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Chemical Composition, Antioxidant and Antimicrobial Activity of The Essential Oils from *Melaleuca Alternifolia* and *Citrus Hystrix*

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Abstract

This investigation aimed to assess essential oil chemical composition, antioxidant, and antimicrobial activity from *Melaleuca alternifolia* and *Citrus hystrix* peel. The essential compositions were identified using gas chromatography-mass spectrometry (GC-MS). Antioxidant activity was evaluated using the DPPH assay. Antimicrobial activity and synergistic effects of combined essential oils were assayed against five selected microbial strains (*Candida albicans*, *Staphylococcus aureus*, *Methicillin-resistant Staphylococcus aureus*, *Staphylococcus pyogenes*, and *Acinetobacter baumannii*. In this research, GC-MS analysis revealed the presence of 25 compounds in *Citrus hystrix* peel essential oil, of which citronella was a major component, whereas terpinen-4-ol was a key constituent among 25 compounds detected in *Melaleuca alternifolia* essential oil. *Melaleuca alternifolia essential oil* exhibited higher antioxidant activity with IC_{50} of 79.044 ± 8.183 µg/mL than *Citrus hystrix* peel essential oil with IC_{50} of 572.075 ± 71.005 µg/mL. Minimum inhibitory concentration values of *Citrus hystrix* peel essential oil were found between 0.250% and 1.000%, whereas minimum inhibitory concentration values of *Melaleuca alternifolia* essential oil were recorded between 0.0078% and 0.125%. The combination of essential oils from *Melaleuca alternifolia* and *Citrus hystrix* peel displayed the powerful synergistic antimicrobial effectiveness against three of the five studied microbial species. The additive antimicrobial effects, however, were documented in blended essential oils against *Staphylococcus aureus* and *Candida albicans*.

Keywords: *Melaleuca alternifolia;* Citrus hystrix; chemical compositions; antioxidant

1. Introduction

For many decades now, natural materials have been studied to replace synthetic products which might be harmful to human health. Previous studies have provided much evidence on the advantages of tea tree and kaffir lime peels in both crude extract and essential oil forms that suggested a study on the cosmeceutical potential of essential oils from *Melaleuca alternifolia* (*M. alternifolia*) (1), (2) and *Citrus hystrix* (*C. hystrix*) peels which are widely known for their antimicrobial and anti-inflammatory activity (3), (4), (5). Since antibiotic-resistant bacteria have been increasing at an alarming rate over the last decade, the combination of different pharmacological activity-proven agents of natural origin could be an effective solution to enhance the effectiveness of drugs and reduce their adverse reactions and toxicity. On the other hand, there could be additionally a possibility of improving pharmacokinetic profiles such as bioavailability or dosage forms (6).

M. alternifolia commonly known as tea tree, is a member of the family Myrtaceae native to Australia. Tea tree essential oil (TTO) has been widely studied in use as an alternative and complementary therapy for various skin conditions over 100 years, owing to the presence of typical bioactive phytochemical organic groups including phenolic, polyphenols, flavonoids, terpenes (7). Besides, numerous previous scientific studies revealed that TTO exerts significant pharmacological properties including antioxidant, antimicrobial, and anti-inflammatory activity. TTO-based commercial cosmeceuticals and health care products are now widely available over the counter in drug stores or chemists' shops. TTO is commonly incorporated as an active ingredient into topical formulations such as tea tree face cream, tea tree medicated gel for acne or purifying tea tree cleansing soap (8).

C. hystrix commonly known as kaffir lime belongs to the family of Rutaceae. The plant is natively distributed in Southeast Asia and has been used as an aromatic spice, cosmetics, and medicine reducing the severity of certain illnesses (9). Kaffir lime essential oil (KLO), a mixture of natural volatile compounds extracted mainly from kaffir lime peels, has been proven to exert antimicrobial activity against a broad spectrum of microorganisms with less toxicity to humans than other existing antibiotics. Reports from previous studies indicated that the pharmacological properties of KLO are attributed primarily to the major phyto-contituents present in KLO including terpinen-4-ol, α-terpineol, 1,8-cineole, citronellal, limonene. (10). Furthermore, KLO has been found potentially effective in inhibiting tyrosinase, which is well known as a copper-containing enzyme responsible for the production of melanin determining skin, eyes and hair color. According to the Food and Agriculture Organization (FAO) statistics, approximately 60% of 998,7 thousand tons of citrus fruit waste were eliminated per year in Vietnam (11). Utilization of such a huge waste would be thus both a challenge and opportunity toward

