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Full Length Research Paper

The proposed mechanism of bactericidal action of eugenol, α-terpineol and γ-terpinene against *Listeria monocytogenes*, *Streptococcus pyogenes, Proteus vulgaris* and *Escherichia coli*

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The mechanism of antimicrobial activity of essential oils components; α- terpineol, γ-terpinene and eugenol was studied to evaluate their effect on the bacterial membrane against four strains of bacteria: *Listeria monocytogenes, Streptococcus pyogenes, Proteus vulgaris* and *Escherichia coli*. The study was done to observe changes in membrane composition by assaying for the leakage of protein and lipid using Bradford and van Handel's method respectively. The oils components were capable of inducing cell lysis by the leakage of protein and lipid contents. Eugenol at 2 × MIC was highly effective toward protein content leakage after 120 min of exposure. Alpha terpineol and γ-terpinene showed similar effect at 2 × MIC under the same condition. Gamma terpinene displayed the highest activity toward lipid content leakage at 2 x MIC while α-terpineol and eugenol showed similar effect after 120 min of exposure. The result revealed that both cell wall and membrane of the treated gram negative and gram positive bacteria were significantly damaged.

Key word: Mechanism of action, essential oil components, lipid content, protein content.

INTRODUCTION

The probable mechanism of action of antibacterial activity of essential oil previously studied was investigated. The antibacterial activity of essential oils studied was supported by the results obtained by gas-liquid chromatographic analysis. The chemical analysis revealed the presence of eugenol in *Ocimum basilicum* and *Pteronia incana* oils, α-terpineol and γ-terpinene was present in *Rosemary officinallis* oil, while α-terpineol was present in *Eucalyptus cinerea* (yet to be published). α-terpineol, γ-terpinene and eugenol were present as major components of the used essential oils. The presence of these components may constitute the effectiveness of the oils based on their structural configuration (γ-terpinene and eugenol) and their relative percentage composition (Marino et al., 2001). However, the basis of the mechanisms of action of essential oils and their components has not been fully established. Recent investigations have been made to elucidate this mechanism in Gram negative and Gram positive bacteria (Ultee et al., 1998). Furthermore, little or no work has been done on the mechanism of actions of α- terpineol, γ-terpinene and eugenol on the protein and lipid leakage of bacterial membrane.

The aim of this study is to investigate the role of eugenol, γ-terpinene and α–terpineol played in the inhibition of *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Escherichia coli* at bactericidal concentration. Likewise, to evaluate the mechanism of inhibition by studying their potential of inducing cell lysis through protein and lipid leakage.

MATERIALS AND METHODS

Bacteria strains used in this study

The reference strains used in this study were chosen based on their pathological effects on human and deterioration of food products: Gram positive bacteria; *Listeria monocytogens* (ATCC 12022) and

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Table 1. Bactericidal activity of essential oil components against selective bacteria.

Test organisms	Nature of inhibition (%)								
	γ-terpinene			α-terpineol			Eugenol		
	MIC	Log kill 1×MIC	Log kill 2×MIC	MIC	Log kill 1×MIC	Log kill 2×MIC	MIC	Log kill 1×MIC	Log kill 2×MIC
L. monocytogenes	0.50	3.175§	7.79§	0.50	1.49	2.40	0.50	3.23§	7.84§
S. pyogenes	0.50	8.30§	7.60§	0.25	1.50	2.79	0.25	3.26§	3.60§
P. vulgaris	0.75	1.48	2.21	0.50	2.94	4.07§	0.50	2.84	7.90§
E. coli	0.50	2.50	3.26§	0.75	4.70§	7.00§	0.25	2.22	7.00§

MIC represents Minimum inhibitory concentrations; § represents bactericidal activity.

Streptococcus pyogenes (ATCC 19615). Gram negative bacteria; *Escherichia coli* (ATCC 87536) and *Proteus vulgaris* (ATCC 43071) were obtained from the Institute of Louis Pasteur, Paris, France.

