ± S.D Control +
ECB39	21.67 ± 0.58	20.00	40.00	28.33 ± 2.89
ECB41	25.00 ± 0.00	20.00	> 40.00	26.33 ± 1.15
ECB57	21.33 ± 1.15	20.00	40.00	21.33 ± 1.53
ECB74	27.67 ± 2.52	20.00	40.00	21.00 ± 1.00
ECB80	21.33 ± 1.15	20.00	40.00	22.67 ± 2.52
ECB86	21.00 ± 1.00	20.00	40.00	20.33 ± 2.52
ECB96	22.66 ± 0.58	20.00	40.00	6.66 ± 0.58
ECB118	25.00 ± 0.00	20.00	40.00	24.33 ± 1.15
ECB122	22.00 ± 0.00	10.00	40.00	7.00 ± 0.00
ECB123	21.00 ± 1.73	20.00	40.00	13.33 ± 0.58
ECB125	22.33 ± 0.58	20.00	> 40.00	22.00 ± 1.00
ECB126	21.33 ± 1.15	20.00	40.00	7.00 ± 0.00
ECB129	22.66 ± 0.58	20.00	40.00	24.33 ± 2.31
ECB130	26.66 ± 1.53	20.00	40.00	17.33 ± 0.58
ECB132	24.33 ± 1.15	10.00	80.00	22.33 ± 4.04
ECB133	21.00 ± 0.00	40.00	> 40.00	21.00 ± 1.00
ECB134	25.00 ± 0.00	20.00	40.00	19.33 ± 1.15

Positive control: (chloramphenicol); inhibition halos: mean data and standard deviation (S.D) (mm), MIC: minimum inhibitory concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL)

Detection of bacteria resistance genes

Using plasmid DNA extraction and RT-PCR technique, we found that ESBL-*E. coli* strains had the expression of antibiotic-resistant gene blaTEM-20 in a 23.52% (4/17 strains) and no expression of blaSHV-2 (Table 2).

Analysis of the chemical composition by GC–MS

GC–MS analysis revealed the presence of two majority terpene compounds (eugenol and caryophyllene) in EOSA (Fig. 2).

The majority compounds of EOSA are eugenol (77.322%, Tr = 8.728) and caryophyllene (16.77% Tr = 9.305).

Discussion

We found that EOSA had an antibacterial activity on ESBL-producing *E. coli*; particularly, ECB132 had a bacteriostatic effect until 12 h. In the same way, this antibacterial activity of *S. aromaticum* (i.e., ethanolic, methanolic extracts) has been observed on strains ATCC such as *E. coli, P. aeruginosa, S. aureus* (Ajiboye et al. 2016) and a bacteriostatic effect on *K. pnemoniae* (Lawal 2019). Therefore, the clove mechanism of action has been described altering the permeability of the membrane, causing oxidative stress enzymes (i.e., catalase and superoxide dismutase) (Ajiboye et al. 2016). For instance, we believe that of a single dose some metabolites of *S. aromaticum* were gradually released over time until their completely released. Otherwise, a single dose in combination or sequential antimicrobial treatments (Paterson et al. 2016) to daily dose, could produce multidrug-resistant bacteria caused by MIC variation on the time, thus considered the antimicrobial dosing in critical change to prevent the bacterial resistance (Kim et al. 2014). Alternatively, natural products need a encapsulation (i.e., biomolecular capping in green synthesis process nanoparticles)

