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Research Article

ESSENTIAL OILS HAVE GREAT POTENTIAL IN MOUNTING AN OXIDATIVE STRESS RESPONSE IN STAPHYLOXANTHIN PRODUCING *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

S. aureus is an etiological agent that possesses a myriad array of virulence factors (VFs). Staphyloxanthin (STX) is one such VF present in *S. aureus* that confers protection against reactive oxygen species produced by host defense and allows the bacteria to persist in the inflammation site. These properties make STX an attractive target for antivirulence therapy, as demonstrated for some plant-derived compounds. Essential oils (EOs) are invigorating and rational approaches to combat the complications arising from multidrug-resistant STX producing *S. aureus* (SPSA). The main objective of this study is to derive novel inferences to effectively determine EOs as antistaphylococcal agents suppressing STX pigment production and activating oxidative stress response in SPSA. From the EOs library, a total of 16 EOs were studied against SPSA isolates, among them, cinnamon (3.125 µL/mL), clove (3.125 µL/mL)), thyme (12.5 µL/mL)), ginger (12.5 µL/mL)), tea tree (12.5 µL/mL)), tulsi (25 µL/mL)), peppermint (25 µL/mL)) and eucalyptus (25 µL/mL)) EO successfully arrests SPSA isolates for inhibiting the production of STX. The inhibitory effects of EOs towards STX production confirmed that *S. aureus* grown in the presence of EOs showed increased susceptibility to H_2O_2 and singlet oxygen. Consequently, the survival ability of the EOs treated organism decreased in freshly isolated human whole blood due to less carotenoid pigment to act as an antioxidant scavenger. Furthermore, the statistical analysis of the results established EOs as a significant protection against SPSA where normal probability test, linear correlation, ANOVA, and two sample T-test successfully analyzed at significance α=0.05. Thus the present findings reveal the potential of EOs as an alternative antivirulence candidate to control *S. aureus* pathogenicity, which may give a new lifeline in this post-antibiotic era.

Keywords: Staphyloxanthin, Essential Oils, Oxidative Stress, Antivirulence, Statistical Analysis.

1. INTRODUCTION

The overuse and misuse of antibiotics have resulted in different drug concentrations in humans, animals, and the environment [1]. In this sense, bacteria are frequently exposed to a subinhibitory concentration of minimum inhibitory concentration (sub-MIC) of antimicrobial agents, and this condition can lead to mutagenesis and the release of microbial virulence factor (VF) [2,3]. *S. aureus* is one such etiological agent that possesses a myriad array of VFs that stands vital in *S. aureus* infection and manipulates the host cell to assist in its survival [4]. These VFs inhibit complement activation, prevent neutrophil function, and recruitment and inhibition of phagocyte function [5, 6].

Staphyloxanthin (STX) is a major VF in *S. aureus* that confers protection against Reactive Oxygen Species (ROS) produced by host defense and allows the bacteria to persist in the inflammation site. It exhibits a complex combination of virulence and resistance genes and is associated with increased morbidity and mortality, and represents a serious concern to global systems worldwide [7]. As per some studies, the STX biosynthesis pathway gets involved in virulence and mainly serves as an antioxidant protecting the bacterium from neutrophil oxidative burst [8]. In addition to the antioxidant properties of STX pigments, it can also alter membrane rigidity, which is important in protecting against non-oxidative host defenses mediated by cationic peptides [9]. The STX pigment contributes to staphylococcal fitness, inhibition of STX biosynthesis is viewed as a potential therapeutic target in treating *S. aureus* infections [10, 11]. The exact influence of STX deficiency on pathogen-host interactions has been extensively studied and indicated that the improved immune response and improved

clinical course of *S. aureus* infections are associated with decreased STX synthesis [12]. These data justify the search for new alternatives to treat *S. aureus* infections targeting STX biosynthesis, and herbal products have been highlighted as attractive candidates [13].

