Original Research Article

Antibacterial Synergism of Blended Essential Oils from *Piper betle* L. and *Melaleuca alternifolia*

ABSTRACT

Aims: To investigate the synergistic antibacterial effects of blended essential oils from *Piper betle* and *Melaleuca alternifolia* against nine different bacterial strains.

Methodology: GC-MS was employed for the analysis of essential oil extracted by hydro-distillation. The synergistic antibacterial effects of blended essential oils from *Piper betle* and *Melaleuca alternifolia* against nine different microbial strains were assayed using standard methods including disc diffusion, agar dilution and checkerboard.

Results: The GC-MS analysis indicated the presence of 13 and 12 chemical compounds for the *Piper betle* and *Melaleuca alternifolia* essential oil, respectively. Essential oil from *Piper betle* consisted mainly of eugenol (39.21%) followed by other components: eugenol acetate (16.42%), 4-allyl-1,2-diacetoxybenzene (12.24%), terpinen-4-ol (6.58%), α-cadinol (6.13%), γ-terpinene (3.46%), and sabinene (2.14%). Meanwhile, the major components of tea tree essential oil were terpinen-4-ol (49.62%), followed by other components: γ-terpinene (18.08%), α-terpinene (9.16%), *p*-cymene (5.89%), α-terpineol (4.94%), terpinolene (3.47%), and α-pinene (2.02%). The synergistic antibacterial effects of blended essential oils from *Piper betle* and *Melaleuca alternifolia* were recorded in seven of the nine bacteria tested, including *Bacillus subtilis* (FICI = 0.250%), *Enterococcus faecalis* (FICI = 0.375%), *Staphylococcus aureus* (FICI = 0.313%), Methicillin-resistant *Staphylococcus aureus* (FICI = 0.281%), *Acinetobacter baumannii* (FICI = 0.375%), *Escherichia coli* (FICI = 0.375%) and *Salmonella sp* (FICI = 0.375%). However, additive effects were only observed in both Gram-negative bacteria including *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* when treated with blended essential oils resulting in FICI value of 0.625% and 0.750%, respectively.

Conclusion: The combination of essential oils from *Piper betle* and *Melaleuca alternifolia* exhibited more significant antibacterial effects against nine studied bacterial strains than single essential oil. Accordingly, the synergistic antibacterial effects were recorded in seven

of the nine bacterial strains tested, whereas the additive effects were only observed in both Gram-negative bacteria.
Keywords: Piper betle, Melaleuca alternifolia, GC-MS, synergism, antibacterial assays.

1. INTRODUCTION

Medicinal plants are always regarded as potential natural sources for new antibacterial drug development. Since drug-resistant bacteria have become a global concern to public health in recent years, the discovery of new antibiotics has always attracted considerable interest from researchers working in several different disciplines. Potential antibacterial agents from plants can be total extracts, fractions, pure secondary metabolites or essential oils. Contemporary dada clearly indicated that approximately 300 essential oils have been commercially used in several different fields ranging from folk medicine, aromatherapy to cosmetics, food preservatives and pharmaceutical industries. The antibacterial properties of essential oils have been traditionally and scientifically known for a long time [1], and since essential oils are a multi-constituent mixture with complex mechanisms of action, the combination of essential oils from different medicinal plants could be one of the potential strategies to enhance the antibacterial effects of blended essential oils against antibiotic-resistant bacteria. In this regard, this study was primarily focused on investigating the potential antibacterial synergism of blended essential oils from *Piper betle* and *Melaleuca alternifolia* against nine different bacterial strains using standard microbiological assays including disc diffusion, agar dilution and checkerboard.

Piper betle L. (*P. betle*), commonly known as betel vine, belongs to genus Piper of the Piperaceae family. Betel vine has been traditionally used as herbal medicine mainly as an antiseptic in dental practice. Betel plants, locally known as Pann (India), Ikmo (Philippines) or Trầu không (Vietnam), are popularly cultivated for their leaves in India, Sri Lanka, Thailand and other Asian countries. Betel leaves, the most commonly used plant parts, are widely chewed as not merely a habit practice and a custom, but also an identity of the traditional cultural values which are known as a symbol of love and happiness. In addition, regular chewing of a mixture of betel leaf, areca nut and lime is believed beneficial to health and well-being in several ways such as preventing halitosis, strengthening the gum, protecting the teeth and even improving the digestive health [2,3]. Phytochemical analysis of betel leaves from different sources of botanical origin revealed the presence of alkaloids, glycosides, saponins, steroids, tannins, flavonoids, volatile oils, proteins and carbohydrates.

Commercial betel leaf-based formulations are now available over the counter in pharmacies or drug stores such as mouthwash, toothpaste, shampoo, soap, hand sanitizer or lady care. Betel leaf essential oil (BLO) is a complex chemical mixture consisting of approximately between 30 to 60 natural volatile compounds [2,3]. These compounds mainly belong to classes of terpenes and phenols in various proportions depending on the botanical origin of the betel leaves. Major components of BLO include eugenol, chavicol, chavibetol, hydroxychavibetol and safrole. Other chemical components of BLO are anethole, estragole, linalool, caryophyllene, *p*-cymene, eucalyptol (1,8-cineole), estragol, sabinene, α-cadinol, γ-terpinene, γ-muurolene, α-muurolene, β-phellandrene, germacrene-D, 4-allyl-1,2-diacetoxybenzene [4,5]. BLO possesses a variety of significant pharmacological properties including antiseptic, antioxidant, antiprotozoal, antifungal, antibacterial and radio-protective properties. Nevertheless, as evidenced from previous studies, the antiseptic property of BLO is attributed chiefly to the major constituents of BLO including chavicol, chavibetol and eugenol. In addition, previous researches showed that BLO exhibits a broad spectrum of antimicrobial activity against pathogenic microorganisms including Gram-positive and Gram-negative bacteria, and fungi such as *Aspergillus*, *Candida*, *Epidermophyton*, *Microsporum*, *Trichophyton*, *Bacillus*, *Escherichia*, *Klebsiella*, *Listeria*, *Pseudomonas*, *Salmonella*, *Staphylococcus* or *Streptococcus* [2-5].