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environmentally friendly sustainable development. In this regard, this study was primarily focused on investigating the chemical composition and synergistic antimicrobial potentials of blended essential oils from *M. alternifolia* and *C. hystrix.*

Antimicrobial.

2. Material and Methods

a. Chemicals and reagents

All chemicals and reagents were provided by Pharmaceutical Chemistry Laboratory and Applied Biochemistry Laboratory of Applied Biochemistry Department of International University HCMC. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich. Mueller-Hinton agar, Tryptic Soy Agar, and Sabouraud Dextrose Agar were obtained from Merck, Germany. All chemicals and reagents were stored in accordance with the most stringent regulations and freshly prepared with distilled water to the desired concentrations for experiment purposes.

b. Extraction of essential oils

Fresh *M. alternifolia* leaves and *C. hystrix* peels were collected from a farm in Southern Vietnam 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 subjected to the microwave-assisted extraction NEOS Milestone for 90 min under the power of 900 W **(12)**, **(13)**. Once the distillation was complete, the obtained essential oil was dehydrated using anhydrous sodium sulphate and then stored in sealed amber vials at 4^{o}C until GC-MS analysis.

c. Gas chromatography- Mass spectrometry

Gas chromatography-Mass spectroscopy (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^{o}C for 1 min and then increased to 80^{o}C at 30^{o}C/min. After that, it was increased to 230^{o}C at 5^{0}C/min, and finally to 280^{o}C at 25^{o}C/min where it was held isothermally for 3 min. The injector temperature was set at 250^{o}C and the rate of Division was 1:30. Fragmentation was done by electron impact (EI+) 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 **(14)**.

d. DPPH free-radical scavenging assay

The antioxidant capacity of the essential oil was quantitatively assessed using DPPH method **(15)**, **(16)**. Ascorbic acid was used as reference standard. The assay was prepared by mixing 500 µL of obtained essential oil with 500 µL of 0.1 mM DPPH and then vortexed vigorously. The mixture was subsequently incubated for 30 min in the dark at room temperature. The absorbance of the mixture was spectrophotometrically measured by Multiskan Microplate Spectrophotometer **(17)** against the blank at 517 nm using Biotek Synergy HT 96-well plate reader (USA) **(18)**. The antioxidant capacity was calculated using the following formula:

$$\%\ Inhibition\ = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

The DPPH free-radical scavenging activity was expressed as IC_{50} which was the concentration of the sample required to inhibit 50% of DPPH free radicals **(19)**.

e. Antimicrobial synergism assay

i. Microbial strains

The antibacterial effectiveness of essential oils from *M. alternifolia* and *C. hystrix P.* were tested against four different microbial strains procured from International University. Gram-positive species were *Staphylococcus aureus* ATCC25923 (*S. aureus*), *Methicillin-resistant Staphylococcus aureus* (*MRSA*), and *Staphylococcus pyogenes* ATCC 19615 (*S. pyogenes*). Gram-negative strain was *Acinetobacter baumannii* (ATCC 19606) (*A. baumannii*). In addition, the fungus *Candida albicans* ATCC10231 (*C. albicans*) from Pasteur Institute Ho Chi Minh City was also subjected to the assay. The identity of the microorganisms assayed in this research was confirmed by morphological studies and standard biochemical tests. The bacteria species were maintained in Blood agar (BA), Mueller Hinton Agar (MHA) and Tryptic Soy Agar (TSA) (BA, MHA, and TSA, Merck, Germany), whereas *C. albicans* was maintained on Sabouraud Dextrose Agar (SDA, Merck, Germany). **(20), (21), (22), (23)**