Essential oil components

Eugenol, γ-terpinene and α-terpineol of essential oil components used were purchased from Sigma-Aldrich Steinheim (Germany). They were used based on the result of GC-MS chromatography obtained from previous study.

Culture media and growth conditions

The bacterial stock cultures were maintained on nutrient agar (Saarchem, Gauteng, SA) plates. A loopful of bacterial cells from the nutrient agar plates was inoculated into 100 ml nutrient broth (Difco, California, USA) in 250 ml side arm Erlenmeyer flask and incubated at 37°C for 16 h with vigorous shaking (orbital incubator, S150, UK). After incubation, the culture was diluted with fresh media to give an $O.D_{600nm}$ of 0.1. One hundred micro litre of the culture cells was added onto the plate and spread into a bacterial lawn using a sterile glass spreader.

MIC determination of eugenol, γ-terpinene and α-terpineol

The Minimum Inhibitory Concentration (MIC) of the essential oils and its components was determined by broth dilution technique as described by Irobi et al. (1996). Essential oil components were diluted with Tween-80 to give concentrations ranging from 0.01 to 2% v/v. 50 µl of standardized 18 h incubated bacterial culture (10^5 CFU/ml) was introduced into test tubes, followed by the addition of essential oils components; eugenol, α-terpineol and γ-terpinene. A set of tubes containing only growth medium inoculated with each of the bacterial strains were set up as controls. All tubes were incubated at 37°C for 24 h. The MIC determined was recorded as the lowest concentration that inhibits the growth of the bacterial strains. Tween-80 was used as the negative control but showed minimal effect on the test bacteria. The culture cells without the essential oils were used as the negative control with no effect.

Protein leakage assay

Protein content in the supernatant obtained by centrifugation of the cell suspension treated with different essential oils components (eugenol, α-terpineol and γ-terpinene) at 1 × MIC and 2 × MIC was measured to determine the leakage of intracellular materials from the cells. The samples were incubated at 37°C for 120 min and at 30 min intervals each suspension was centrifuged at 7000 rpm. Protein amounts were determined at 595 nm using Coomassie brilliant blue G-250 by the method of Bradford (1976). The concentration of protein leakage was extrapolated from Bovine serum albumin (BSA) which was used as a standard.

Lipid leakage assay

Lipid leakage was measured using a method described by van Handel (1985). Bacterial cultures were harvested after standardization (1.2×10^8 CFU/ml) by centrifugation at 10000 rpm. The cell suspension treated with 1 × MIC and 2 × MIC concentrations of eugenol, α-terpineol and γ-terpinene was further incubated at 37°C for 30 min. At each time interval each suspension was centrifuged at 10000 rpm for 10 min. The absorbance of the duplicate samples after the addition of vanillin-phosphoric acid reagent followed by vortexing was measured at 525 nm after 30 min. The concentration of lipid leakage was estimated from the triolein standard curve.

RESULTS

Antibacterial and time-kill regimes of eugenol, γ-terpinene and α-terpineol

The result of the time–kill studies is summarized in Table 1. The data are presented in terms of the log-CFU/mL and are judged relative to the convectional definition of bactericidal activity, that is, 3-log-CFU/mL or greater reduction in the initial inoculum within 24 h (Yaki and Zurenko, 2003). At 2 × MIC, γ-terpinene demonstrated bactericidal activity against all the strains tested except *P. vulgaris*. It was bactericidal at 1× MIC against *L. monocytogenes*, *S. pyogenes* and bacteriostatic to others. α-terpineol was bactericidal against *E. coli* at both 1 × MIC and 2 × MIC and only at 2 × MIC for *P. vulgaris*. The compound was bacteriostatic at both 1 × MIC and 2 × MIC against *L. monocytogenes* and *S. pyogenes*. Eugenol on the other hand demonstrated bactericidal activity against the entire test bacteria at 2 × MIC where as, at 1 × MIC it was bactericidal against *L. monocytogenes* and *S. pyogenes* and bacteriostatic against the other test organisms. All three components of essential oils possessed bactericidal and bacteriostatic activities at different concentrations.