Plant-derived phytochemicals essential oils (EOs) are major secondary metabolites showing antioxidant, antifungal, anticancer, antiviral, insecticidal, anti-inflammatory, and antimicrobial properties [14]. Similarly, several EOs have been shown to be effective antimicrobial agents capable of inhibiting various multidrug resistance (MDR) strains and enhancing antibiotic activity [15,16]. In addition, several researchers have also revealed that EOs can act as immune enhancers. EOs possess a phenolic group containing bioactive components, which increases antioxidative properties and can interact with several bacterial targets such as membranes, protein synthesis, efflux pumps inhibition, and virulence-related pathways such as biofilm formation and toxin production [17,18]. Thus this study focused on deriving novel inferences to effectively determine EOs as antistaphylococcal agents suppressing STX pigment production and activating oxidative stress response in STX producing *S. aureus* (SPSA)

2. MATERIAL AND METHODS

2.1. Antimicrobial agents and chemicals

All microbial media and antibiotic discs were purchased from Hi-Media Laboratory Mumbai, India. Also, cinnamon, tulsi, lemongrass, ginger, tea tree, sunflower seed, peppermint, orange, thyme, clove, lemon, eucalyptus, sesame, castor, linseed, and mustard EO were collected based on their availability and antimicrobial property.

2.2. Bacterial strains and growth conditions

135 clinical samples (pus, blood, and urine) were collected from different hospitals and pathology laboratories from the Chandrapur region. The samples were consecutive and collected on transport media swabs and brought to the microbiology laboratory within 24 h. On receipt, the swabs were inoculated onto Trypton Soya Broth (TSB) and incubated for 18 h at 37°C. The broth cultures were then sub-cultured on Baird Parker Agar (BPA) and Mannitol Salt Agar (MSA) (Hi-Media, India) used for selective isolation of *S. aureus*. All *S. aureus* colonies were screened for STX production by transferring them on Nutrient Agar Media (NA) and incubated for 24 h at 37°C and at room temperature for 24 h to get pigmented colonies [19].

2.3. The antimicrobial activity of EOs

The antimicrobial activity of 16 different EOs were determined against the standard microbial strains of *S. aureus* (ATCC 6538, ATCC 25923, and ATCC 25913) and clinical SPSA isolates (n=44) by disc diffusion method (DDM). The MIC and minimum bactericidal concentration (MBC) of the EOs were carried out, and MIC_{50} and MIC_{90} and MBC_{50} and MBC_{90} were determined accordingly by the National Committee for Clinical Laboratory Standards [20]. Experiments were performed in duplicate.

2.4. Effect of EOs on STX production

The effect of EOs on STX production was estimated by qualitative and quantitative assay [21]. The SPSA isolates (n=25) suspension were incubated with a sub-inhibitory concentration of EOs (n=8) (½ and ¼ MIC) at 37°C for 24 h. These overnight cultures were centrifuged at 5000 rpm for 10 min, and supernatant and pellets were separated. The separated pellets were suspended with 1ml of phosphate-buffered saline (PBS) and recentrifuged at 5000 rpm for 10 min; this process was repeated 2-3 times. The bacterial pigmented pellets were visualized and recorded.

In quantitative estimation, total carotenoid pigments in each pellet were extracted using ethanol (0.2 ml). All pellets were suspended in ethanol and incubated at 40°C for 30 min. The ethanol phase and cell debris were separated by centrifugation (5000 rpm for 10 min). The process was repeated for complete carotenoid extraction. Next, the ethanol was allowed to evaporate, and the final dried pigment was suspended in ethyl acetate, and the absorbance was determined at 462 nm [22].

2.5. Oxidative stress response of EOs

The oxidative susceptibility test and whole blood killing assay were carried out to determine the oxidative stress response of EOs on SPSA isolates [21, 22].

2.5.1. Oxidative susceptibility test

Microbial suspension prepared from overnight cultures of SPSA (n=25) was incubated with a subinhibitory concentration of EOs (⅛, ¼, ½, 1X, and 2X MIC). Each culture received H_2O_2 to reach a final concentration of 1.5% (v/v) for 1 h and were incubated at 37°C. The viable bacterial cell count was enumerated on the Muller Hinton Agar (MHA) plate. A modified method was performed for the singlet oxygen assay described elsewhere [10, 21]. In brief, EOs-treated and untreated SPSA were prepared and incubated in 96-well plates in

the presence of 10 µg/mL methylene blue at 37°C for 1hr. The plates were placed exactly 20 cm from a 100w light source. The samples were collected at 0, 30, and 60 min intervals, cultured on MHA, and enumerated viable cells.

2.5.2. Whole blood killing assay

The microbial suspension was prepared as per the oxidative susceptibility test. The cell pellets were separated and washed with PBS, adjusted to an inoculum 10^{-4} CFU, and mixed with 75µL of freshly drawn whole human blood in a heparinized tube. The suspension was then incubated at 37°C for 4 h, and viable cells were then counted using MHA agar plate.