Melaleuca alternifolia (*M. alternifolia*), commonly known as tea tree, is a member of the family Myrtaceae native to Australia. Tea tree essential oil (TTO), a mixture of natural volatile compounds extracted mainly from tea tree leaves, has been widely used as an alternative and complementary therapy for a variety of skin conditions such as acne, wounds or contact dermatitis. TTO possesses various significant biological activities including antibacterial, antiviral, antifungal and anti-inflammatory properties. TTO is composed of approximately 100 various chemical compounds. Chemical analysis of TTO reveals the presence of terpinen-4-ol, *p*-cymene, γ-terpinene, α-terpineol, terpinolene, α-pinene, α-terpinene and 1,8-cineole. Other chemical components of BLO are sabinene, limonene, aromandendrene, β-thujene, β-myrcene, β-pinene [6-8]. As indicated from previous literature, the antibacterial and antifungal properties of TTO are attributed mainly to terpinen-4-ol and α-terpineol; however, α-terpineol often contributes an insignificant proportion of TTO which is commonly accounted to form approximately between 2 and 5% of the total composition of essential oil. In addition, several previous scientific studies showed that TTO exhibits a wide spectrum of antimicrobial activity against pathogenic microorganisms including Gram-positive and Gram-negative bacteria, viruses, protozoa and fungi such as *Aspergillus*, *Candida*, *Bacillus*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Haemophilus*, *Mycobacterium*, *Probionibacterium*,

Salmonella, *Staphylococcus*, *Streptococcus*, Herpes simplex zoster (HSV), *Leishmania* or *Trypanosoma* [6-8]. Nowadays, TTO is commonly incorporated as an active ingredient of many topical formulations for treatment of skin infectious diseases. TTO-based cosmetics and skin care products are now widely available over the counter in pharmacies or chemists shops, such as Australian tea tree cream, Australian tea tree oil cleansing soap, Australian tea tree essential oil 25 mL or Australian tea tree stimulating body wash [9].

2. MATERIALS AND METHODS

2.1 Plant Materials and Essential Oil Extraction

Fresh *P. betle* leaves were collected from a local garden and washed several times with running water to remove any impurities. The plant was identified by Associate Professor Dr. Tran Van Minh of the Institute of Tropical Biology, Vietnam and deposited in the herbarium of Applied Biochemistry Laboratory, Department of Applied Biochemistry, School of Biotechnology, International University, Vietnam National University-Ho Chi Minh City, Vietnam with voucher No. HB-BIO-04-02-22. The plant leaves were immediately air-dried and cut into small pieces. The extraction of essential oil was carried out by hydro-distillation for 3 h using a Clevenger-type apparatus [10]. Meanwhile, fresh *M. alternifolia* leaves and young twigs were harvested from a farm in Mekong Delta and washed thoroughly with running water to eliminate any possible contaminants. The plant materials were then air-dried and cut into small pieces prior to the extraction of essential oil. The plant materials were also subjected to hydro-distillation for 2 h. Once the distillation was complete, the obtained essential oil was dehydrated using anhydrous sodium sulphate and then stored in sealed opaque brown bottles at 4^0C until GC-MS analysis.

2.2 GC-MS Analysis

GC-MS was employed for the analysis of obtained essential oil. GC-MS analyses were performed using SCION SQ 456-GC equipped with a Rxi-5ms RESTEX column (30 m x 0.25 mm x 0.25 µm). Helium was used as carrier gas at a constant flow rate of 1 mL/min. The oven temperature was initially programed at 50^0C for 1 min and then increased to 80^0C at 30^0C/min. After that, it was increased to 230^0C at 5^0C/min, and finally to 280^0C at 25^0C/min where it was held for 3 min. The injector temperature was set at 250^0C and the rate of Division was 1:30 [11]. Fragmentation was done by electron impact under a field of 70eV. The mass spectra were recorded over the mass range of 50-500 amu with the full-scale mode at a rate of 1s/scan.

2.3 Physicochemical Analysis

The determination of physicochemical parameters of single essential oil for specific gravity, acid value, ester value and saponification value was performed as per standard test methods [12]. The obtained essential oil was also subjected to organoleptic evaluation including appearance, color, odor and taste.

2.4 Microbial Strains

The synergistic antibacterial effectiveness of blended essential oils from *P. betle* and *M. alternifolia* were tested against nine different bacterial strains. Gram-positive species were *Bacillus subtilis* (*B. subtilis*), Enterococcus *faecalis* (*E. faecalis*), *Staphylococcus aureus* (*S. aureus*) and Methicillin-resistant *Staphylococcus aureus* (MRSA). Gram-negative strains included *Acinetobacter baumannii* (*A. baumannii*), *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Pseudomonas aeruginosa,* (*P. aeruginosa*) and *Salmonella sp*. The identity of the microorganisms assayed in this research was confirmed by morphological studies and standard biochemical tests [13].

2.5 Microbial Assay

2.5.1 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was conducted to investigate the sensitivity of a microorganism to essential oil using disc diffusion technique on agar media. For each sample, 100 µL of essential oil 5% was prepared for the assay while DMSO 1% was used as a negative control and Ampicillin (10 mg/mL) as a positive control. The test was carried out by applying a standardized microbial inoculum of approximately 10^6 CFU/mL to the surface of Mueller-Hinton agar (MHA) plate. Agar plates were then incubated for 24 h at 35-37^{0}C prior to determination of results. The AST activity was assessed by measuring the growth inhibition zone (in millimetres, mm). The results were reported qualitatively as susceptible (++), intermediate (+) or resistant (-) [14,15].

2.5.2 Minimum Inhibitory Concentration Assay

Agar dilution method was employed for the determination of minimum inhibitory concentration (MIC). Two-fold serial dilutions of essential oil were prepared by dilution with dimethyl sulfoxide (DMSO) to produce a series of decreasing concentration (8%, 4%, 2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125% and 0.0156%). MHA plates were inoculated with a standardized inoculum of microbial according to McFarland standard. The plates were

then incubated at 35-37^{0}C for 24 h. MIC value was defined as the lowest concentration of essential oil that completely inhibited visible growth of the tested microorganisms [16,17]. Ampicillin (10 mg/mL) was employed as a positive control while DMSO 1% was used as a negative control.