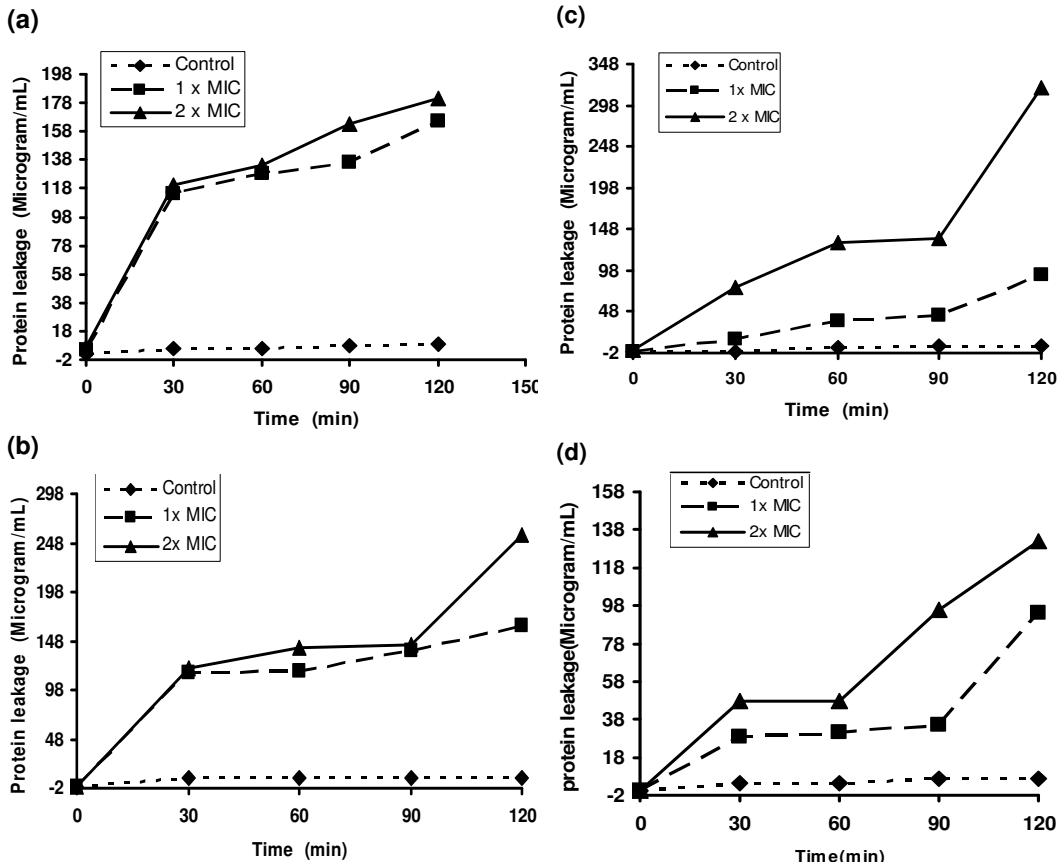

Figure 1. Protein leakage in the test organisms. **(a)** The effect of eugenol on *L. monocytogenes*; **(b)** effect of eugenol on *S. pyogenes*; **(c)** effect of eugenol on *P. vulgaris*; and **(d)** effect of eugenol on *E. coli.*