2.6. Statistical analysis

All assays were performed in triplicate in at least two independent experiments. Statistical analyses were performed using the MiniTab 2017.

3. RESULTS

3.1. Identification of SPSA isolates

A total of 80 *S. aureus* isolates were isolated from clinical samples (pus, urine, and blood). All these isolates were studied for STX pigment production, and noted 44 isolates of *S. aureus* were SPSA isolates.

3.2. EOs enhances the activity of drugs toward SPSA

A total of 16 EOs were studied against SPSA that showed a highly variable susceptible pattern (Fig.1). In addition, the antioxidant activity of EOs was studied, in which cinnamon, clove, thyme, tea tree, tulsi, ginger, peppermint, and eucalyptus EO displayed potent antioxidants activity than lemon, orange, lemongrass, linseed, castor, mustard, sunflower, and sesame EO. Accordingly, EOs antioxidant activity and sensitivity were compared, and noted that EOs with good anti-oxidant properties displayed good inhibitory activity (Table 1). The EOs showed a good correlation with antioxidant activity, taken for MIC and MBC study (cinnamon, clove, thyme, tulsi, tea tree, ginger, peppermint, and eucalyptus EO). Similarly, the EOs whose sensitivity pattern was negatively correlated (n= 3) and the sensitivity pattern showing only a 5% correlation (n=5) with antioxidant activity were rejected for further study.

Among the eight EOs, MIC_{50} and MIC_{90} value of cinnamon EO 3.125 µL/mL and 6.25 µL/mL concentration respectively demonstrated excellent antistaphylococcal activity. The resistance gradually decreased with clove, thyme, tea trees, ginger, tulsi, peppermint, and eucalyptus EO. The MIC and MBC of cinnamon, clove, thyme, and peppermint EOs were similar, whereas tulsi, ginger, tea tree, and eucalyptus EO were different.

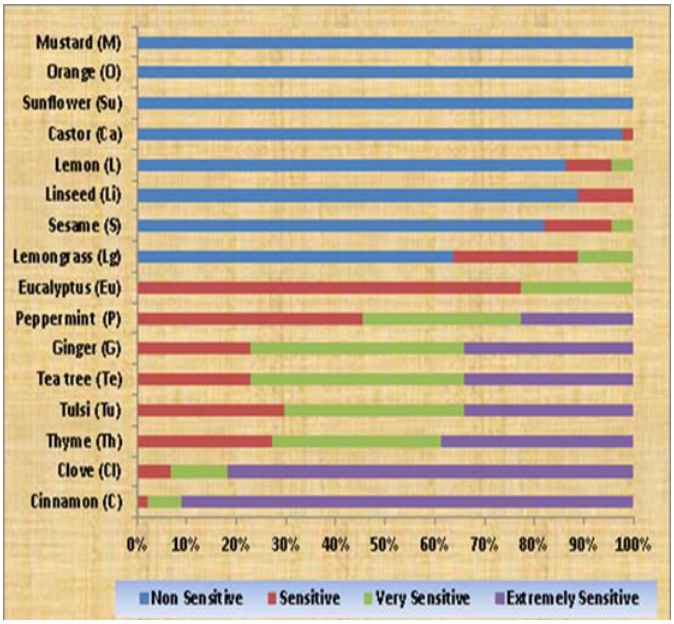

Fig. 1: Sensitivity Pattern of EOs against SPSA isolates

Table 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			$p>0.05^{*}$	

3.3. Effect of EOs on STX production

The qualitative and quantitative assays were performed to determine the effects of EOs on STX production. It was noted that EOs treated SPSA cells produced less pigment than untreated SPSA. The STX pigment biosynthesis from EOs treated SPSA isolates was not measured at 462 nm, indicating that STX-biosynthesis in SPSA isolates was inhibited at a subinhibitory concentration of EOs. A one-way ANOVA test analysis verified the significance value ($p<0.05$) of STX

biosynthesis from EOs treated SPSA at a subinhibitory concentration at significance level α=0.05, which indicated that selected EOs successfully inhibited STX biosynthesis in SPSA isolates. In addition, the normal probability plot of the STX biosynthesis at a subinhibitory concentration of EOs (½ MIC and ¼ MIC) was plotted in the ANOVA test, indicating the observed data is significant (Fig.2).