ii. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) **(24)** was conducted to investigate the sensitivity of a microorganism to essential oil using disk diffusion method on agar media **(25)**. For each sample, paper disks with 6 mm diameter were soaked with 100 uL of the essential oil for the assay while DMSO 1% was used as a negative control and gentamicin (10 ug) and fluconazole (10 ug) as positive controls for bacteria and fungi, respectively. 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 millimeter, mm). The positive control used for bacterial species was Gentamicin 10 ug and Fluconazole 10 ug for fungi species (ASD04300 and ASD03931, Bioanalyse, Turkey)

iii. Minimum inhibitory concentration assay

The MIC of EOs was determined by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines using 96-well plates **(26)**, **(27)**, **(28)**. Two-fold serial dilutions of essential oil were prepared by dilution with DMSO to produce a series of decreasing concentrations (4.00, 2.00, 1.00, 0.50, 0.25, 0.125, 0.0625 and 0.0313%). The assay

was undertaken in 96 flat-bottom well plates. Fresh overnight cultures of bacteria and fungi were adjusted with media to an inoculum concentration of 1.0 x 10^6 CFU/per well.

Gentamicin was used as a positive control against test bacteria **(29)**, **(30)** whilst fluconazole was selected as a positive control for fungus **(31)**. Negative control of untreated media was prepared under the same investigational conditions. Plates were then incubated with shaking for 48 h at incubation temperatures of 37°C for the bacterial plates and 33°C for the *Candida* plates. Optical densities were observed at wavelength 600 nm (OD_{600}). MIC value was defined as the lowest concentration of essential oil that completely inhibited visible growth of the tested microorganisms.

iv. Synergy checkerboard assay

The fractional inhibitory concentration (FIC) was determined by the checkerboard assay **(32)**. The 96-well plate was prepared with nutrient medium, microbial solution, and testing mixture or control solution. Two-fold serial dilutions of essential oil were prepared by dilution with DMSO to achieve a series of decreasing concentration from 4% (v/v) (2.00, 1.00, 0.50, 0.25, 0.125, 0.0625, 0.0313, 0.0156, 0.0078, and 0.0039%). The plate was incubated for 24-36 h at 37ºC for the bacteria and 33ºC for the fungus. The plate was then measured using a spectrophotometer at 600 nm. All experiments used to calculate the FIC index (FICI) were performed similarly to that of MIC. The FICI values were calculated using the following equation **(33)**:

$$\mathbf{FICI} = \frac{\mathbf{MIC_{A\ in\ combination}}}{\mathbf{MIC_{A\ alone}}} + \frac{\mathbf{MIC_{B\ in\ combination}}}{\mathbf{MIC_{B\ alone}}}$$

The interaction was categorized as follows: Synergism as FICI ≤ 0.5; additive effect as 0.5 < FICI ≤ 1; indifference as 1 < FICI ≤ 4; and FICI > 4 was considered to be antagonism.

f. Statistical analysis

All experiments were performed in triplicates, with the results expressed as mean ± SD of three independent experiments. Data analysis was done by ANOVA test for a single factor using SPSS **(34)**. All differences with a p value < 0.05 were considered statistically significant.

3. Results and discussion

a. Chemical composition of essential oil

The chemical composition of KLO is shown in Table 1 and Figure 1. A total of 25 different compounds representing approximately 99.834% of the total oil were qualitatively and quantitatively identified (trace components below 0.05% were not listed). The chemical composition of KLO which was determined by GC-MS revealed the presence of citronellal (42.505%) followed by other components: terpinen-4-ol (10.656%), β-pinene (8.136%), α-terpineol (7.365%), limonene (6.846%), and sabinene (4.192%). Meanwhile other compounds made contributions of less than 3% each to the total composition of essential oil.

