

# Mechanism of Action of Essential Oils and their Major Components

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## Abstract

The essential oil of lemongrass, palm rosa and eucalyptus were found to be good antimicrobial agents. To a large extent the results suggest their potential use as chemotherapeutic agents, food preserving agents, and disinfectants. However before considering these compounds as chemotherapeutic agents against human/animal diseases, it is important to study their cytotoxic and mutagenic effects. Studies were then carried to investigate the probable mechanism by which these compounds act against Gram negative (*E. coli*) and Gram-positive (*Staphylococcus aureus*) bacteria. The leakage of potassium ions from the cell suspension of bacteria and change in absorption maxima in presence of the test compounds was monitored. The results indicate that, in presence of crude essential oils the leakage of bacterial cellular material was higher than that showed in presence of the individual major components of essential oils, which is due their ability to disrupt the permeability barrier of microbial membrane structures, although the presence of additional mechanisms or targets cannot be ruled out.

**Key words:** Plant essential oils, anti bacterial, anti fungal, potassium leakage, absorption maxima

## INTRODUCTION

Laboratories across the world have found literally thousands of phytochemicals, which have inhibitory effects on all types of microorganisms in vitro. It is well known that various types of secondary metabolites produced by plants are responsible for the biological activities of these phytochemicals [1]. Though much is known about the chemistry and the antimicrobial activity of several phytochemicals, very few reports are available on the possible mechanism of action. The bioactive compounds isolated from plants are substances whose chemical structures are widely different, with only rare exceptions, from those of the antibiotics derived from bacteria, actinomycetes, fungi, etc. [2, 3]. Most of the studies on phytochemicals and their antimicrobial activity are not followed by investigations on the mechanism of action of these compounds. This is regrettable, because the antimicrobial agents isolated from higher plants may act as regulators of intermediary metabolism by activating or blocking an enzyme reaction, removing or neutralizing an inhibitor influencing nutrient uptake from the medium, acting as a depressor of or otherwise affecting enzyme synthesis at nuclear or ribosomal level, changing membrane structures or substituting a limiting factor in intermediary metabolism [4, 5]. In this context it is desirable that the possible mode of action of these phytochemicals is studied. However, of late we could see an increase in the number of reports studying some aspects of mode of action of phytochemicals.

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Most of the plant secondary metabolites with antibacterial activity are lipophilic compounds. Lipophilic compounds were reported to act predominantly by dissipating the pH gradient across the cytoplasmic membrane. Food preservatives such as lactic acid, benzoic acids and some other lipophilic drugs were shown to act on the cytoplasmic membrane by disturbing hydrophobic interaction between the lipids and proteins [6, 7]. The cytoplasmic membranes of bacteria provide a barrier to the passage of small ions such as  $H^+$ ,  $K^+$ ,  $Na^+$  and allow cells to control the entry and exit of different compounds. This permeability barrier role of cell membranes is integral to many cellular functions, including the maintenance of the energy status of the cell, other membrane coupled energy-transducing processes, solute transport, regulation of metabolism and control of turgor pressure [8, 9, 10]. Hence any compound which damages the cytoplasmic membrane of bacteria, will have bactericidal activity. For phenols and phenolic compounds an injury of membrane functions has been proposed as a mechanism of action [11, 12]. The observation by two different groups [13, 14] that the toxicity of phenols correlates well with hydrophobicity indicates cell membrane, which is rich in lipid content, as the possible main target of phenolic antimicrobial agents. Independent research reports by Hugo & Bloomfield and Lee et al., [15, 16, 17] have shown that fenitrothion which is toxic to bacteria shows an increase in permeability of cytoplasmic membrane to protons with a consequent dissipation of the proton motive force (PMF) and an uncoupling of oxidative phosphorylation. There is a close relationship between the leakage of  $UV_{260}$  absorbing material by bacteria and the bactericidal activity of fenitrothion. In general, the mode of action by which essential oils, which are lipophilic in nature, act seems to involve the cytoplasmic membrane of bacteria [18]. However, enzymes and DNA of the bacteria have also been mentioned as possible targets [19].

Knobloch et al., [20.] have studied more than 40 terpenoids isolated from essential oils and investigated their possible influence on the reaction mechanisms of primary energy metabolism, in particular NADH and succinate dehydrogenase activities, membrane-bound respiratory electron flow and oxidative phosphorylation [21, 22]. All reactions investigated were inhibited by terpenoids, with thymol and carvacrol being the most effective. Studies by Ultee et al., Cristani et al., and Nazzaro et al., [23, 24, 25] with liposome model systems confirmed that cyclic terpenes accumulate in the membrane, affecting membrane integrity and resulting in the dissipation of proton motive force which is in accordance with the earlier report by Sikkema et al., [26].