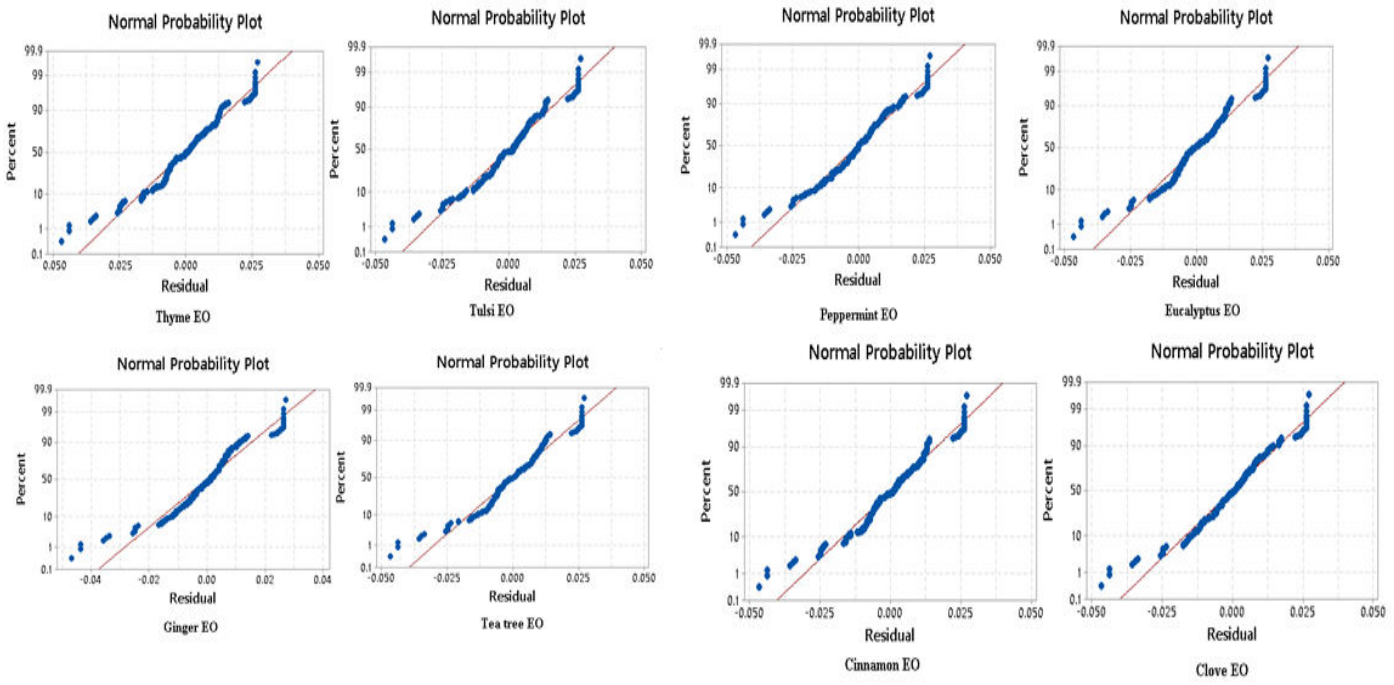

Fig. 2: Normal probability plot of cinnamon, clove, thyme, ginger, peppermint, tea tree, tulsi, and eucalyptus EO

3.4. Oxidative stress response

Next, we sought to determine whether the EOs-treated SPSA reduced the survival ability in the oxidative stress response by oxidant susceptibility assays and whole blood killing assay, *in vitro* and *ex vivo*, respectively.

3.4.1. Oxidant susceptibility assays

SPSA isolates were exposed with subinhibitory concentrations of selected EOs for 18 h. The treated cells were collected, followed by incubation with H_2O_2. The susceptibility of EOs-treated cells compared with untreated cells to oxidants was studied *in vitro*. The results indicated that the normal untreated cells of the pathogen survived better than those of the treated organisms in H_2O_2. After incubation with EOs at 2X MIC, 1XMIC, ½ MIC, and ¼ MIC of cinnamon, clove, and thyme EO, SPSA with reduced pigmentation were more susceptible to oxidative killing. In contrast, pathogens treated with EOs at ⅛ MIC and DMSO-treated SPSA were able to survive under the H_2O_2 conditions tested. Similarly, after incubation with EOs at 2X MIC, 1XMIC and ½ MIC, of tea tree, ginger, tulsi, peppermint, and eucalyptus EO resulting SPSA with reduced pigmentation were more susceptible to oxidative killing, whereas EOs at ¼ MIC, ⅛ MIC, and DMSO-treated SPSA were able to survival under the H_2O_2 conditions tested (Fig.3).