On the other hand, the essential oil from *M. alternifolia* consists of 25 compounds accounting for 99.958% of the total oil (trace components below 0.05% were not listed) (Table 2 and Figure 2)**.** The major components of TTO were terpinen-4-ol (29.983%), followed by other components: eucalyptol (25.670%), α-terpineol (14.130%), γ-terpinene (9.085%), and limonene (4.115%). Meanwhile, other substances contributed less than 3% each to the total composition of essential oil. Noticeably, a comparison of constituents between KLO and TTO shows the presence of two major compounds, namely terpinen-4-ol, α-terpineol and limonene, in both essential oils, but at significantly different concentrations. Nevertheless, since these three compounds are well known as major molecules substantially responsible for antibacterial activity of both KLO 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 **(35), (36), (37), (38).**

Table 1. Chemical composition of essential oil from *Citrus hystrix*

No.	Retention time (min)	Identified compound	Quantity (%)	Molecular mass	Retention index
1	4.069	α-Pinene	0.651	136	906
2	4.666	Sabinen	4.192	136	889
3	4.768	(-)-β-Pinene	7.716	136	939
4	4.867	β-Pinene	0.420	136	908
5	5.411	Terpinolene	1.039	136	863
6	5.654	Limonene	6.846	136	920
7	6.234	γ-Terpinen	2.340	136	887
8	6.516	Linalool oxide	0.692	170	841
9	6.878	Terpinolene	1.550	136	922
10	7.047	3-Carene	3.411	136	858
11	8.219	Carveol, dihydro-	1.926	154	828
12	8.357	Citronellal	42.505	154	900
13	8.515	Isopulegol	1.287	154	893
14	8.725	Camphol	0.244	154	827
15	8.998	4-Terpinenyl acetate	10.656	196	827
16	9.294	α-Terpineol	7.365	154	923
17	10.098	Citronellol	3.390	156	885
18	10.751	Pseudolimonen	0.241	136	812
19	13.229	Citronellol acetate	1.209	198	896
20	13.987	Ylangene	1.045	204	818
21	15.105	Caryophyllene	0.226	204	879
22	17.546	δ-Cadinene	0.524	204	847
23	18.381	Nerolidol, acetate	0.168	264	828
24	19.735	Cadinene	0.191	204	863
25	19.970	β-Guaiene	0.167	204	846

Table 2. Chemical composition of essential oil from *Melaleuca alternifolia.*

No.	Retention time (min)	Identified compound	Quantity (%)	Molecular mass	Retention index
1	4.068	α-Pinene	1.243	136	914
2	4.759	L-β-Pinene	0.443	136	938
3	4.866	β-Myrcene	0.841	136	888
4	5.414	Terpinolene	3.412	136	895
5	5.561	o-Cymene	1.324	134	944
6	5.664	Limonene	4.115	136	917
7	5.760	Eucalyptol	25.670	154	923
8	6.246	γ-Terpinene	9.085	136	932
9	6.880	Isoterpinolene	1.943	136	881
10	8.710	cis-Ocimenol	0.205	154	795
11	9.032	4-Terpinenyl acetate	29.983	196	828
12	9.313	α-Terpineol	14.130	154	920
13	15.584	Aromandendrene	0.286	204	869
14	16.925	δ-Guaiene	0.627	204	935
15	17.546	δ-Cadinene	0.408	204	786
16	18.647	β-Guaiene	0.224	204	841
17	19.027	γ-Gurjunene	1.874	204	882
18	19.200	Leden	1.601	204	859
19	19.354	β-Gurjunene	0.591	204	810
20	19.639	Guaia-6,9-diene	0.572	204	843
21	19.693	Copaene	0.607	204	799
22	19.803	β-Vatirenene	0.164	202	815
23	19.851	β-Copaene	0.215	204	795
24	19.877	α-Copaene	0.207	204	833
25	19.984	Valencene	0.188	204	831

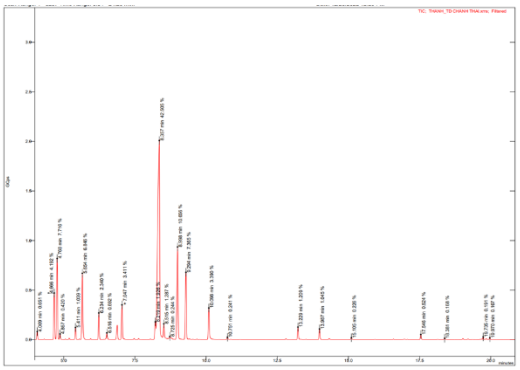


Fig 1. GC-MS Chromatogram of *Citrus hystrix* peel essential oil.