Studies by Cox et al., and Nazzaro et al., [25, 27] on tea tree oil, which is found to contain cyclic monoterpenes, showed that the antibacterial activity of this oil is related to its ability to disrupt the permeability barrier of cell membrane structures of bacteria, and the accompanying loss of chemiosmotic control.

Some of the studies showed that Gram-negative bacteria are less sensitive to lipophilic compounds than Gram-positive bacteria [28]. Studies by Sikkema et al., [26] indicated that the higher tolerance of Gram-negative bacteria to lipophilic compounds is related to the resistance shown by the outer membrane of Gram-negative bacteria to these molecules.

In light of the above knowledge, it was proposed to investigate the effect of these compounds on cell permeability of Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. We have selected the essential oils lemon grass; palm rosa, eucalyptus and major components citral, geraniol and citronellal for the present studies. Studies were undertaken to monitor the response of *E. coli* and *Staphylococcus aureus* against these compounds in terms of permeability control i.e., leakage of potassium ions and  $UV_{260}$  and  $UV_{280}$  absorbing substances from the cell suspension in presence of the test compounds.

## MATERIALS

Chemicals (analytical grade) were purchased from M/s Qualigens and Hi Media laboratories India Ltd.

**Test compounds:** Three essential oils have been used in the present investigation viz- lemongrass oil, palm rosa oil, eucalyptus oil. These were obtained by steam distillation at CIMAP, Boduppal, Hyderabad. The major components used in the present investigation viz. Citral, Citronellal and Geraniol were obtained from M/s Sigma Chemicals.

## METHODS

### Potassium Efflux

Preparation of Bacterial cell suspension: *E. coli* and *S. aureus* were grown overnight in nutrient broth at 37°C with shaking at 120 rpm. Bacterial cells were then harvested and washed once with 10mM EDTA and then twice with distilled water by centrifuging each time at 6000 rpm for 15 minutes at 4°C, and resuspended such that the absorbance of the final suspension was 2.0 at A<sub>450</sub>.

The cell suspension was incubated for half an hour at room temperature and the potassium concentration in the cell free supernatant, obtained after centrifuging the cell suspension at 6000 rpm for 15 minutes, was determined by flame photometry. This served as control.

### Effect of Essential Oils and Major Components on Potassium Efflux

The cell suspension was prepared as described above. After incubation for half an hour at room temperature, the test compounds viz. lemon grass, palmrosa, eucalyptus and major components citral, geraniol and citronellal were added at MBC values to the cell suspension separately. At regular intervals of 15 minutes, aliquots of the samples were drawn and extra cellular potassium ion levels were measured as described above.

### Leakage of UV<sub>260</sub> And UV<sub>280</sub> Absorbing Material

The method of Heipieper et al., [29] was followed to determine the leakage of UV<sub>260</sub> absorbing material. The cell suspension was prepared as described above and the absorbance of the cell free supernatant was determined at 260nm using Spectronic UV-spectrophotometer. This served as control for the leakage studies of UV<sub>260</sub> absorbing material.

The absorbance at 280nm was also measured and this served as control for the leakage studies of UV<sub>280</sub> absorbing material.

### Effect of Essential Oils and Major Components on Leakage of UV<sub>260</sub> and UV<sub>280</sub> Absorbing Material

The procedure followed was similar to the measurement of potassium ion efflux. Samples treated with test compounds viz. lemon grass, palm rosa, eucalyptus and major components citral, geraniol and citronellal at MBC values were drawn at regular intervals and the A<sub>260</sub> and A<sub>280</sub> of the supernatant obtained after centrifugation was measured.

## RESULTS

The effect of essential oils of lemongrass, palmrosa, eucalyptus and major components citral, geraniol and citronellal on the membrane permeability of *E. coli* and *S. aureus* cells was studied at their minimum bactericidal concentration in terms of leakage of potassium ions, and UV 260 and UV280 absorbing material.