2.5.3 Synergy Checkerboard Assay

The antibacterial synergism of blended essential oils from *P. betle* and *M. alternifolia* against nine selected bacterial strains were evaluated by the checkerboard method followed by the calculation of fractional inhibitory concentration index (FICI) using the formulas as described below:

FIC of BLO = MIC of BLO in combination/MIC of BLO alone

FIC of TTO = MIC of TTO in combination/MIC of TTO alone

FICI = FIC of BLO + FIC of TTO

The interaction was categorized as follows: Synergism as FICI ≤ 0.5, additive effect as 0.5 < FICI ≤ 1, indifference as 1 < FICI ≤ 2, and FICI > 2 was considered to be antagonism [18-20].

2.6 Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed in terms of Mean ± Standard Error of Mean (SEM). Statistical analysis was performed by SPSS and analysis of variance (ANOVA) with the level of significance $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Organoleptic and Physicochemical Properties

Table 1: Organoleptic and physicochemical properties of single essential oil from *Piper betle* and *Melaleuca alternifolia*

Organoleptic property/ Physicochemical parameter	Description/Value	
	BLO	TTO
Appearance	Oily liquid	
Solubility	Immiscible in water, but soluble in non-polar organic solvents	
Color	Light yellow	Nearly colorless to pale yellow
Odor	Creosote-like note	Camphor-like note

Taste	Pungent and sharp	Bitter and spicy
Specific gravity	0.97 ± 0.01	0.89 ± 0.01
Acid index (mg KOH/g)	7.09 ± 0.17	1.49 ± 0.16
Ester index (mg KOH/g)	12.05 ± 0.56	16.39 ± 0.16
Saponification index (mg KOH/g)	19.14 ± 0.42	17.88 ± 0.01

Note: The data are presented as mean ± SEM; $p < 0.05$.

3.2 Chemical Composition of Essential Oil

Table 2: Chemical composition of essential oils from *Piper betle* and *Melaleuca alternifolia*

BLO		TTO	
Identified compound	**Quantity (%)**	**Identified compound**	**Quantity (%)**
Eugenol	39.21	Terpinen-4-ol	49.62
Eugenol acetate	16.42	γ-Terpinene	18.08
4-Allyl-1,2-diacetoxybenzene	12.24	α-Terpinene	9.16
Terpinen-4-ol	6.58	*p*-Cymene	5.89
α-Cadinol	6.13	α-Terpineol	4.94
γ-Terpinene	3.46	Terpinolene	3.47
Sabinene	2.14	α-Pinene	2.02
γ-Muurolene	2.06	Limonene	1.34
Germacrene-D	1.65	Aromandendrene	0.99
β-Phellandrene	1.27	β-Thujene	0.69
Eucalyptol	1.21	β -Myrcene	0.64
α-Muurolene	1.17	β-Pinene	0.52
Caryophyllene	0.92		

The organoleptic and physicochemical properties of essential oils from BLO and TTO are presented in Table 1. Although organoleptic evaluation is highly subjective, it may provide reliable information and is helpful in evaluating the quality of given essential oils. The obtained BLO is light yellow oily liquid, insoluble in water and soluble in non-polar organic solvents. It resembles to the smell of creosote, and with a sharp, pungent taste. Meanwhile, the obtained TTO is nearly colorless or pale yellow oily liquid, non-soluble in water but soluble in non-polar organic solvents. It has a camphoraceous odor, and with a spicy, bitter taste. As can be seen from Table 2, the essential oil from *P. betle* contains 13 compounds representing approximately 95% of the total oil. The chemical composition of BLO which was determined by GC-MS revealed the presence of eugenol (39.21%) followed by other

components: eugenol acetate (16.42%), 4-allyl-1,2-diacetoxybenzene (12.24%), terpinen-4-ol (6.58%), α-cadinol (6.13%), γ-terpinene (3.46%), and sabinene (2.14%). Meanwhile other compounds made contributions less than 2% each to the total composition of essential oil. As reported from previous literature, the components of essential oil were influenced by several factors, including plant species, genotypes, climatic conditions, cultivation, ages of the plants, harvesting time, botanical origin and experimental conditions. Thus, the biological activity of essential oil will likely vary depending on various factors, particularly the diversity of microbial strains and the complexity of single molecules present in essential oils. Generally, based on the most common molecules present in BLO obtained from different parts of Asia, constituents of BLO can be classified into eight different chemotypes, including anethole, chavicol, chavibetol, eugenol, eugenol acetate, germacrene-D, isoeugenol, and safrole [2,3]. However, in reality, major constituents of each essential oil are attributed to commonly between 2 and 4 chemotypes. In this research, the obtained BLO reveals the absence of five chemotypes including anethole, chavicol, chavibetol, isoeugenol, and safrole. However, eugenol which has been recognized as the most powerful component notably responsible for various biological properties particularly antiseptic potency was accounted to compose approximately 40% of the total constituents of BLO.

On the other hand, the essential oil from *M. alternifolia* consists of 12 compounds accounting for almost 97% of the total oil. The major components of TTO were terpinen-4-ol (49.62%), followed by other components: γ-terpinene (18.08%), α-terpinene (9.16%), *p*-cymene (5.89%), α-terpineol (4.94%), terpinolene (3.47%), and α-pinene (2.02%). Meanwhile, other substances contributed lower than 2% each to the total composition of essential oil. TTO is a mixture of more than 100 natural volatile compounds. The concentration and chemical constituents of TTO may be extremely variable depending on several factors such as chemotypes, places of cultivation, climate conditions or extraction methods. Basically, TTO has been categorized into six chemotypes; each chemotype has been characterized by typical chemical constituents. Of which, the terpinen-4-ol chemotype is specified as type 1, followed by terpinolene as type 2 and four 1,8-cineole chemotypes [6]. Notably, the chemical constituents of TTO are regulated by International Standard ISO 4730:2017 for “Essential oil of *Melaleuca,* Terpinen-4-ol type (Tea Tree oil)”, providing specific quantity ranges (in percentage) of 15 major components among more than 100 components identified in pure Australian TTO. Organoleptic characteristics and physical properties are also included in ISO description. As regulated, commercial TTO products to be marketed must be always of terpinen-4-ol chemotype, type 1, typically consisting of at least 35% terpinen-4-ol and eucalyptol (1,8-cineole) must be less than 10% [21]. In practice, however, approximately