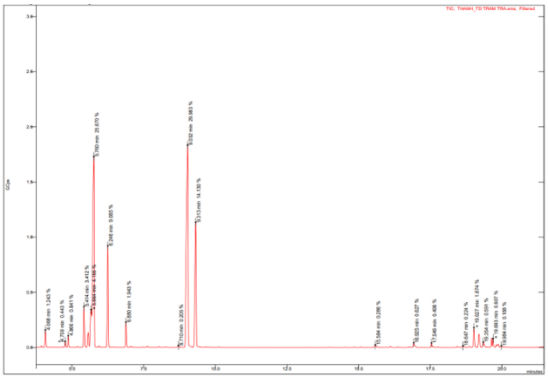


Fig 2. GC-MS Chromatogram of *Melaleuca alternifolia* essential oil.

b. DPPH free-radical scavenging activity

In this section, the IC_{50}, IC_{50} means, and SD were calculated from three sub-calibration curves from each of the triplication. Overall, the amount of ascorbic acid, TTO and KLO that required to inhibit 50% of DPPH radical were 1.999 ± 0.245 µg/mL, 79.044 ± 8.183 µg/mL and 572.075 ± 71.005 µg/mL, respectively. As summarized in Table 3, ascorbic acid was recorded with IC_{50} value of 1.999 ± 0.245 µg/mL, firmly denoting the most powerful antioxidant activity as compared to both essential oils (KLO and TTO). There was a statistically significant difference in IC_{50} between KLO and TTO. Accordingly, IC_{50} of TTO was found to be approximately 40 times higher than that of ascorbic acid, whereas IC_{50} of KLO was documented approximately 7 times higher than that of TTO and 286 times when comparing to ascorbic acid

Table 3. DPPH free-radical scavenging activity.

Sample	Calibration curve	R^2	* IC_{50} (µg/mL)
Ascorbic Acid	y = 3.2375x + 46.101	0.9249	1.999 ± 0.245
TTO	y = 0.6505x + 1.124	0.9995	79.044 ± 8.183
KLO	y = 0.0667x + 11.954	0.9417	572.075 ± 71.005

* Presented as Means ± SD (n=3).