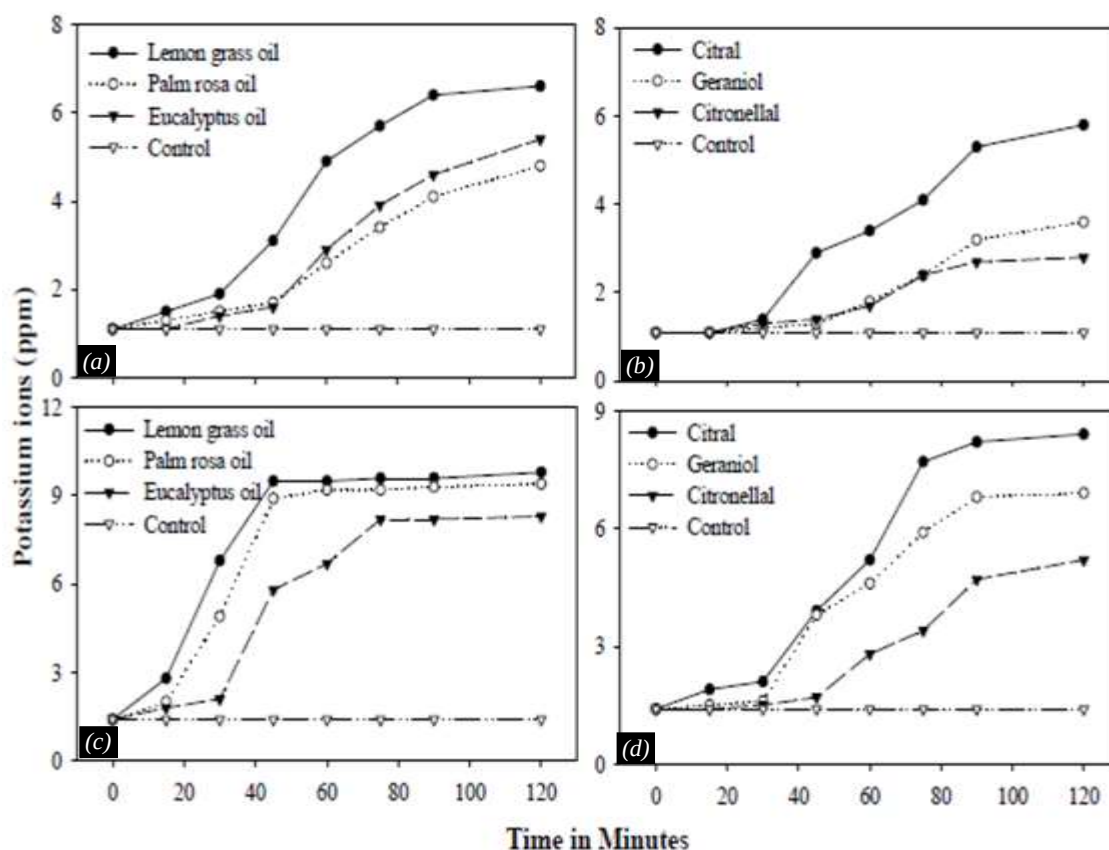
### Efflux of Potassium Ions

*Escherichia coli* and *S. aureus* cells were treated with the test compounds at minimum bactericidal concentration and the extracellular potassium ion concentration was measured both in the presence and absence of test compounds at regular time intervals. In the absence of test compounds, the extracellular concentration of K<sup>+</sup> ions of *E. coli* and *S. aureus* cell suspension was 1.1 ppm and 1.4 ppm respectively.

The *E. coli* cell suspension when treated with test compounds viz. lemon grass oil, palm rosa oil, eucalyptus oil, citral, geraniol and citronellal at their minimum bactericidal concentration showed an increase in the extracellular concentration of potassium ions compared to the control which implicates increased cell permeability in the presence of test compounds. The results are presented in Figure 1a and 1b.

In the presence of lemon grass oil, leakage of potassium ions from *E. coli* cell suspension could be seen after 30 minutes of incubation. In the presence of palm rosa oil and eucalyptus oil, the leakage could be seen after 45 minutes of incubation (Figure 1a). Citral, the major component of lemon grass oil induced leakage of  $K^+$  ions from the *E. coli* cell suspension after 30 minutes. Citronellal and geraniol induced leakage of  $K^+$  ions after 60 minutes, and the leakage of  $K^+$  ions in presence of these two compounds is low compared to the other test compounds (Figure 1b).