50% of the commercial products did not meet the ISO specifications. In this study, the obtained TTO reveals the absence of four 1,8-cineole chemotypes. However, terpinen-4-ol which has been confirmed as the principal active component significantly responsible for various biological activities especially antibacterial property was accounted to form approximately 50% of the total composition of TTO. Noticeably, a comparison of constituents between BLO and TTO shows the presence of two major compounds, namely terpinen-4-ol and γ-terpinene, in both essential oils, but at significantly different concentrations (Table 2). Nevertheless, since these two compounds are well known as major molecules substantially responsible for antibacterial activity of both BLO and TTO, the synergistic antibacterial efficacy may be likely variable depending on the ratio of essential oils in combination prepared for the assays against microbial strains.

3.3 AST and MIC Values

Table 3: AST and MIC values of essential oils from *Piper betle* and *Melaleuca alternifolia*

Microorganism	AST and MIC Value			
	BLO		TTO	
	AST	MIC (%)	AST	MIC (%)
Bacillus subtilis	+	0.125	++	0.500
Enterococcus faecalis	++	0.125	++	0.125
Staphylococcus aureus	++	0.125	+	0.500
Methicillin-resistant *Staphylococcus aureus*	+	2.000	+	0.500
Acinetobacter baumannii	++	0.125	++	0.125
Escherichia coli	+	0.125	+	0.125
Klebsiella pneumoniae	++	0.125	+	0.125
Pseudomonas aeruginosa	+	2.000	+	2.000
Salmonella sp	++	0.125	++	0.125

Note: susceptible (++); intermediate (+).
The data are presented as mean ± SEM; $p < 0.05$ compared with the control.

The results detailed in Table 3 clearly show that BLO and TTO were found effective against all bacterial strains tested, but at different levels. Differences in the susceptibility of the tested microorganisms to BLO and TTO were qualitatively identified. Five bacterial strains were found susceptible to BLO and four were intermediate, whereas four microorganisms were confirmed susceptible to TTO and five were intermediate. MIC values of both BLO and TTO were varying between 0.125% and 2.000%. BLO was able to powerfully inhibit the growth of *B. subtilis, E. faecalis, S. aureus, A. baumannii, E. coli, K. pneumoniae* and *Salmonella sp* with the same lowest MIC value of 0.125%, whereas both MRSA and *P. aeruginosa* displayed the least sensitivity to BLO with the same highest MIC value of

2.000%. TTO, on the other hand, efficaciously inhibited the growth of *E. faecalis, A. baumannii, E. coli, K. pneumoniae* and *Salmonella sp* with the same lowest MIC value of 0.125%. TTO also showed potent inhibition against *B. subtilis, S. aureus* and MRSA with the same MIC value of 0.500%. Among nine bacteria tested, however, *P. aeruginosa* exhibited the lowest sensitivity to TTO with the highest MIC value of 2.000%.

Numerous studies have been conducted on elucidating the mechanisms of action of essential oils. In general, the antibacterial mechanisms of essential oil are attributed to a variety of factors, including the characteristics of essential oil chemotypes, the quantity of major single chemical constituents present in essential oils and the diversity of microbial strains. Therefore, it might not be possible to postulate a single mode of action of essential oil against various microorganisms. As reported from previous literature, most of the researches were mainly involved in the physicochemical properties of pure single molecules and the characteristics of investigated microorganisms. Concerning the nature of chemical constituents, plant essential oils are not true oils, because they are not composed of lipids. Instead, essential oils truly possess a complex mixture of naturally occurring volatile compounds, mainly consisting of terpenoids, phenolics and phenylpropanoids. These compounds, being hydrophobic molecules, are capable of achieving a potent binding affinity to different lipophilic molecular structures such as lipids, proteins, lipoproteins, glycoproteins or glycolipids. It has been previously reported that numerous components present in various essential oils have been identified as key molecules responsible for biological activities and subsequently subjected to investigating the antibacterial mechanism of action [22-26]. As scientifically reviewed elsewhere, in general, the most commonly possible antibacterial mechanism of essential oils is involved in the bacterial cell wall and plasma membrane compositions which are identified as the main target sites of essential oils. Owing to the hydrophobicity nature of key constituents present in essential oils, hydrocarbons are capable of partitioning into the cell wall and cytoplasmic membrane of microorganisms. Different molecules present in essential oils may have different interactions with cell plasma membrane with regards to the types of interactions and strengths of binding affinity. Therefore, the disturbance of cell plasma membrane integrity and permeability may be likely to cause negative impacts in several different ways which can be briefly described as follows: (1) the leakage of essential metabolites; (2) efflux of RNA and DNA from the cytoplasm; (3) loss of vital ions such as Na^+, K^+, Ca^{2+} or Mg^{2+}; (4) depletion of proton pumps in the plasma membrane; (5) depletion of ATP during biosynthesis; (6) disruption of cell respiration; (7) inactivation of various bacterial enzymes such as ATPase, amylase, histidine carboxylase or protease; (8) turbulence of electron transport system; (9) coagulation of

cellular components in the cytoplasmic membrane; (10) blockage of protein expression; (11) inhibition of nucleic acid synthesis and function or (12) inhibition of the production and secretion of toxic bacterial metabolites [27-29]. These series of events may be responsible for a number of serious sequelae. Finally, a consequence of any of these impairments may be induction of bacterial cell lysis and cell death.