Evaluation of protein leakage

Eugenol, γ-terpinene and α-terpineol were assessed for their ability to induce cellular protein leakage in *P. vulgaris* and *E. coli* (Gram negatives) as well as *L. monocytogenes* and *S. pyogenes* (Gram positives). The three test components were observed to induce protein leakage in all the test organisms at 1 × MIC and 2 × MIC leading to incremental concentration of protein in the cell free media at different time intervals (Figures 1 - 3) up to as much as 325 µg/ml. Both the Gram negative and Gram positive test bacteria showed a similar trend of protein leakage when treated with eugenol, γ-terpinene and α-terpineol. Eugenol however, had the highest damaging effect on cell walls and caused protein leakage in the range of 120 – 325 µg/ml at 2 × MIC concentration (Figure 1). The protein leakage after treatment with γ-terpinene ranged from 60 – 225 µg/ml at 2 × MIC. The effect of α-terpineol ranged from 70 – 120 µg/ml at 2 × MIC and 50 – 90 µg/ml at 1× MIC against the test bacteria (Figure 3). Protein leakage could be used as an indicator of the membrane damage caused by chemical and physical agents. It has been suggested that the cytoplasmic membrane is also a target for eugenol action and the results evidencing the protein leakage corroborated this hypothesis.

Lipid leakage assessment

Eugenol, γ-terpinene and α-terpineol were assessed for their ability to induce cellular lipid leakage in *P. vulgaris* and *E. coli* as well as *L. monocytogenes* and *S. pyogenes*. This was determined by measuring the amount of lipid leakage after treatment with eugenol, γ-terpinene and α-terpineol for a period of 120 min. The three test compounds were observed to induce lipid leakage in all the test organisms at 1 × MIC and 2 × MIC leading to incremental concentration of lipid in the cell free media at different time interval. Treatment of bacteria with eugenol showed lipid leakage ranging from 120 - 220 µg/ml at 2 × MIC and 80 - 170 µg/ml at 1× MIC (Figure 5). The essential oil constituent γ-terpinene caused lipid leakage ranging from 150 - 550 µg/ml at 2× MIC (Figure 4). α-terpineol damaged cell walls causing lipid leakage between 110 - 450 µg/ml at 2 × MIC within a period of 120 min (Figure 6).

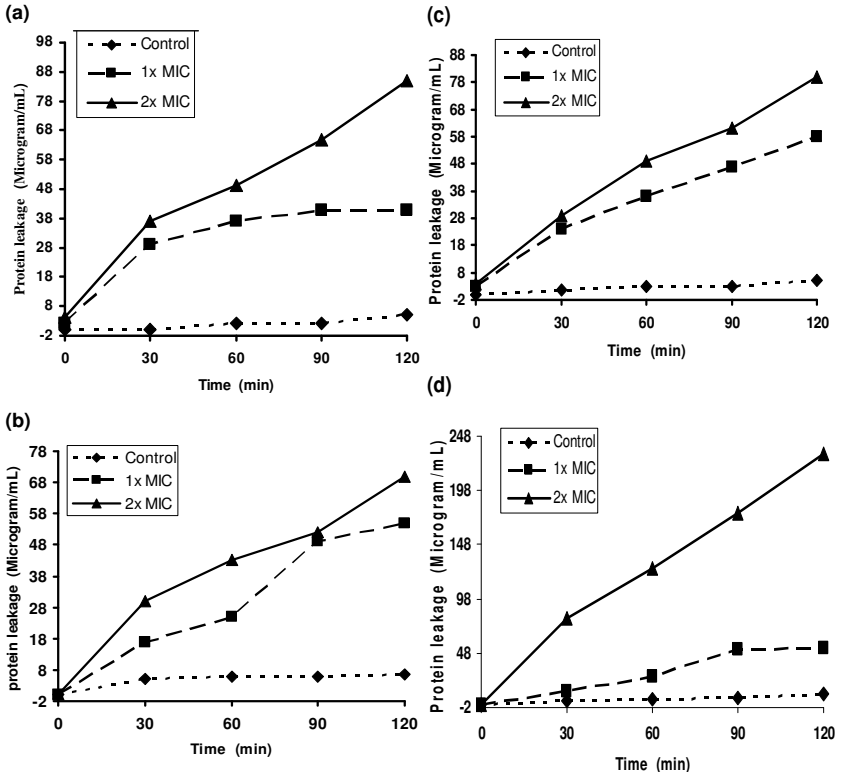

Figure 2. Protein leakage in the test organisms. **(a)** The effect of γ-terpinene on *L. monocytogenes*; **(b)** effect of γ-terpinene on *S. pyogenes*; **(c)** effect of γ-terpinene on *P. vulgaris*; and **(d)** effect of γ-terpinene on *E. coli.*