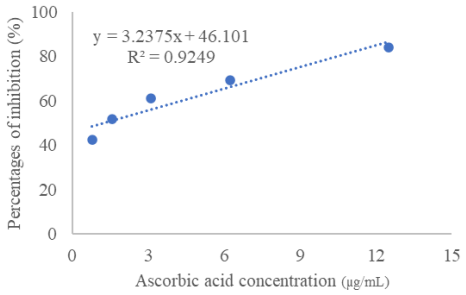


Fig 3. Calibration curve of Ascorbic acid

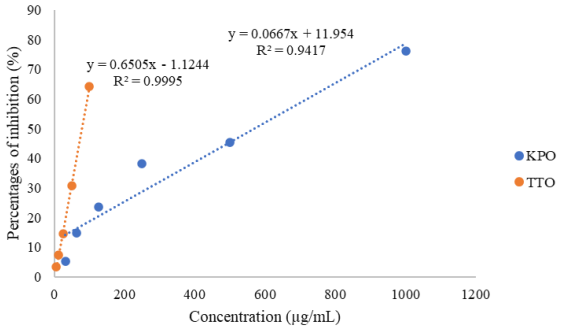


Fig 4. Calibration curves of KPO and TTO

c. Antimicrobial susceptibility values

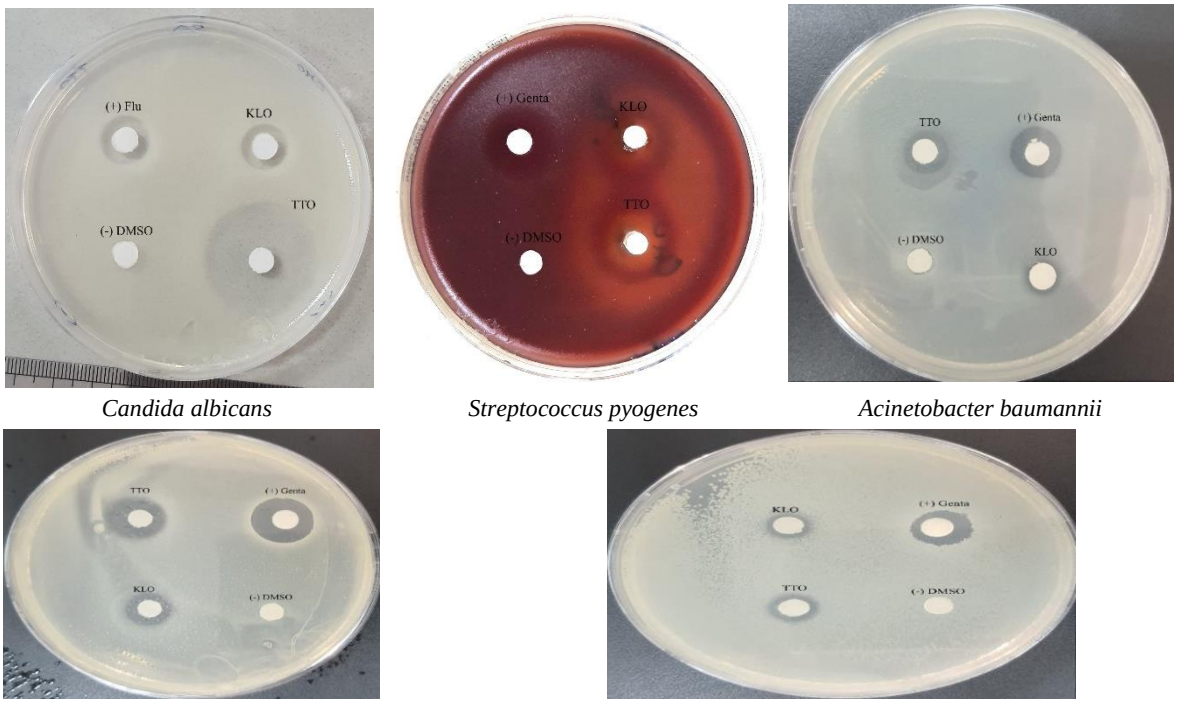
In general, KLO displayed less effective in antimicrobial susceptibility assay against five selected microbial strains than that of TTO. The inhibitory zone diameter of KLO was recorded in the range between 10.67 ± 0.58 and 16.33 ± 0.58 mm, whereas the inhibitory zone diameter of TTO was documented between 13.67 ± 0.58 and 35.00 ± 1.00 mm) (Table 4 and Figure 5). The most highly significant difference was observed in *C. albicans* in which the inhibitory zone diameter of TTO against *C. albicans* was 35.00 ± 1.00 mm which was approximately two and a half times greater than that of KLO (13.67 ± 1.15 mm). TTO exhibited better inhibition against the tested microbial species than their positive controls. KLO exerted smaller inhibition zone diameters compared to positive controls against almost tested species, except *A. baumannii*. There was a significant difference on inhibition zone diameter within each species (*A. baumannii* $p = 0.016$, *S. aureus* $p = 0.001$, MRSA $p = 0.016$, *S. pyogenes* $p = 0.013$, *C. albicans* $p = 0.0001$)

Table 4. Inhibitory zone diameter of essential oils against five selected microbial species.

Microbial species	*Inhibitory zone diameter (mm)		
	** Positive control	** KLO	** TTO
S. aureus	[a]16.67 ± 1.53	11.00 ± 1.00	16.67 ± 0.58
MRSA	[a] 13.33 ± 0.58	11.67 ± 0.58	14.67 ± 1.15
A. baumannii	[a] 9.33 ± 0.58	10.67 ± 0.58	13.67 ± 0.58
S. pyogenes	[a] 17.33 ± 1.00	16.33 ± 0.58	19.33 ± 0.58
C. albicans	[b] 22.00 ± 1.73	13.67 ± 1.15	35.00 ± 1.00

* Presented as Means ± SD (n = 3). ** Significant difference within each species ($p < 0.05$)

[a] Positive control zone diameter performed by standard gentamicin 10 µg each. [b] Positive control zone diameter performed by standard fluconazole 10 µg each.