A further experiment tested the viability of EOs-treated SPSA after incubation with singlet oxygen. Samples were collected at various time intervals, and viable SPSA was enumerated. The bacterial population of 1% DMSO-treated SPSA after exposure to 10 µg/mL methylene blue for 1 h remained unchanged (Fig. 4). In contrast, EOs-treated SPSA cells were less able to survive under this extreme condition. When SPSA treated with EOs at the MIC was tested for its survival ability after exposure to methylene blue for 30-60 min, the bacterial population decreased by at least 2 logs. EOs-treated SPSA at subinhibitory concentrations also failed to grow under singlet oxygen conditions.

3.4.2. Whole blood killing assay

This experiment investigated the killing of EOs-treated SPSA by human whole blood. The results showed that

the number of 1% DMSO-treated culture cells did not change. In contrast, increased susceptibility of the EOs-treated SPSA to killing by human whole blood was observed. After incubation at subinhibitory concentrations of EOs tested, the resulting pathogen with reduced pigmentation was less able to survive in freshly isolated human whole blood, as the organisms contained less carotenoid pigment to act as an antioxidant scavenger (Fig. 5). Our *in vitro* and *ex vivo* results suggested that STX pigment is necessary and sufficient to promote oxidative stress resistance, offering a novel target for antibiotic therapy.