Among the test compounds, lemongrass oil displayed high activity in terms of inducing  $K^+$  ion leakage from *E. coli* cell suspension. In the presence of lemongrass oil efflux of potassium ions from *E. coli* cell suspension could be observed from 30 minutes of incubation and after 120 minutes of incubation the concentration of extracellular  $K^+$  ions was 6.6ppm. In the presence of palm rosa oil and eucalyptus oil, after 120 minutes of incubation the extra cellular  $K^+$  ions was 4.8ppm and 5.4ppm respectively (Figure 1a). Similarly in presence of citral, geraniol and citronellal, after 120 minutes of incubation the extra cellular  $K^+$  ion concentration was 5.8ppm, 2.8ppm and 3.6 ppm respectively (Figure 1b).



**Figure 1.** Efflux of potassium ions from the cell suspension of *E. coli* and *S. aureus* treated with essential oils and their major compounds, (a, b) Efflux of potassium ions from the cell suspension of *E. coli* treated with lemon grass, palm rosa, eucalyptus oils and citral, geraniol, citronellal, respectively, (c, d) Efflux of potassium ions from the cell suspension of *S. aureus* treated with lemon grass, palm rosa, eucalyptus oils and citral, geraniol, citronellal, respectively.

In case of *S. aureus* cell suspension, all the test compounds induced a high leakage of potassium ions. The leakage was induced after 15 minutes of incubation in presence of lemon grass oil and palm rosa oil, and after 30 minutes of incubation in presence of Eucalyptus oil (Figure 1c). In the presence of citral an increase in leakage could be seen from 30 minutes. In the presence of geraniol also an increase in leakage could be observed from 30 minutes of incubation, while in presence of citronellal an increase in leakage could be observed after 45 minutes of incubation (Figure 1d). In the presence of lemongrass oil the extracellular  $K^+$  concentration after 120 minutes of incubation was 9.8ppm. In the presence of palm rosa oil and eucalyptus oil, the extracellular  $K^+$  concentration after 120 minutes of incubation was 9.4ppm and 8.3ppm respectively (Figure 1c). Similarly in the presence of citral, geraniol and citronellal, the extracellular  $K^+$  concentration after 120 minutes of incubation was 8.4 ppm, 6.9 ppm and 5.2 ppm, respectively (Figure 1 d).

#### **Leakage of UV<sub>260</sub> and UV<sub>280</sub> – Absorbing material**

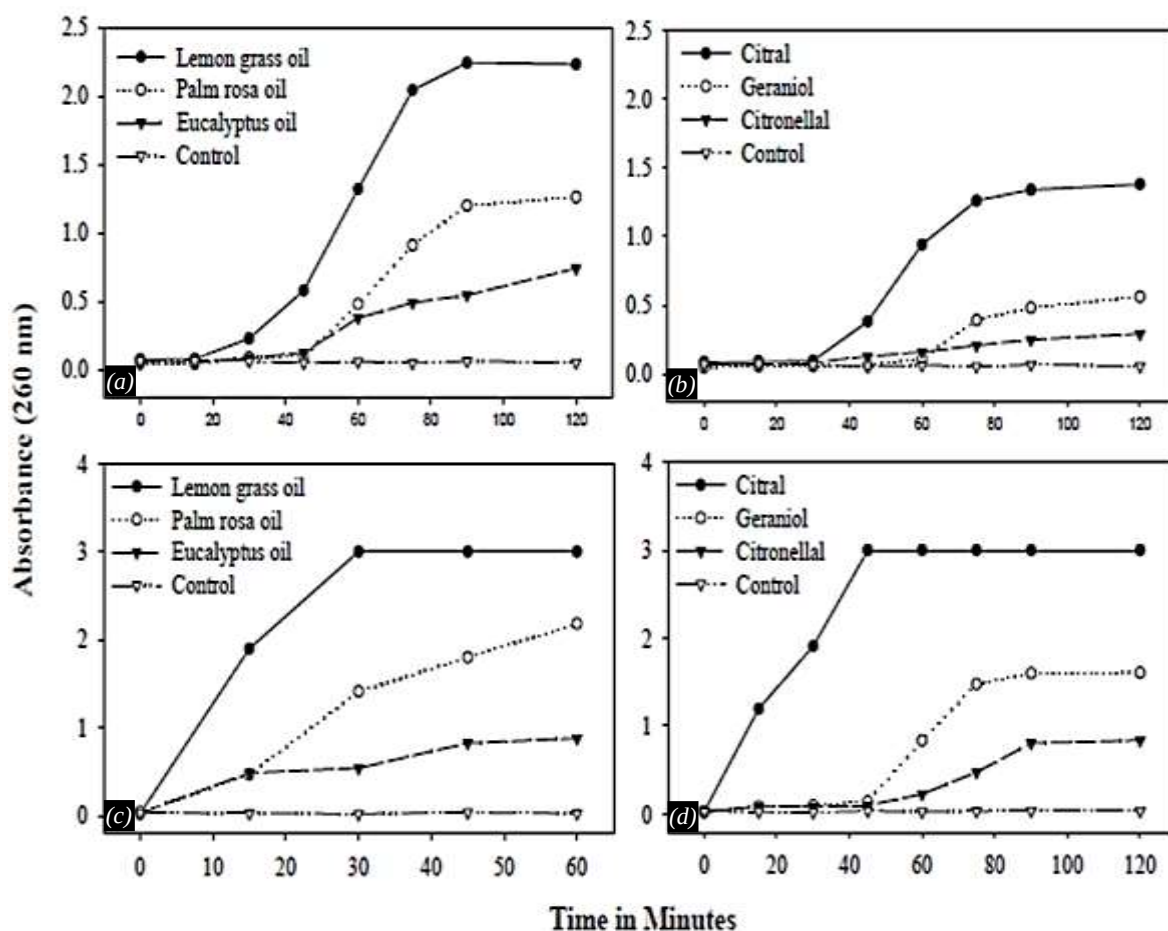
The leakage of UV<sub>260</sub> absorbing material (mainly nucleic acid material) was monitored in presence of test compounds against *E. coli* and *S. aureus*. In the absence of test compounds the OD of UV<sub>260</sub> absorbing material was between 0.012 to 0.064 for *E. coli* cell suspension, and 0.013 to 0.053 for *S. aureus* cell suspension.