Candida albicans | *Streptococcus pyogenes* | *Acinetobacter baumannii*

Staphylococcus aureus | MRSA

Including TTO zone; KLO zone; (+) Genta zone: Gentamicin as a positive control against bacterial species; (+) Flu zone: Fluconazole as a positive control against *Candida albicans*; (-) DMSO: negative control.

Fig 5. Inhibitory zone diameter of essential oils against five selected microbial species.

d. Minimum inhibitory concentration values

The results detailed in Table 5 clearly show that both KLO and TTO were found effective against all microbial strains tested, but at different levels. MIC values of both KLO and TTO were generally varying between 0.0078% and 1.000%. However, MIC values of KLO were generally higher than that of TTO. Accordingly, MIC values of KLO were found between 0.25% and 1.0%, whereas MIC values of TTO were recorded between 0.0078% and 0.125%. TTO was able to powerfully inhibit the growth of *C. albicans* with the lowest MIC value of 0.0078%, whereas MRSA displayed the least sensitivity to TTO with the highest MIC value of 0.500%. Meanwhile, KLO exhibited the lowest MIC value of 0.250% against *C. albicans*, followed by *S. aureus* with MIC value of 0.500% and three remaining bacterial strains with the same highest MIC value of 1.000%.

Table 5. MIC and FICI values of blended essential oils from *Melaleuca alternifolia* and *Citrus hystrix*.

Species	Sample	MIC (%,v/v)		FIC (%)			Interaction
		MIC alone	MIC in combination	FIC_{TTO}	FIC_{KLO}	FICI	
Staphylococcus aureus	TTO	0.125	0.0625	½	¼	0.750	Additive
	KLO	0.500	0.125				
MRSA	TTO	0.500	0.0625	⅛	¼	0.375	Synergistic
	KLO	1.000	0.250				
Acinetobacter baumannii	TTO	0.250	0.0313	⅛	⅛	0.250	Synergistic
	KLO	1.000	0.125				
Streptococcus pyogenes	TTO	0.0625	0.0078	⅛	¼	0.375	Synergistic
	KLO	1.000	0.250				
Candida albicans	TTO	0.0078	0.0039	½	½	1.000	Additive
	KLO	0.250	0.125				

e. Fractional inhibitory concentration values

The combination of KLO and TTO exhibited significant results as summarized in Table 5. Synergistic antimicrobial effects of blended essential oils from *C. hystrix* and *M. alternifolia* against five different microbial strains showed the considerable reduction in MIC values of both KLO and TTO at different levels, resulting in FICI values varying between 0.250% and 1.000%. In general, the mixture of essential oils from *C. hystrix* and *M. alternifolia* reduced the MIC values of KLO and TTO by 2-8-fold, as gains. There was a 2-fold reduction in MIC value for *C. albicans*, 2-4-fold for *S. aureus*, 4-8-fold for MRSA and *S. pyogenes*, and 8-fold for *A. baumannii*. Meanwhile, the synergistic antibacterial effects of blended essential oils from *C. hystrix* and *M. alternifolia* were recorded in three of the five microbial strains tested, including MRSA (FICI = 0.375%), *A. baumannii* (FICI = 0.250%), and *S. pyogenes* (FICI = 0.375%). On the contrary, additive effects were observed in *S. aureus* and *C. albicans* when treated with blended essential oils resulting in FICI value of 0.750% and 1.000%, respectively.