Moreover, the complexity of antibacterial mechanism of essential oils is even attributed to the difference in susceptibility of Gram-positive and Gram-negative bacteria to a variety of essential oils. In general, Gram-negative bacteria exhibit more natural resistance to various types of essential oils than Gram-positive strains due to the difference in the cell membrane structures and compositions. Gram-negative bacteria possess hydrophilic lipopolysaccharide (LPS)-based outer membrane which play a key role in structural integrity of the bacterial cell membrane and also considerably contribute to the negative charge of the cell membrane on the basis of negatively charged carbohydrates and phosphates bonded in the polysaccharides. A thick layer of LPS which is surrounding the cell wall of Gram-negative bacteria establishes a physical barrier limiting the diffusion of macromolecules and hydrophobic compounds, thereby facilitating more resistance to the lypophilicity nature of essential oils. In contrast, Gram-positive cell wall is mainly composed of peptidoglycan, but lack of lipopolysaccharides, which may be likely more vulnerable to essential oils and their components. It should be noted, however, there has been no general principles relevant to properly assessing Gram susceptibility. Practically, some studies have shown no significant difference in susceptibility between Gram-positive and Gram-negative bacteria to essential oils and their components; even in some cases, Gram-negative strains were found greater susceptible than some Gram-positive species to certain essential oils and their constituents. Such conflicting studies may gain considerable attention from researchers in various fields and even trigger more investigation for further understanding the antibacterial mechanism of action of essential oils and their components [30,31]. It is noteworthy to know that some essential oils may be capable of inhibiting the cell-cell signal communication between bacterial cells. This communication, commonly known as quorum sensing, is one of the vital biochemical processes in the biofilm formation. The biofilm is typically composed of a complex mixture of highly polar biomolecules mainly consisting of proteins, nucleic acids and polysaccharides from resident bacteria. The biofilm formation is best known as part of microbial action against antibiotics, owing to the formation of physical barriers to limit the infiltration of antibiotics. Therefore, the inhibition of biofilm formation could be one of the capacities of various potential essential oils in fighting against bacterial infections. Noticeably, the antibacterial mechanisms of eugenol and terpinen-4-ol have been intensively

investigated using relevant modern techniques, such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM) or measurement of release of 260-nm absorbing materials and proteins. By such means, the physiological and morphological changes have been observed and the biochemical parameters have been recorded. Generally, the antibacterial mechanism of both components against various pathogenic microorganisms might be likely related to the disruption of cell wall and the damage of cytoplasmic membrane. Additionally, eugenol was able to cause the leakage of intracellular components and effectively inactivated various bacterial enzymes such as ATPase, amylase, protease or histidine carboxylase. Terpinen-4-ol, on the other hand, could strongly induce the release of Ca^{2+}, Mg^{2+} and enzyme lactate dehydrogenase (LDH); and powerfully inhibited the synthesis of protein, DNA and ATPase. Furthermore, both components were found to be capable of effectively inhibiting the biofilm formation resulting in increased number of dead cells [32-37].

3.4 FICI Values

Table 4: FICI values of blended essential oils from *Piper betle* and *Melaleuca alternifolia*

Microorganism	Essential oil	MIC (%)		FIC (%)			Interaction
		MIC alone	MIC in combination	FIC BLO	FIC TTO	FICI	
Bacillus subtilis	BLO	0.125	0.0156	1/8 MIC	1/8 MIC	0.250	Synergistic
	TTO	0.125	0.0156				
Enterococcus faecalis	BLO	0.125	0.0313	1/4 MIC	1/8 MIC	0.375	Synergistic
	TTO	0.125	0.0156				
Staphylococcus aureus	BLO	0.125	0.0313	1/4 MIC	1/16 MIC	0.313	Synergistic
	TTO	0.500	0.0313				
Methicillin-resistant *Staphylococcus aureus*	BLO	2.000	0.0625	1/32 MIC	1/4 MIC	0.281	Synergistic
	TTO	0.500	0.1250				
Acinetobacter baumannii	BLO	0.125	0.0313	1/4 MIC	1/8 MIC	0.375	Synergistic
	TTO	0.125	0.0156				
Escherichia coli	BLO	0.125	0.0313	1/4 MIC	1/8 MIC	0.375	Synergistic
	TTO	0.125	0.0156				
Klebsiella pneumoniae	BLO	0.1250	0.0625	1/2 MIC	1/8 MIC	0.625	Additive
	TTO	0.125	0.0156				
Pseudomonas aeruginosa	BLO	2.000	1.0000	1/2 MIC	1/4 MIC	0.750	Additive
	TTO	2.000	0.5000				
Salmonella sp	BLO	0.125	0.0313	1/4 MIC		0.375	Synergistic

	TTO	0.125	0.0156		1/8 MIC		

The data are presented as mean ± SEM; $p < 0.05$ compared with the control.

The combination of BLO and TTO exhibited significant results as detailed in Table 4. Synergistic antibacterial effects of blended essential oils from *P. betle* and *M. alternifolia* against nine different microbial strains showed the considerable reduction in MIC values of both BLO and TTO at different levels, resulting in FICI values varying between 0.250% and 0.750%. In general, the blended essential oils from *P. betle* and *M. alternifolia* reduced the MIC values of BLO and TTO by 2-32-fold, as gains. There was an 8-fold reduction in MIC value for *B. subtilis*, 2-4 fold for *P. aeruginosa*, 2-8 fold for *K. pneumoniae,* 4-8 fold for *E. faecalis, A. baumannii, E. coli* and *Salmonella sp*, 4-16 fold for *S. aureus,* and 4-32 fold for MRSA. Meanwhile, the synergistic antibacterial effects of blended essential oils from *P. betle* and *M. alternifolia* were recorded in seven of the nine bacteria tested, including *B. subtilis* (FICI = 0.250%), *E. faecalis* (FICI = 0.375%), *S. aureus* (FICI = 0.313%), MRSA (FICI = 0.281%), *A. baumannii* (FICI = 0.375%), *E. coli* (FICI = 0.375%) and *Salmonella sp* (FICI = 0.375%). In contrast, additive effects were observed in both Gram-negative bacteria including *K. pneumoniae* and *P. aeruginosa* when treated with blended essential oils resulting in FICI value of 0.625% and 0.750%, respectively.