The cell suspension of *E. coli* in the presence of test compounds showed an increase in absorbance of extra cellular UV<sub>260</sub> material giving an indication of membrane leakage. Lemongrass oil was the most effective compound in terms of inducing leakage of UV<sub>260</sub> absorbing material. In the presence of lemon grass oil there was an increase in the UV<sub>260</sub> absorbing material from 30 minutes of incubation, and a maximum absorbance of 2.24 was recorded after 90 minutes of incubation. Palm rosa oil induced leakage of UV<sub>260</sub> absorbing material from 45 minutes of incubation, and a maximum absorbance of 1.26 was recorded after 120 minutes of incubation. Similarly eucalyptus oil induced leakage of UV<sub>260</sub> absorbing material after 45 minutes of incubation reaching a maximum of 0.74 OD after 120 minutes (Figure 2a). Among major components citral showed highest activity. In the presence of citral leakage of UV<sub>260</sub> material started after 30 minutes of incubation, with a maximum absorbance of 1.38 recorded after 120 minutes of incubation. Geraniol induced leakage of UV<sub>260</sub> absorbing material after 60 minutes with a maximum of 0.56 OD recorded after 120 minutes. In the presence of citronellal leakage of UV<sub>260</sub> absorbing material was slow and steady. The leakage of UV<sub>260</sub> absorbing material in presence of citronellal reached a maximum of 0.289 OD after 120 minutes. Thus, citronellal induced very low leakage of UV<sub>260</sub> material from the cell suspension of *E. coli* (Figure 2b).

The cell suspension of *S. aureus* was very sensitive to all the test compounds in terms of leakage of UV<sub>260</sub> absorbing material. In the presence of lemon grass oil the leakage of UV<sub>260</sub> absorbing material started immediately and a maximum absorbance of 3.0 was recorded after 30 minutes of incubation indicating the quick action of lemon grass oil (Fig 2c). Palm rosa oil induced the leakage of UV<sub>260</sub> absorbing material after 15 minutes of incubation and a maximum absorbance of 2.18 was recorded after 60 minutes of incubation. Eucalyptus oil induced the leakage from 15 minutes of incubation with a maximum absorbance of 0.882 recorded after 60 minutes of incubation. The results show that these three essential oils are very quick in their action against *S. aureus* (Figure 2c). In presence of the major component citral, leakage of UV<sub>260</sub> absorbing material could be seen immediately and a maximum absorbance of 3.0 was recorded after 45 minutes of incubation. In presence of geraniol an increase in the leakage could be observed from 45 minutes reaching a maximum absorbance of 1.61 after 120 minutes. In presence of citronellal, increase in the leakage could be observed from 45 minutes with a maximum absorbance of 0.84 recorded after 120 minutes of incubation (Figure 2d).