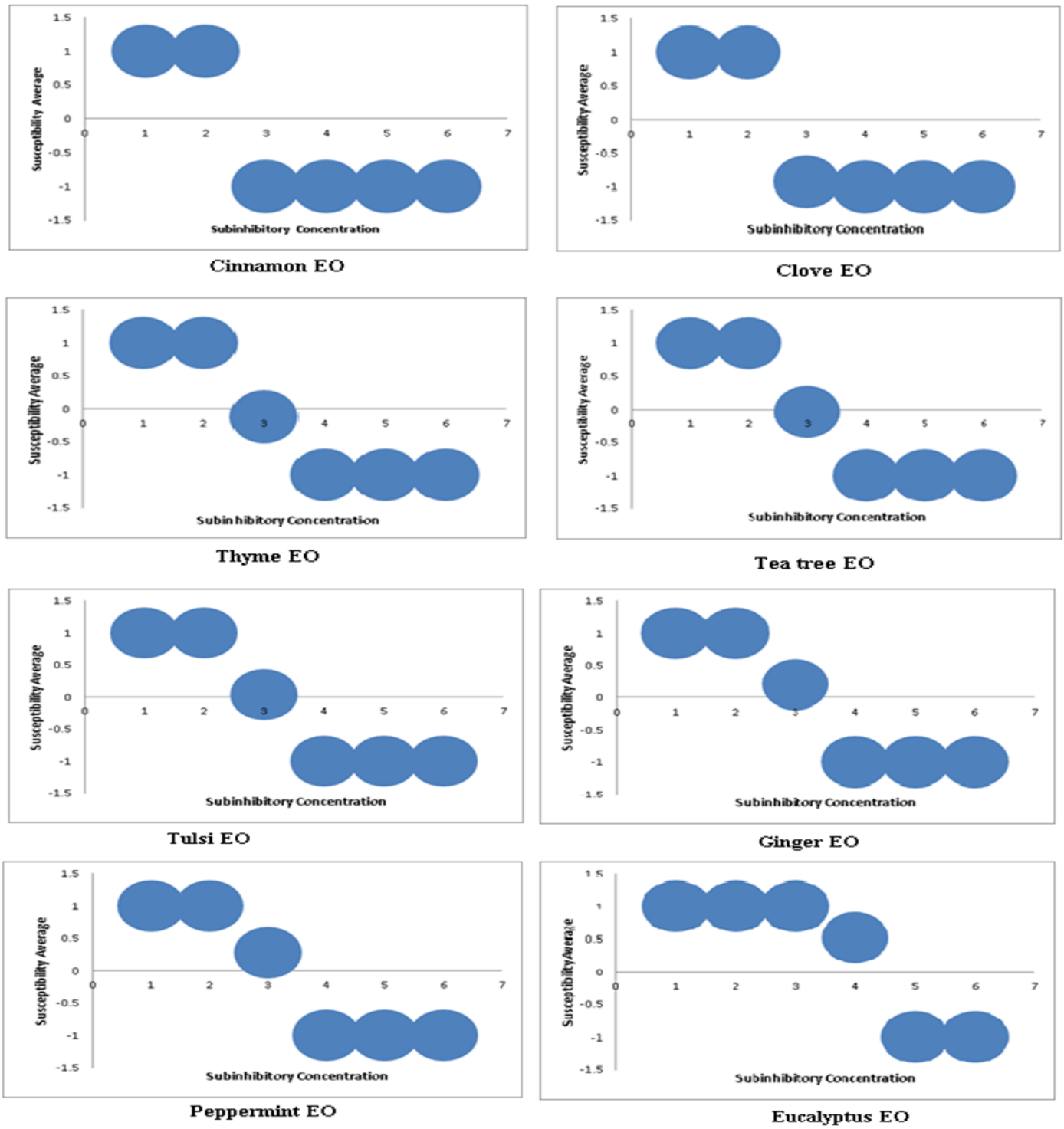

Fig. 3: Survival ability of SPSA isolates after treatment with a sub-inhibitory concentration of EOs in H_2O_2