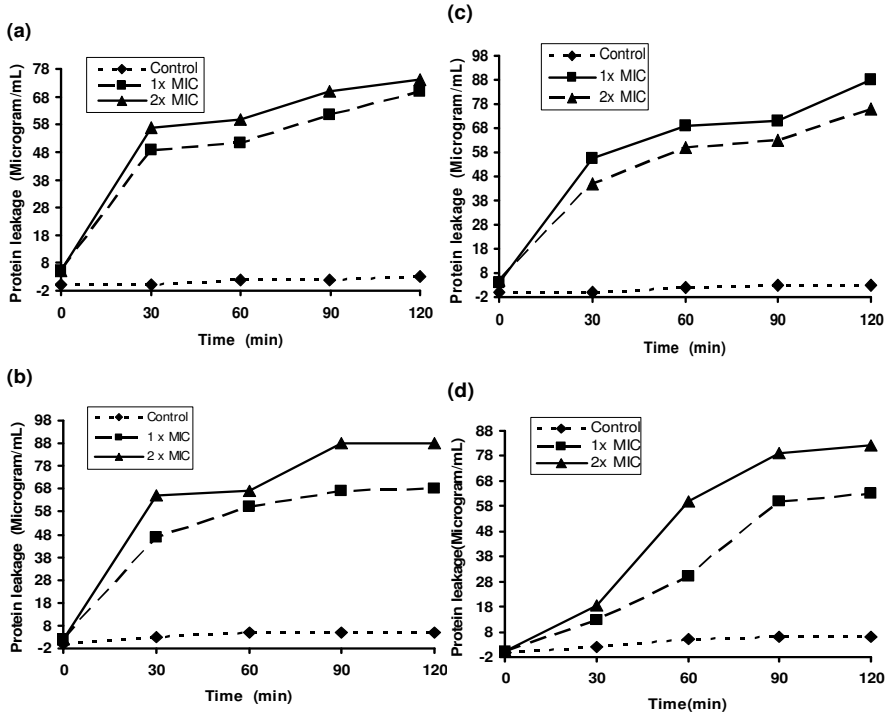

Figure 3. Protein leakage in the test organisms. **(a)** The effect of α-terpineol on *L. monocytogenes*; **(b)** effect of α-terpineol on *S. pyogenes*; **(c)** effect of α-terpineol on *P. vulgaris*; and **(d)** effect of α-terpineol on *E. coli.*