The combination of essential oils has been explored historically for hundreds of years for both aromatic and therapeutic benefits. Aromatherapy, one of the most popular alternative and complementary therapies, is the practice of blending essential oils topically and aromatically for overall health and well-being. However, there has been not enough scientific research to elucidate their effectiveness in human health. In recent years, numerous efforts have been made to exploit the combination of essential oils in various different fields including cosmetics, food preservatives and pharmaceutical industries. In general, the combination of essential oils has been primarily focused on enhancing the therapeutic efficiency and minimizing the dose of essential oils, thus decreasing the toxicity and adverse reactions. In addition, the interactions between molecules may enhance the solubility and dissolution rate leading to the potential development of new dosage forms and possible improvement of bioavailability. There has been currently an increasing demand for synergistic antibacterial effects of blended essential oils due to not only increasing antibiotic-resistant bacteria at an alarming rate over the last decade, but also negative concerns regarding safety in use of synthetic chemical-based drugs. As mentioned above, the mode of action of individual essential oil has been partially elucidated; however, the mechanism of action of multiple essential oils basically remains unclear, owing to the limited numbers of

investigation on the synergistic antibacterial effects of combined essential oils. The diversified and complicated nature of phytoconstituents of essential oils and the complexity of chemical molecules present in blended essential oils would be a big challenge in elucidating the mechanisms of action. Different chemical molecules may possess different characteristics with regards to chemotypes, ring structures, functional groups, physical and chemical properties. As a result, bioactive compounds may have different mechanisms acting on various different target sites. Thus, it is difficult to predict the synergistic antibacterial effects of blended essential oils against various microbial strains. As scientifically reported elsewhere, numerous studies have been conducted on the synergistic antibacterial effects of essential oils blended with other available substances including commonly used antibiotics, and plant-derived components (such as plant extracts, essential oils or single molecules isolated from plants) [38-41]. In principles, the interactions between components from essential oils may lead to antagonistic, indifferent, additive or synergistic effects. Therefore, microbiological assays would be helpful in not only determining the potency of antibacterial synergism of blended essential oils but also considerably contributing to the elucidation of antibacterial mechanism of action. In this investigation, neither indifferent nor antagonistic effect was detected in any of tested combination of BLO and TTO against nine bacterial strains selected for the assay. Instead, the combination of BLO and TTO has demonstrated the powerful synergistic antibacterial effectiveness against seven of the nine studied bacterial strains. Of the seven interactions synergistically detected, four strains were Gram-positive and three others were Gram-negative bacteria. It is noteworthy to know that additive effects were only detected against both Gram-negative bacteria including *K. pneumoniae* and *P. aeruginosa*. This result might partly support the postulation on the difference in susceptibility of Gram-positive and Gram-negative bacteria to antibacterial agents. Noticeably, among five Gram-negative bacterial strains tested, *P. aeruginosa* was found to be intermediate to both single BLO and TTO with the same highest MIC value of 2.000% (Table 3), apparently confirming the considerable variation of both lipid and polysaccharide components in LPS which may be likely attributed to the notable difference in genera and species of Gram-negative bacteria involved; and even in some cases, to the presence of particular bacterial strain factors. Furthermore, these findings were even consolidated by previous researches as *P. aeruginosa* was also found to be less susceptible to both single BLO and TTO [42-45], thereby partially reflecting the complexity of natural constituents of essential oils and the diversity of biological system of living microorganisms, especially the characteristics of bacterial cell wall and cytoplasmic membrane and the proper function of outer membrane and LPS of Gram-negative bacteria

in facilitating resistance against hydrophobicity nature of various molecules present in essential oils.

4. CONCLUSION

In this study, BLO was shown to consist of 13 compounds, of which eugenol was dominant, whereas TTO was composed of 12 compounds, of which terpinen-4-ol was a major constituent. Overall, the combination of essential oils from *Piper betle* and *Melaleuca alternifolia* had more significant antibacterial effects against all of the nine studied bacterial strains than single essential oil. In general, the blended essential oils from *Piper betle* and *Melaleuca alternifolia* reduced the MIC values of BLO and TTO by 2-32-fold, as gains. The synergistic antibacterial effects were recorded in seven of the nine bacterial strains tested, whereas the additive effects were only observed in both Gram-negative bacteria including *Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* This promising research scientifically contributes to the herbal medicine database system and promotes the significant potential of synergistic antibacterial efficacy of blended essential oils from BLO and TTO that might be exploited for the development of plant-based therapeutic agents for the topical treatment against skin infectious diseases. Nevertheless, further studies are necessary to investigate comprehensively the possible synergistic effects of blended essential oils from BLO and TTO against not only other bacteria but also various microorganisms such as viruses, fungi, yeasts, molds or protozoa. Furthermore, the synergistic interactions need to be characterized at a molecular level for a better understanding of the synergistic mechanism, which would be core fundamentals of biochemistry and molecular biology for future research.

REFERENCES

[1] Chouhan S, Sharma K, Guleria S. Antimicrobial Activity of Some Essential Oils-Present Status and Future Perspectives. Medicines (Basel). 2017;4(3):58.
Doi: 10.3390/medicines4030058. PMID: 28930272; PMCID: PMC5622393.

[2] Guha P, Nandi S. Essential Oil of Betel Leaf (*Piper betle* L.): A Novel Addition to the World Food Sector. 2019.
DOI:10.1007/978-3-030-16546-8_5.

[3] Nayaka NMDMW, Sasadara MMV, Sanjaya DA, Yuda PESK, Dewi NLKAA, Cahyaningsih E, et al. Piper betle (L): Recent Review of Antibacterial and Antifungal Properties, Safety Profiles, and Commercial Applications. Molecules. 2021;26(8):2321.
DOI.org/10.3390/molecules26082321.

[4] Vandana D, Shalini T. Review study on potential activity of *Piper betle.* Journal of Pharmacognosy and Phytochemistry. 2014;3(4):93-98.
Available: https://www.phytojournal.com/vol3Issue4/Issue_nov_2014/17.1.pdf.

[5] Karunanithi S, U Eswaran GM, Guha P, Srivastav PP. A Review on Piper betle L.: Antioxidant, Antimicrobial, Extraction and Application in Food Product Development. Acta Scientific Nutritional Health. 2023;7(1):49-61.
DOI:10.31080/ASNH.2023.07.1170.