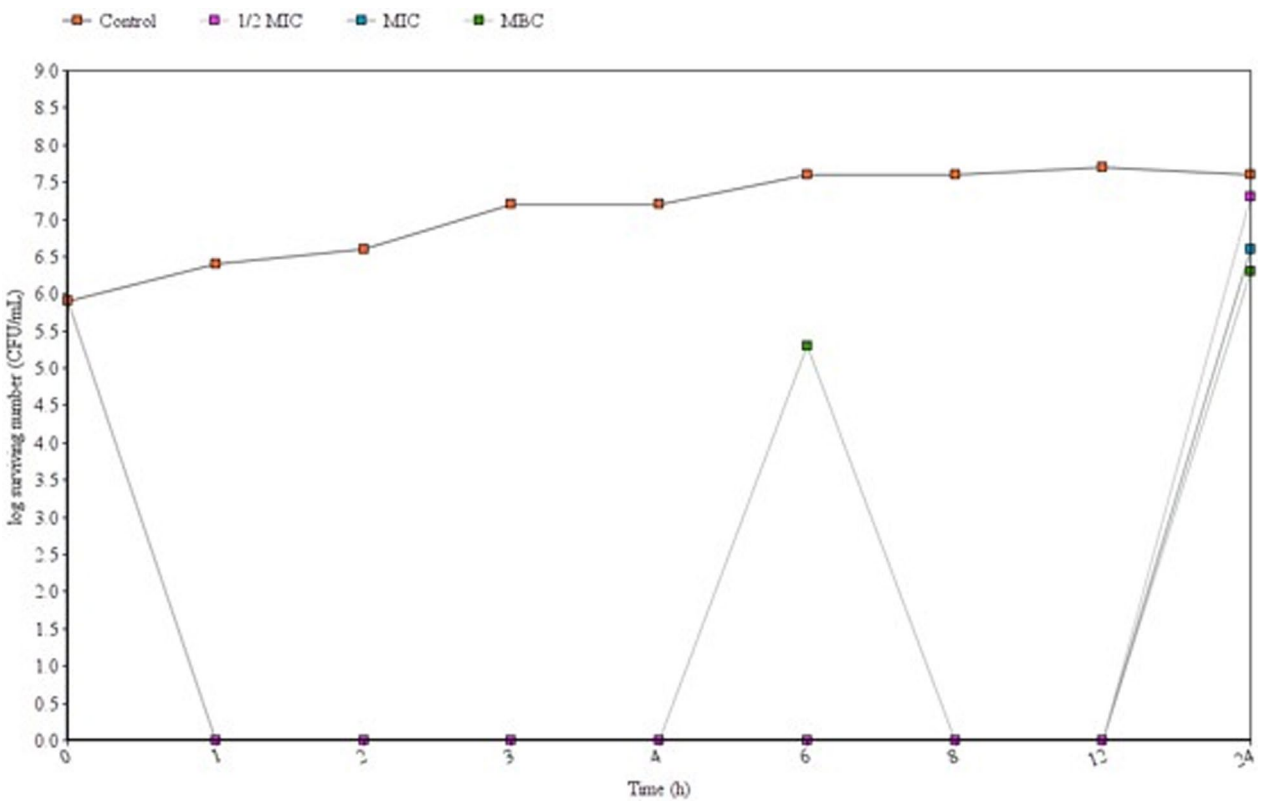

Fig. 1 Bactericidal kinetic of ESBL-*E. coli* (ECB132) exposed to EOSA. The concentrations were: 1/2 MIC: 5 mg/mL; MIC: 10 mg/mL; MBC: 80 mg/mL. CFU: colony-forming unit; hours (h); control (chloramphenicol); MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration. For kinetic curve the times measured were 0, 2, 4, 6, 8, 12 h with a decrease of bacteria except for MBC at 6 h until 24 h an increasing of bacteria survival in comparison with control

Table 2 Antibiotic-resistant gene blaTEM-20 expression in ESBL-*E. coli*

Gene/strain	ECB41	ECB132	ECB86	ECB126
TEM-20 (225 bp)				

ECB: ESBL-*E. coli* strain. 23.52% (4/17 strains) have antibiotic-resistant gene blaTEM-20 expression in plasmidic DNA, not blaSHV-2

to prevent the compounds volatilization and reduce the toxicity and concentration (Roy et al. 2019).

On the other hand, our EOSA had a yield of 6.35%. Yield difference can be influenced by the harvesting season, the geographical origin (Nana et al. 2015) and extraction method that involves the particle size of ground cloves on the yield (Guan et al. 2007).

The EOSA studied in this research contains eugenol and caryophyllene as major compounds, and these we believe that had the antimicrobial activity on ESBL-producing *E. coli*. Most of the earlier studies reported inhibitory potential of herbal compounds against antibiotic-resistant ATCC strains, and there are not enough studies with antibiotic resistance clinical strains and their gene expression. Similarly, the antibacterial activity of clove compounds is well known: 1) eugenol on ESBL-QR *E. coli* (quinolone resistant) has demonstrated a synergistic interaction with cefotaxime, and ciprofloxacin (MIC = 0.91–7.34 μg/mL), and shrinkage of cell surfaces with reduced cytoplasm among in others ESBL-QR strains (Dhara and Tripathi 2020). And 2) caryophyllene as β-caryophyllene was able to alter membrane permeability and integrity of *B. cereus*, leading to membrane damage and intracellular content leakage, which eventually caused cell death (MIC = 2.5% –v/v–) (Moo et al. 2020).