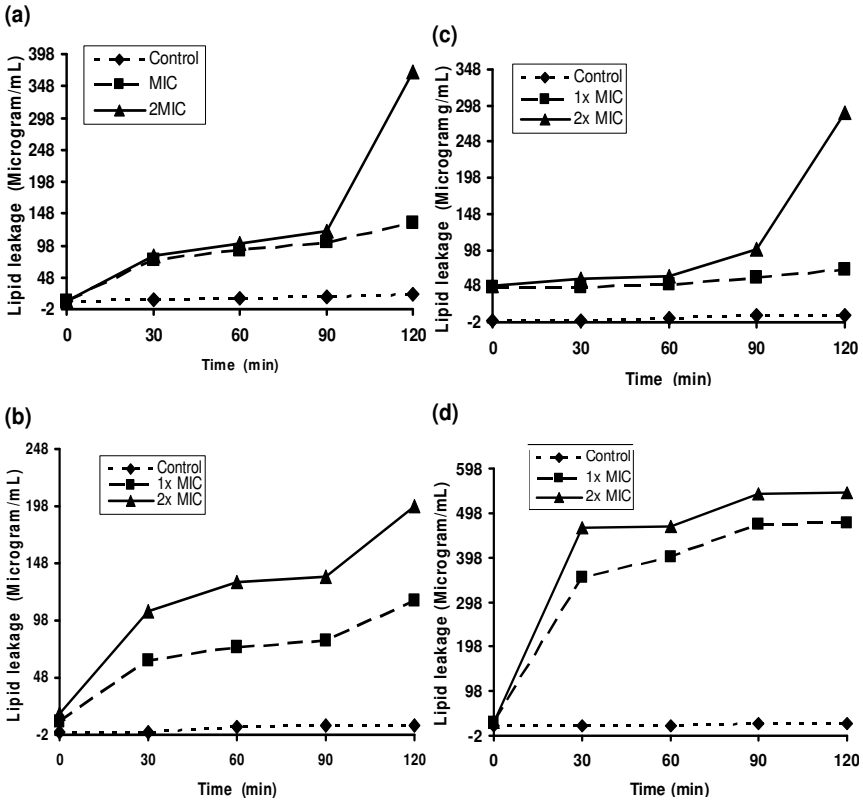

Figure 4. Lipid leakage in the test organisms. **(a)** The effect of γ-terpinene on *L. monocytogenes*; **(b)** effect of γ-terpinene on *S. pyogenes*; **(c)** effect of γ-terpinene on *P. vulgaris*; and **(d)** effect of γ-terpinene on *E. coli.*

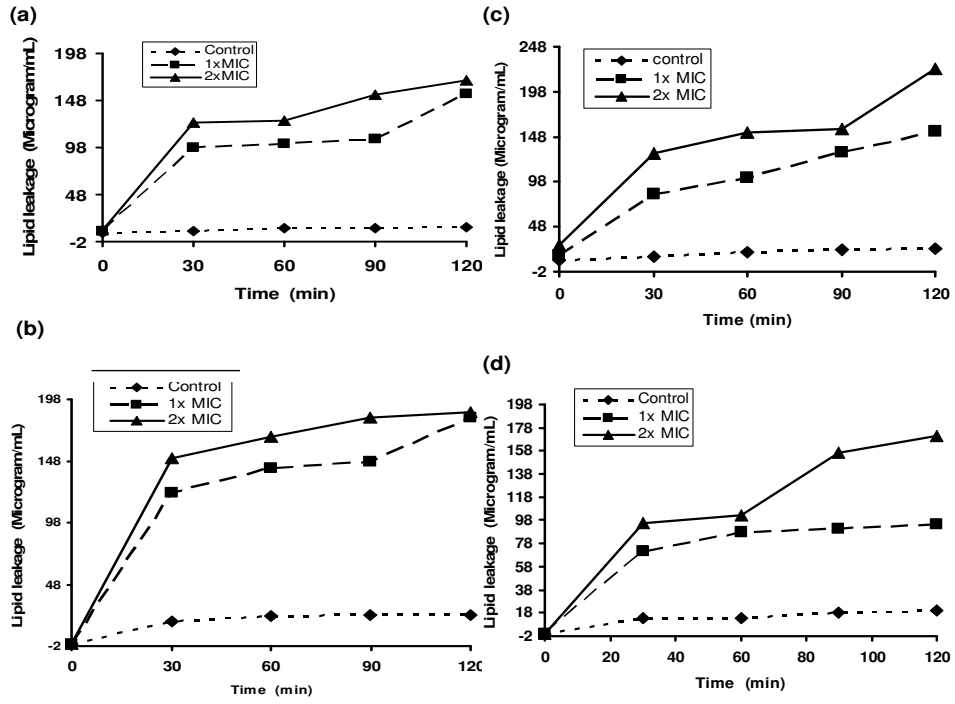

Figure 5. Lipid leakage in the test organisms. **(a)** The effect of eugenol on *L. monocytogenes*; **(b)** effect of eugenol on *S. pyogenes*; **(c)** effect of eugenol on *P. vulgaris*; and **(d)** effect of eugenol on *E. coli.*