[6] Carson CF, Hammer KA, Riley TV. *Melaleuca alternifolia* (Tea Tree) Oil. A Review of Antimicrobial and Other Medicinal Properties. Clinical Microbiology Reviews. 2006;19(1):50-62.
DOI: 10.1128/CMR.19.1.50-62.2006. PMID: 16418522; PMCID: PMC1360273.

[7] de Groot AC, Schmidt E. Tea tree oil: contact allergy and chemical composition. Contact Dermatitis. 2016;75(3):129-43.
DOI: 10.1111/cod.12591. Epub 2016 May 13. PMID: 27173437.

[8] Zhang X, Guo Y, Guo L, Jiang H, Ji Q. *In Vitro* Evaluation of Antioxidant and Antimicrobial Activities of *Melaleuca alternifolia* Essential Oil. BioMed Research International. 2018; Article ID 2396109.
DOI.org/10.1155/2018/2396109.

[9] Australian Tea Tree
https://www.biggreensmile.com/brands/australian-tea-tree.aspx?brand=Australian%20Tea%20Tree

[10] Moshe B. Small and efficient distillation apparatus for extraction of essential oils from plant matter. United States Patent Application Publication. 2008;Pub. No.: US 2008/0128260A1.
Available: https://patents.google.com/patent/US20080128260A1/en.

[11] Scion Instruments 456-GC Reference Manual.
Available: https://www.manualslib.com/manual/1626438/Scion-Instruments-456-Gc.html.

[12] AOAC. Official Methods of Analysis of the Association of Official Analytical Chemists. 15th Ed. Washington DC. 1990;1:1223.

[13] Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996.
Available: https://www.ncbi.nlm.nih.gov/books/NBK7627/.

[14] Laboratory Guide: Methodologies for Antimicrobial Susceptibility Testing (Manual). Committee on Trade and Investment (CTI), Sub-Committee on Standards and Conformance (SCSC). 2020.
Available: https://www.apec.org/Publications/2020/05/Laboratory-Guide-Methodologies-for-Antimicrobial-Susceptibility-Testing.

[15] Mark L. Antimicrobial Susceptibility Testing. American Association for Clinical Chemistry (AACC). 2017.
Available: https://www.aacc.org/science-and-research/clinical-chemistry-trainee-council/trainee-council-in-english/pearls-of-laboratory-medicine/2017/antimicrobial-susceptibility-testing.

[16] Kowalska-Krochmal B, Dudek-Wicher R. The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. Pathogens. 2021;10(2):165. DOI: 10.3390/pathogens10020165. PMID: 33557078; PMCID: PMC7913839.

[17] Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). 2009. Available: https://onlinelibrary.wiley.com/doi/10.1046/j.1469-0691.2000.00142.x.

[18] van Vuuren S, Viljoen A. Plant-based antimicrobial studies--methods and approaches to study the interaction between natural products. Planta Med. 2011;77(11):1168-82. DOI: 10.1055/s-0030-1250736. Epub 2011 Jan 31. Erratum in: Planta Med. 2012 Feb;78(3):302. PMID: 21283954.

[19] Faleiro ML, Miguel MG. Chapter 6 - Use of Essential Oils and Their Components against Multidrug-Resistant Bacteria. In: Mahendra KR, Kateryna VK, editors. Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and their Components. Academic Press; 2013. DOI:10.1016/B978-0-12-398539-2.00006-9.

[20] Kalpna DR, Mital JK, Sumitra VC. Chapter 11 - Medicinal Plants as Alternative Sources of Therapeutics against Multidrug-Resistant Pathogenic Microorganisms Based on Their Antimicrobial Potential and Synergistic Properties. In: Mahendra KR, Kateryna VK, editors. Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and their Components. Academic Press; 2013. DOI:10.1016/B978-0-12-398539-2.00011-2.

[21] International Standard. Essential oil of *Melaleuca*, terpinen-4-ol type (Tea Tree oil). iTeh STANDARD PREVIEW (standards.iteh.ai) ISO 4730:2017. Third edition 2017-02. Available:https://cdn.standards.iteh.ai/samples/69082/302bafb843a64a95b8c74676e282d921/ISO-4730-2017.pdf.

[22] Su R, Bai X, Liu X, Song L, Liu X, Zhan X, et al. Antibacterial Mechanism of Eugenol Against Shigella sonnei and Its Antibacterial Application in Lettuce Juice.Foodborne Pathogens and Disease. 2022:779-786. DOI.org/10.1089/fpd.2022.0046.

[23] Mak KK, Kamal MB, Ayuba SB, Sakirolla R, Kang YB, Mohandas K, et al. A comprehensive review on eugenol's antimicrobial properties and industry applications: A transformation from ethnomedicine to industry. Phcog Rev. 2019;13:1-9. Available: https://www.phcogrev.com/sites/default/files/PhcogRev_2019_13_25_1.pdf.

[24] Bai X, Li X, Liu X, Xing Z, Su R, Wang Y, et al. Antibacterial Effect of Eugenol on Shigella flexneri and Its Mechanism. Foods. 2022;11:2565. DOI.org/10.3390/foods11172565 PMID: 36076751; PMCID: PMC9455010.

[25] Shapira S, Pleban S, Kazanov D, Tirosh P, Arber N. Terpinen-4-ol: A Novel and Promising Therapeutic Agent for Human Gastrointestinal Cancers. PLoS One. 2016;11(6):e0156540. DOI: 10.1371/journal.pone.0156540. PMID: 27275783; PMCID: PMC4898785.

[26] Cordeiro L, Figueiredo P, Souza H, Sousa A, Andrade-Júnior F, Medeiros D. Terpinen-4-ol as an Antibacterial and Antibiofilm Agent against *Staphylococcus aureus*. Int. J. Mol. Sci. 2020;21:4531.
DOI.org/10.3390/ijms21124531 PMID: 32630600; PMCID: PMC7350221.

[27] Li WR, Li HL, Shi QS, Sun TL, Xie XB, Song B, et al. The dynamics and mechanism of the antimicrobial activity of tea tree oil against bacteria and fungi. Applied Microbiology and Biotechnology. 2016;100(20):8865-8875.
DOI: 10.1007/s00253-016-7692-4. PMID: 27388769.