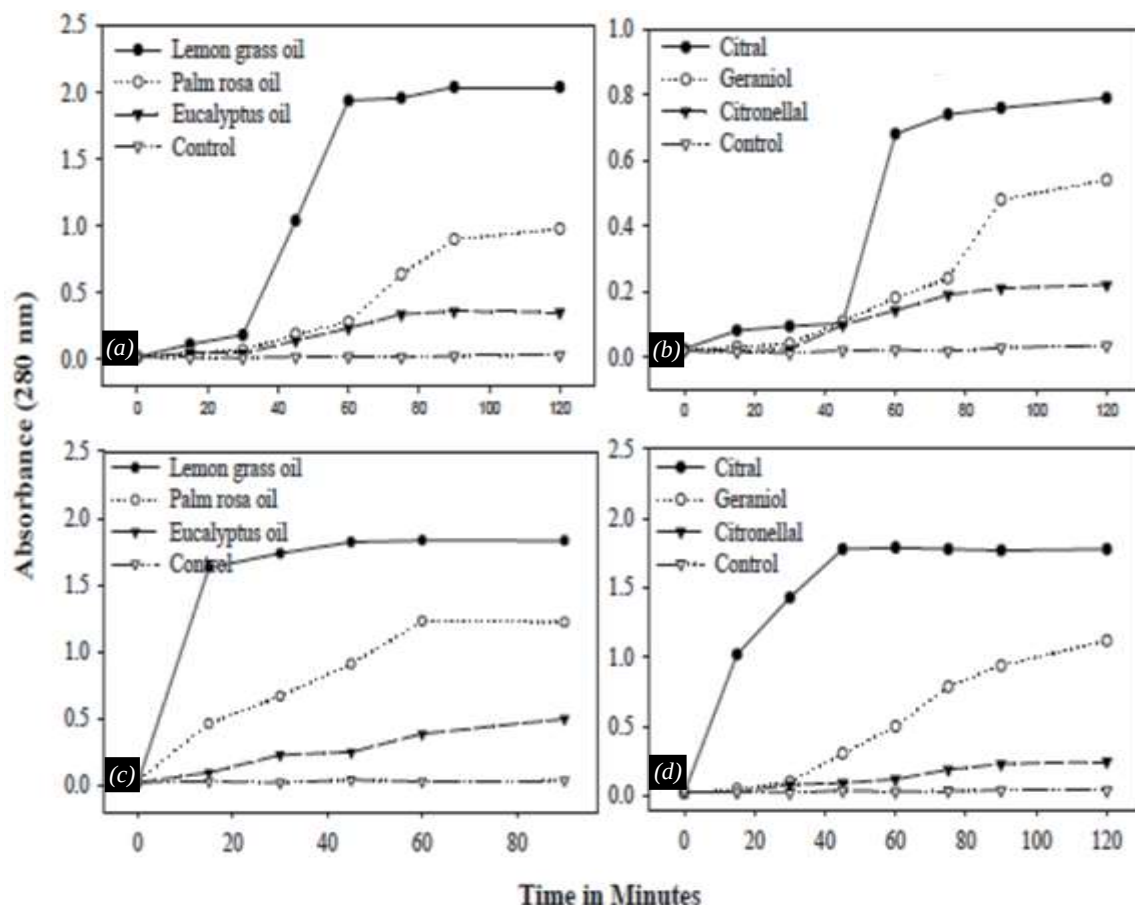
The leakage of UV<sub>280</sub> absorbing material (mainly proteins) was also monitored. In the absence of test compounds the OD of UV<sub>280</sub> material was between 0.015-0.034 for *coli* cell suspension, and 0.019-0.049 for *S. aureus* cell suspension. Control cultures that were not treated with test compounds,

showed negligible increase in the  $UV_{280}$  absorbing material, whereas the treated cultures showed a rapid increase in presence of certain compounds.