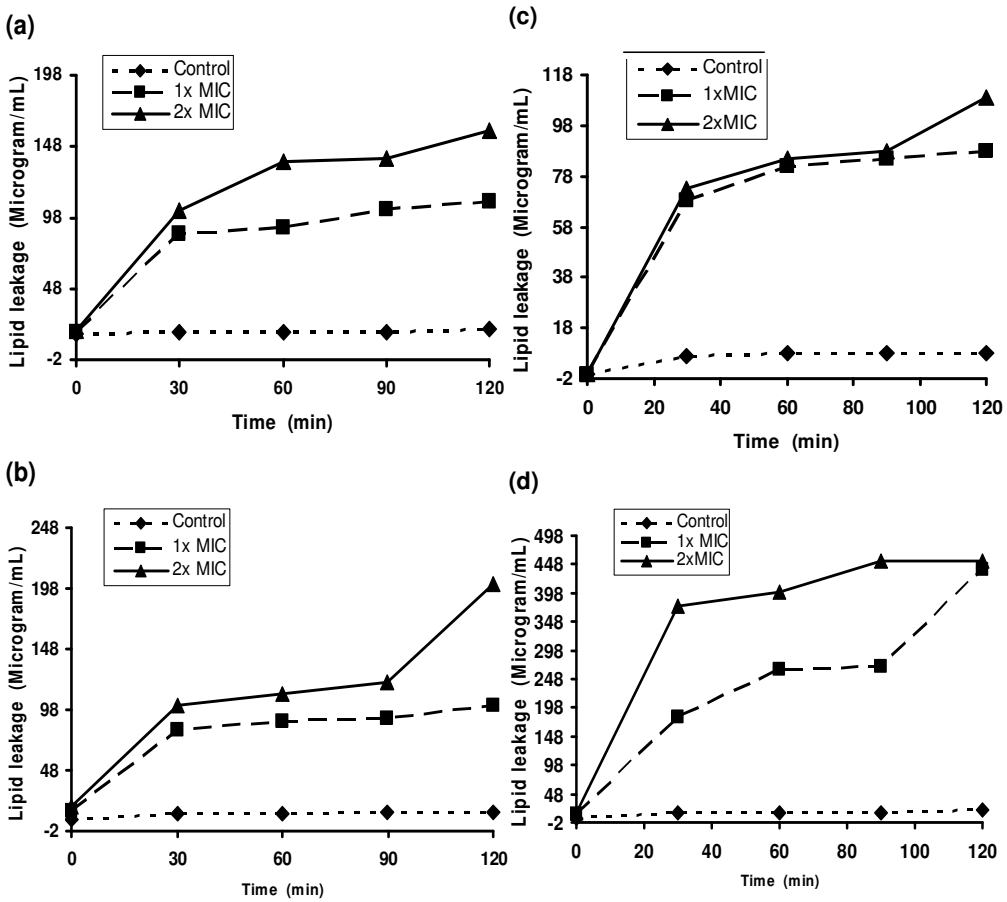

Figure 6. Lipid leakage in the test organisms. **(a)** The effect of α-terpineol on *L. monocytogenes*; **(b)** effect of α-terpineol on *S. pyogenes*; **(c)** effect of α-terpineol on *P. vulgaris*; and **(d)** effect of α-terpineol on *E. coli.*