[28] Yang SK, Tan NP, Chong CW, Abushelaibi A, Lim SH, Lai KS. The Missing Piece: Recent Approaches Investigating the Antimicrobial Mode of Action of Essential Oils. Evolutionary Bioinformatics. 2021;17.
DOI:10.1177/1176934320938391 PMID: 34017165; PMCID: PMC8114247.

[29] Tang C, Chen J, Zhang L, Zhang R, Zhang S, Ye S, et al. Exploring the antibacterial mechanism of essential oils by membrane permeability, apoptosis and biofilm formation combination with proteomics analysis against methicillin-resistant staphylococcus aureus. International Journal of Medical Microbiology. 2020;310(5):151435.
DOI.org/10.1016/j.ijmm.2020.151435.

[30] Puškárová A, Bučková M, Kraková L, Pangallo D, Kozics K. The antibacterial and antifungal activity of six essential oils and their cyto/genotoxicity to human HEL 12469 cells. Sci Rep. 2017;7(1):8211.
DOI: 10.1038/s41598-017-08673-9. PMID: 28811611; PMCID: PMC5557807.

[31] Zaïri A, Nouir S, Zarrouk A, et al. Chemical composition, Fatty acids profile and Biological properties of Thymus capitatus (L.) Hoffmanns, essential Oil. Sci Rep. 2019;9: 20134.
DOI.org/10.1038/s41598-019-56580-y.

[32] Mak KK, Kamal MB, Ayuba SB, Sakirolla R, Kang YB, Mohandas K, et al. A comprehensive review on eugenol's antimicrobial properties and industry applications: A transformation from ethnomedicine to industry. Phcog Rev 2019;13(25).
Available: https://www.phcogrev.com/sites/default/files/PhcogRev_2019_13_25_1.pdf.

[33] Angane M, Swift S, Huang K, Butts CA, Quek SY. Essential Oils and Their Major Components: An Updated Review on Antimicrobial Activities, Mechanism of Action and Their Potential Application in the Food Industry. Foods. 2022;11: 464.
DOI.org/ 10.3390/foods11030464. PMID: 35159614; PMCID: PMC8833992.

[34] Mohammadi Nejad S, Özgüneş H, Başaran N. Pharmacological and Toxicological Properties of Eugenol. Turk J Pharm Sci. 2017;14(2):201-206.
DOI: 10.4274/tjps.62207. Epub 2017 Aug 15. PMID: 32454614; PMCID: PMC7227856.

[35] Elbestawy MKM, El-Sherbiny GM, Moghannem SA. Antibacterial, Antibiofilm and Anti-Inflammatory Activities of Eugenol Clove Essential Oil against Resistant *Helicobacter pylori*. Molecules. 2023;28(6):2448.
DOI.org/10.3390/molecules28062448. PMID: 36985419; PMCID: PMC10058968.

[36] Carson CF, Mee BJ, Riley TV. Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. Antimicrob Agents Chemother. 2002;46(6):1914-20.

DOI: 10.1128/AAC.46.6.1914-1920.2002. PMID: 12019108; PMCID: PMC127210.

[37] Cordeiro L, Figueiredo P, Souza H, Sousa A, Andrade-Júnior F, Medeiros D, et al. Terpinen-4-ol as an Antibacterial and Antibiofilm Agent against *Staphylococcus aureus*. Int J Mol Sci. 2020;21(12):4531.
DOI: 10.3390/ijms21124531. PMID: 32630600; PMCID: PMC7350221.

[38] Fadila M, Tajelmolk A. Evaluation of antibacterial activity and synergistic effect between antibiotic and the essential oils of some medicinal plant. Asian Pacific Journal of Tropical Biomedicine. 2016;6(1):32-37.
DOI.org/10.1016/j.apjtb.2015.09.024.

[39] Sharma K, Guleria S, Razdan V, Babu V. Synergistic antioxidant and antimicrobial activities of essential oils of some selected medicinal plants in combination and with synthetic compounds. Industrial Crops and Products. 2020;154:112569.
DOI.org/10.1016/j.indcrop.2020.112569.

[40] Huang X, Lao Y, Pan Y, Chen Y, Zhao H, Gong L, et al. Synergistic Antimicrobial Effectiveness of Plant Essential Oil and Its Application in Seafood Preservation: A Review. Molecules. 2021;26(2):307.
DOI.org/10.3390/molecules26020307.

[41] Fahimi S, Hajimehdipoor H, Shabanpoor H, Bagheri F, Shekarchi M. Synergic antibacterial activity of some essential oils from Lamiaceae. Research Journal of Pharmacognosy. 2015;2(3):23-29.
Available:https://www.researchgate.net/publication/313049591_Synergic_antibacterial_activity_of_some_essential_oils_from_Lamiaceae.

[42] Suppakul P, Sanla-Ead N, Phoopuritham P. Antimicrobial and antioxidant activities of betel oil. Kasetsart Journal - Natural Science. 2006:40.91-100.
Available:https://www.researchgate.net/publication/233727986_Antimicrobial_and_antioxidant_activities_of_betel_oil.

[43] Longbottom CJ, Carson CF, Hammer KA, Mee BJ, Riley TV. Tolerance of *Pseudomonas aeruginosa* to *Melaleuca alternifolia* (tea tree) oil is associated with the outer membrane and energy-dependent cellular processes. Journal of Antimicrobial Chemotherapy. 2004;54(2):386–392.
DOI.org/10.1093/jac/dkh359. Epub 2004 Jul 14. PMID: 15254026.

[44] Papadopoulos CJ, Carson CF, Hammer KA, Riley TV. Susceptibility of pseudomonads to Melaleuca alternifolia (tea tree) oil and components. J Antimicrob Chemother. 2006;58(2):449-51.
DOI: 10.1093/jac/dkl200. Epub 2006 May 30. PMID: 16735435.

[45] Carson CF, Papadopoulos CJ, Hammer KA, Riley TV. Tolerance of Pseudomonas aeruginosa to Tea Tree Oil. A report for the Rural Industries Research and Development Corporation. Publication No. 05/126 Project No. UWA-79A. 2005.
Available: https://agrifutures.com.au/wp-content/uploads/publications/05-126.pdf.