ESBL-producing *E. coli* clinical strains, evaluated in this study, had the expression of antibiotic-resistant gene blaTEM-20 in a 23.52% (4/17 strains) and no expression of blaSHV-2. In this sense, the incidence of antibiotic-resistant genes in ESBL-*E. coli* in the health sector is increasingly concerning, for example, 84% blaSHV and 85% blaTEM were found in a Hospital in Guatemala (Herrera García et al. 2019). Furthermore, from

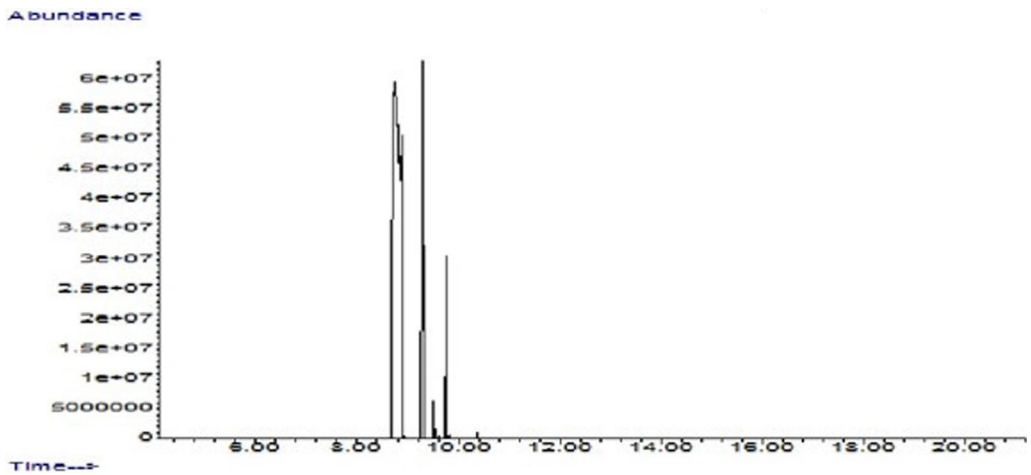

Fig. 2 GC mass chromatogram of EOSA. The majority compounds of EOSA are eugenol (77.322%, Tr = 8.728) and caryophyllene (16.77% Tr = 9.305)

outpatients in Tabriz, Iran could be carriers of ESBL-*E. coli* with resistant genes, 2% of blaSHV and 4% of blaTEM (Dizage 2019). However, the combination between natural compounds and antibiotics (i.e., eugenol-cefotaxime) represents an important management to suppress the synthesis at transcript level in antibiotic-resistant genes (i.e., acrB) (Dhara and Tripathi 2020).

Therefore, EOSA could be a potential such as adjuvant to combat certain infections caused by multidrug-resistant bacteria (i.e., ESBL-*E. coli*).

Conclusions

In our study the EOSA was obtained by hydrodistillation and had an antimicrobial activity in ESBL-producing *E. coli* strains, particularly a bacteriostatic effect. The 23.52% of these strains expressed antimicrobial-resistant gene (blaTEM-20) in plasmid DNA until 12 h of a single dose. Therefore, EOSA could be a potential adjuvant, mainly to combat certain infections related to ESBL-producing *E. coli*. Then, the perspectives to the future researches could be to demonstrate and elucidate the mechanism of action for the different secondary metabolites. The latter will allow to innovate new therapeutic alternatives as adjuvants, to investigate the effect of diverse cells or microorganisms, and their possible suppression of antibiotic-resistant genes.

Abbreviations

ANOVA: Analysis of variance; AROs: Antimicrobial-resistant organisms; ATCC: American Type Culture Collection; DNA: Deoxyribonucleic acid; ECBx: Number of extended-spectrum β-lactamase-producing strain isolated; EOs: Essential oils; EOSA: *Syzygium aromaticum* Essential oil; ESBL: Extended-spectrum β-lactamase-producing strain; FES: Facultad de Estudios Superiores; GC–MS: Gas chromatography–mass spectrometry; IDT: Integrated DNA Technologies; MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration; n: Total number of extended-spectrum β-lactamase-producing strain isolated for experiment; NIST/NCBI: PubMed Compound of the National Center of Biotechnology Information; QR: Quinolone resistant; RT-PCR: Reverse transcription polymerase chain; SD: Standard deviation; SHV-2: TEM-20; arcB: Antibiotic-resistant genes; Tr: Time retention; WHO: World Health Organization.

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Authors' contributions

The authors equally contributed in all the article parts. The authors wrote, read and approved the final manuscript.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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