DISCUSSION

The results of Minimum inhibitory concentration (MIC) showed that two gram positive bacteria, *L. monocytogenes* and *S. pyogenes* were less sensitive to inhibition of eugenol, α-terpineol and γ-terpinene at concentration between 0.25 to 0.50% (Table 1) than the two gram negative bacteria, *P. vulgaris* and *E. coli* at 0.50 to 0.75%. The difference in sensitivity to the essential oils components is supported by other researchers including Shelef (1983) and Smith- Palmer et al. (1997). During the time–kill test, eugenol and γ-terpinene showed similar activity which was higher than α–terpineol at the concentrations tested. *E. coli* and *P. vulgaris* seemed to be very sensitive to the oil components. Reduction in the order of 3 to 8 log10 was obtained after 20 h of incubation at 2 × MIC, with *E. coli* showing the highest reduction of 7.9 log10 reductions after 20 h of incubation followed by *P. vulgaris* showed 3 log10 reductions. At 1 × MIC, four strains of bacteria mentioned above showed 3 to 4 log10 reduction of bacterial growth (Table 1). It is not known exactly why gram negative bacteria should be more susceptible to the time kill study at the same concentration, although the MIC was of closer range. However, this may be related to the outer membrane composition (Nikaido and Vaara, 1985).

Further evaluation was carried out to ascertain the effect of essential components against the bacterial membrane components. The result of lipid leakage showed that eugenol, α-terpineol, and γ-terpinene were effective against both Gram negative and Gram positive bacteria. γ-terpinene displayed a stronger effect on the lipid component of cell membranes of *L. Monocytogenes* and *E. coli,* at both 1 × MIC and 2 × MIC after 120 min of incubation than eugenol and α- terpineol (Figure 4 and 5). The effectiveness of γ-terpinene might be the result of its phenolic structure which interferes with the lipid bilayer of the outer membranes (Janssen et al., 1987). α-terpineol and eugenol showed similar effect on the lipid content of cell membrane of both Gram positive and Gram negative bacteria after 120 min of incubation. All the organisms tested were very susceptible to the effect of essential oil components. The different effects observed could be due to the hydrophobicity of the essential oils components

which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Sikkema et al., 1994).

The evaluation of protein leakage of the three essential oil components; eugenol, γ-terpinene and α-terpineol showed a strong effect on the protein leakage of both the Gram negative and Gram positive bacteria. Eugenol displayed a stronger effect on the protein leakage of cell membranes of *P. vulgaris* followed by *S. pyogenes* and *L. monocytogenes* at 2 × MIC (Figure 1a-d), while *L. monocytogenes* and *S. pyogenes* at 1 × MIC showed high leakage of protein after 120 min of incubation than γ-terpinene and α-terpineol (Figures 1 - 3). *E. coli* and *L. monocytogenes* treated with γ-terpinene and α-terpineol respectively at 2 × MIC showed high content of lipid leakage (Figures 2d and 3c). Both the Gram negative and Gram positive test bacteria showed a similar trend of protein leakage when treated with eugenol, γ-terpinene and α-terpineol. Eugenol however, had the highest damaging effect on cell walls and caused protein leakage in the range of 120 – 325 µg/ml at 2 × MIC concentration (Figure 1). A much lower protein leakage from *L. monocytogenes* cells treated with α- terpineol and high protein leakage from *P. vulgaris* treated with eugenol was observed among the three essential oil components tested (Figure 3a). However, γ-terpinene showed higher effect on the test bacteria than α-terpineol of the bacteria tested. Eugenol was very active despite its relatively low capacity to dissolve in water, which is in agreement with published data (Lattaoui and Tantaoui-Elaraki, 1994).

In conclusion, this study showed that essential oils components used in this study had bactericidal effect against the both gram positive and gram negative bacteria by disrupting their outer membrane. An important characteristic is their hydrophobicity, which enable them to partition the lipids of bacteria cell membrane disturbing the cell structure and rendering them more permeable. The present investigation provides support to the effectiveness of antibacterial properties of the essential oils tested. Especially in the light of the current trend in finding alternative remedies against increasing numbers of pathogenic bacteria that are resistant to current antibiotics. However, more studies are still needed to validate the mechanism of action of essential oils components.

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