**Figure 2.** Leakage of  $UV_{260}$  absorbing material from the cell suspension of *E. coli* and *S. aureus* treated with essential oils and their major compounds, (a, b) Leakage of  $UV_{260}$  absorbing material from the cell suspension of *E. coli* treated with lemon grass, palm rosa, eucalyptus oils and citral, geraniol, citronellal, respectively, (c, d) Leakage of  $UV_{260}$  absorbing material from the cell suspension of *S. aureus* treated with lemon grass, palm rosa, eucalyptus oils and citral, geraniol, citronellal, respectively

In the presence of lemon grass oil, the cell suspension of *E. coli* showed leakage of  $UV_{280}$  material from 15 minutes. The leakage was very rapid with absorbance of  $UV_{280}$  absorbing material increasing from 0.184 at 30 minutes to 1.04 at 45 minutes (Figure 3a). The maximum OD of  $UV_{280}$  absorbing material in presence of lemon grass was 2.04 after 90 minutes of incubation. The leakage in presence of palm rosa could be observed from 30 minutes reaching a maximum absorbance of 0.98 after 120 minutes of incubation. In presence of eucalyptus oil the leakage was slow and steady. The leakage of  $UV_{280}$  absorbing material in presence of eucalyptus could be observed from 30 minutes of incubation and a maximum absorbance of 0.361 was recorded after 90 minutes of incubation (Fig 3a). The major component citral induced leakage of  $UV_{280}$  absorbing material from 45 minutes, and the leakage was rapid with a maximum absorbance of 0.79 recorded after 120 minutes of incubation. Geraniol and citronellal induced leakage of  $UV_{280}$  absorbing material from 30 minutes of incubation with a maximum absorbance of 0.54 and 0.220 respectively, recorded after 120 minutes of incubation (Figure 3b).



**Figure 3.** Leakage of UV<sub>280</sub> absorbing material from the cell suspension of *E. coli* and *S. aureus* treated with essential oils and their major compounds, (a, b) Leakage of UV<sub>280</sub> absorbing material from the cell suspension of *E. coli* treated with lemon grass, palm rosa, eucalyptus oils and citral, geraniol, citronellal, respectively, (c, d) Leakage of UV<sub>280</sub> absorbing material from the cell suspension of *S. aureus* treated with lemon grass, palm rosa, eucalyptus oils and citral, geraniol, citronellal, respectively.

The cell suspension of *S. aureus* was very sensitive to the test compounds in terms of leakage of UV<sub>280</sub> absorbing material. In the presence of lemon grass oil the leakage was very quick. The leakage commenced immediately in presence of lemon grass oil with a maximum absorbance of 1.834 recorded after 60 minutes of incubation. In the presence of Palm rosa oil also the leakage of UV<sub>280</sub> could be observed immediately with a maximum absorbance of 1.234 recorded after 60 minutes of incubation. In presence of eucalyptus oil the leakage of UV<sub>280</sub> material could be observed from 30 minutes with a maximum absorbance of 0.496 recorded after 90 minutes of incubation (Fig 3c). The major component citral induced leakage of UV<sub>280</sub> material immediately from the cell suspension of *S. aureus* with a maximum absorbance of 1.79 recorded after 60 minutes of incubation. Geraniol induced leakage from 30 minutes of incubation with a maximum of absorbance 1.12 recorded after 120 minutes of incubation. Citronellal induced a very low leakage of UV<sub>280</sub> material. The leakage in presence of citronellal was very slow with a maximum absorbance of 0.248 recorded after 120 minutes of incubation (Figure 3d).

## DISCUSSION

Our present investigation has shown that the essential oils lemon grass, palm rosa and eucalyptus, and their major components citral, geraniol and citronellal show high antimicrobial activity compared to the other compounds tested [30]. It would be ideal if the possible mechanism of action of these compounds is also known as this will have implications for its spectrum of activity, selective toxicity, development of resistance etc. Hence it was aimed to study the probable mechanism by which these compounds act on bacteria. For our studies we have selected one Gram-negative bacterium *E. coli*, and one Gram-positive bacterium *S. aureus*.

It is well known that the correct and precise functioning of the cytoplasmic membrane, which is rich in hydrophobic lipid molecules, is indispensable to the cell. Therefore, any compound that disrupts or compromises the cytoplasmic membrane will have a lethal effect on cells. It is already known that interactions with the hydrophobic structures of bacteria play a key role in the antimicrobial actions of hydrocarbons [25, 26, 31]. Numerous investigations by various authors have pointed out that the ability of various essential oils to act as anti bacterial agent's stems from their high lipophilic character [18]. Studies by Cox et al., and Lopez-Romero et al., [27, 32] have shown that exposing *E. coli* and *S. aureus* at MBC concentrations of tea tree oils leads to increased permeability of bacterial cytoplasmic membranes, which was indicated by potassium ion leakage. Carson et al., [33] have also shown that tea tree oil induces leakage of UV<sub>260</sub> absorbing material indicating a gross and irreversible damage of cytoplasmic membrane in *S. aureus*. Many other studies have implicated membrane damage by essential oils as principal contributor to their antibacterial ability [34, 35].