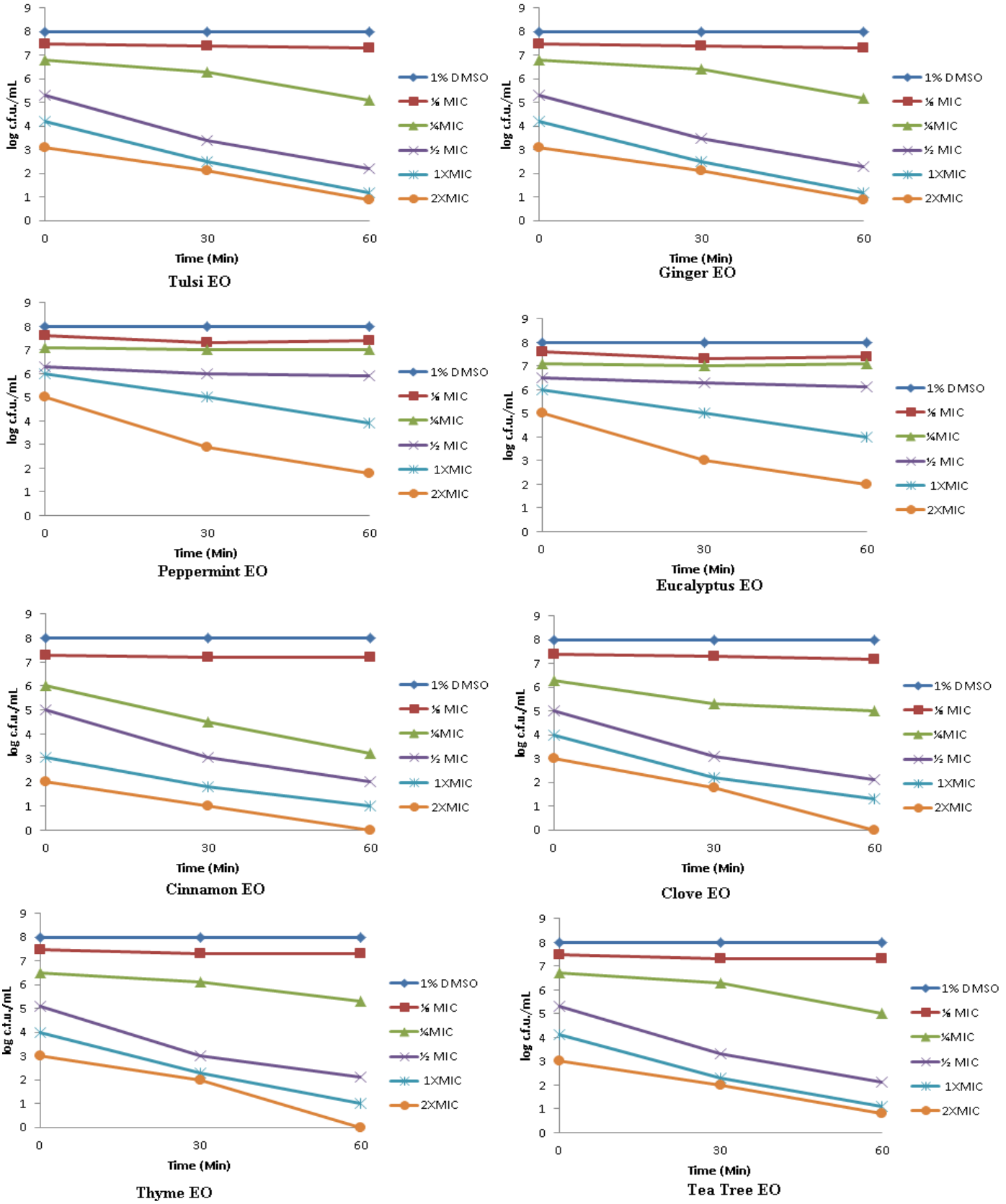

Fig. 4: Survival of SPSA isolates treated with a sub-inhibitory concentration of EOs (2X MIC, 1XMIC, ½MIC, ¼MIC, ⅛MIC and 1%DMSO) after incubation with 10 µg/mL methylene blue for 0, 30 and 60 min.