Loss of cytoplasmic material, leakage of cellular potassium ions from the cell suspension were taken as indicators for gross and irreversible damage to cytoplasmic membrane. It was speculated that the essential oils and their major components in the present study, owing to their extremely lipophilic character may disrupt the membrane integrity resulting in the leakage of intracellular components into the extra cellular medium. Until now different studies have shown that an efflux of potassium ions is a first indication of membrane damage in bacteria [26, 36, 37]. Potassium ion is the major cytoplasmic cation of growing bacterial cells involved in several key functions. It plays a role in the activation of cytoplasmic enzymes, maintenance of turgor pressure and possibly regulation of cytoplasmic pH. Hence the effect of these compounds on leakage of K<sup>+</sup> ions and UV<sub>260</sub> and UV<sub>280</sub> absorbing material into extra cellular medium was studied. The data on effects of lemon grass oil, palm rosa oil, eucalyptus oil and their major components on the leakage of potassium ions and UV<sub>260</sub> and UV<sub>280</sub> absorbing material showed that these oils and their components did affect the membrane integrity of *E. coli* and *S. aureus*.

The test compounds at minimum bactericidal concentration caused an increased efflux of K<sup>+</sup> ions into the extra cellular medium of the cells. In terms of absolute value of K<sup>+</sup> ion leakage, lemon grass oil caused the highest efflux of K<sup>+</sup> ions from *E. coli* cell suspension compared to the other two essential oils. It could be observed that lemon grass oil, which showed highest antibacterial activity of the tested essential oils [30] was the most effective in causing K<sup>+</sup> ion efflux.

One interesting observation is that in presence of crude essential oils the leakage of bacterial cellular materials was higher than that showed in presence of the individual major components of essential oils. The results of minimum bactericidal concentration, time course of lethal action of these compounds also suggested the same. This strengthens the hypothesis that components other than major component within the oil also affect the susceptibility of microorganisms. The results also point that the essential oils are more active against *S. aureus*, than *E. coli*. The reason for this could be due to the higher levels of tolerance shown by the outer membrane of Gram-negative bacteria to lipophilic compounds, as indicated [26] and very recently by [38]. However before coming to any conclusion about the preferential activity of essential oils are to be studied.

When we compare the time course lethal action of essential oils, eucalyptus oil and palm rosa oil was similar to certain extent, while there is much difference observed in case of inducing leakage of cellular material in presence of these oils. Given the heterogeneous composition of these essential oils, it seems unlikely that there is only one mechanism of action or that only one component is responsible for the antimicrobial action. So, it is possible that in addition to membrane damaging effects, there could be additional targets for these essential oils to act and this could be the reason for the small differences observed when we compare various results of our study. There were few reports showing [20,39] that terpenoids derived from essential oils act on primary energy metabolism, in particular NADH and succinate dehydrogenase activities, membrane bound respiratory electron flow and oxidative phosphorylation. Hence, studies on these additional targets might provide clear answer to the differences seen in the results.

However, the loss of UV<sub>260</sub> and UV<sub>280</sub> absorbing material and increased K<sup>+</sup> ion efflux from the cell suspensions of bacteria in presence of all the test compounds suggest that cytoplasmic membrane of bacteria is compromised and damaged irreversibly by treatment with these compounds. The other observation of presence of correlation between the time course of lethal action and damage to cell membrane in majority of the cases suggest that membrane damage is one of the likely causes of cell death. Further, work on the effect of these compounds on microbial energy transduction, electron microscopy studies will give additional information about the mechanism of action.

## CONCLUSION

In conclusion, our observations confirm that the antimicrobial activity of essential oils result from their ability to disrupt the permeability barrier of microbial membrane structures, although the presence of additional mechanisms or targets cannot be ruled out. The mode of action may be same against *E. coli* and *S. aureus*, and could be similar to that of other broad-spectrum membrane active disinfectants and preservatives, such as phenol derivatives, chlorhexidine, para benzoic acid derivatives and tea tree oil [27,40].

## Declaration

**Funding Source:** No funding is available for the present study.

**Conflict of Interest:** None of the authors (MNP, VK, SKP and JNLL) expressed conflict of interest.

**Ethical Approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

**Data Availability Statement:** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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