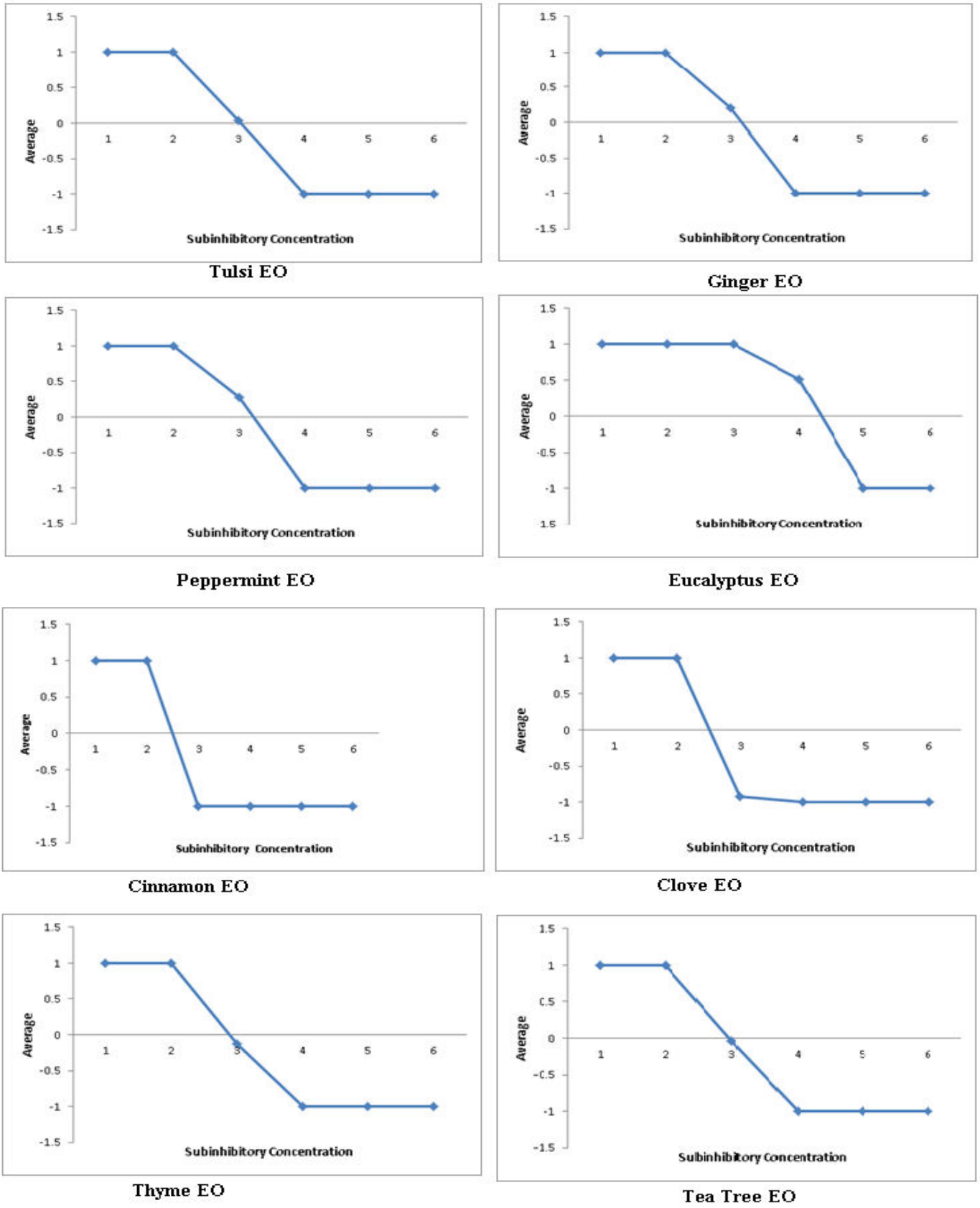

Fig. 5: Survival ability of SPSA isolates after treatment with a sub-inhibitory concentration of EOs in Whole blood

4. DISCUSSION

STX, known as a golden carotenoid pigment distributed in *S. aureus*, the structure of which contains numerous conjugated double bonds to resist the ROS produced by neutrophils and macro-phages, has been deemed an important VF with antioxidant properties [8,10]. Therefore, blocking the STX biosynthetic pathway is an effective therapeutic strategy for complicated *S. aureus* infections [21, 23].

Plant-derived compounds such as EOs can potentially inhibit the STX biosynthesis in SPSA due to the immunomodulatory activity [24]. Thus EOs can be used as an alternative to treat infectious or immune diseases. In the present study total of 16 EOs have been studied against SPSA isolates. Among them, eight EOs such as cinnamon, clove, thyme, ginger, tea tree, tulsi, peppermint, and eucalyptus have successfully arrested SPSA isolates growth. The blocking of STX biosynthesis in SPSA isolates was studied by performing the qualitative and quantitative assay.

The result noted that EOs treated SPSA were unable to produce STX pigment compared to untreated SPSA at MIC concentration of EOs. Moreover, a subinhibitory concentration of EOs able to block the STX biosynthesis pathway. The present result was concerned with Leejae et al. [21] they reported that rhodomyrton had been inactivated the STX biosynthesis pathway by blocking the CrtN enzymes. The present study can conclude that EOs could block the CrtN enzyme activity, resulting from the pigmented cell to non-pigmented SPSA isolates.

According to many researchers, STX could protect *S. aureus* from oxidative stress by ROS, and non-pigmented *S. aureus* was vulnerable to immune clearance [25, 26]. A hydrogen peroxide (H_2O_2) assay and human whole blood killing assay were frequently used methods to imitate ROS *in vitro* [27]. In the present study, oxidant susceptibility assay and whole blood killing assay were used to imitate ROS *in vitro* to verify the efficiency of EOs. As shown in Fig.3, blocking SPSA pigment formation led to an increase in the susceptibility of the pathogen to H_2O_2 killing. Moreover, the treated organism was killed more efficiently by singlet oxygen compared with untreated cells (Fig.4). Next, investigate the innate immune clearance of pigment after incubation with EOs using an *ex vivo* assay system-human whole blood survival. Untreated cells of SPSA survive better than the EOs treated cells in freshly isolated human whole blood in a heparinized tube (Fig.5). In addition, a previous report of the scanning electron microscope (SEM) and transmission electron microscope (TEM) showed a physical and morphological effect of EOs, confirmed the oxidative stress response on SPSA isolates [28].

The inhibition of SPSA carotenogenesis made the organism more susceptible to killing by 1.5% H_2O_2 and decreased human whole blood survival [10]. Moreover, non-pigmented *S. aureus* cells were more sensitive to innate immune clearance in the mouse infection model. According to Gappu et al.[29] the non-pigmented ΔcrtM mutant accumulated intracellular ROS, whereas pigmented cells are ROS quencher. However, ROS accumulation can damage biological molecules such as DNA, amino acids, and protein. These phenomena strongly indicate pigmented strains have some advantages over non-pigmented strains.

5. CONCLUSION

The overall study concludes that cinnamon, clove, thyme, ginger, tea tree, tulsi, peppermint, and eucalyptus EO are promising antivirulence drugs that successfully mount an oxidative stress response in SPSA. These EOs are effective immune enhancers that seemed to be a new lifeline in the post-antibiotic era. Furthermore, the exact mechanism of bioactive compounds of EOs, to activate an oxidative stress response in SPSA has to study. Also, the combination study of EOs bioactive components from the same EO and different EO and their mechanism must be checked.

Conflict of interest

There is no conflict of interest.

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