As reported from previous literature, in general, the most commonly possible antibacterial mechanism of essential oils is related to the bacterial cell wall and plasma membrane compositions which are identified as the major target sites of essential oils. Compounds present in essential oils, being hydrophobic molecules, are able to penetrate into the cell wall and cytoplasmic membrane of microorganisms. As shown in Table 1, the major components present in KLO are citronella and terpinen-4-ol whilst dominant compounds present in TTO were terpinen-4-ol and eucalyptol. These compounds, however, have been intensively subjected to investigating antimicrobial activity, including their mode of action. Terpinen-4-ol could powerfully inhibit the synthesis of protein, DNA and ATPase, and cause the release of vital ions such as Ca^{2+} and Mg^{2+}. Eugenol, on the other hand, was capable of inactivating a variety of bacterial enzymes such as amylase, protease, or ATPase, and cause the leakage of intracellular components. Both components had a capacity of inhibiting the biofilm formation resulting in increased number of dead cells **(39), (40), (41)**.

While the mechanism of action of single essential oil and their molecules has been partly elucidated, the mode of action of multiple essential oil generally remains unclear. Basically, the practice of combining essential oils is chiefly exploited for the synergistic pharmacological effectiveness resulting from the synergism of diversified and complicated nature of phytoconstituents present in blended essential oils. In view of the alternative and complementary therapy, the combination of different medicinal plants in general or essential oils in particular is a crucial therapeutic philosophy of herbal medicine where the combination of key components from different medicinal plants or essential oils is believed to be more effective than single isolated constituents **(42)**.

Considering the effectiveness of current synthetic drugs, however, an increasing antibiotic-resistant microorganisms would be of concern to health care providers, clinicians, and scientists. In principle, the interactions between components from essential oils may lead to antagonistic, indifferent, additive or synergistic effects **(43)**. Microbiological assays thus 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 research, neither antagonistic nor indifferent interaction was detected in any of tested combination of KLO and TTO against five microbial strains selected for the assay. The combination of KLO and TTO, conversely, displayed the powerful synergistic antimicrobial effectiveness against three of the five studied microbial species. The additive antimicrobial effects, however, were documented in blended essential oils against *S. aureus* and *C. albicans* **(44), (45)**. It should be noted, nevertheless, the MIC values of both KLO and TTO were the lowest recorded in the assay against *C. albicans*, but no synergistic interaction was detected in the combination of essential oils, apparently confirming the difference in molecular biological structure and function between bacteria and fungi. This indicates that essential oils do not necessarily have to be combined based purely on independent noteworthy antimicrobial activity. **(46)**.

4. Conclusion

In this study, GC-MS analysis revealed the presence of 25 compounds in KLO, of which citronella was a major component, whereas terpinen-4-ol was a key constituent among 25 compounds detected in TTO. Overall, the combination of essential oils from *Melaleuca alternifolia* and *Citrus hystrix* had more significant antimicrobial effects against all the five studied microbial strains than single essential oil. The synergistic antibacterial effects were recorded in three of the five microbial strains tested. 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 KLO and TTO that might be exploited for the development of plant-based therapeutic agents for the topical treatment against skin infectious diseases.

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5. Conflict of interest

There are no conflicts to declare.

6. References

[1] Hammer K.A., Riley T.V., Carson C.F. Antifungal activity of the components of Melaleuca alternifolia (tea tree) oil. Journal of Applied Microbiology. 2003;95(4):853–60.

[2] Palombo E.A. Traditional Medicinal Plant Extracts and Natural Products with Activity against Oral Bacteria: Potential Application in the Prevention and Treatment of Oral Diseases. Review article Evidence-Based Complementary and Alternative Medicine. 2011;1–16.

[3] Sreepian A., Chanthong C., Prathit P., Sreepian PM. Antibacterial activity of essential oil extracted from Citrus hystrix (Kaffir Lime) peels: An in vitro study. Trop Biomed. 36(2). 2019 Jun 1;531-541.

[4] Mohideen M., Mahadi N. N. S. J, Azlan A. Y. H. N., Suhaimi N. A. N, Kamaruzaman N. A. Antibacterial Properties of Essential Oil Extracted from Kaffir Lime (Citrus Hystrix) Peel. Biomed Pharmacol Journal. 15(1). 2022;

[5] Srifuengfung S., Bunyapraphatsara N.C, Junyaprasert V. B. Antibacterial oral sprays from Kaffir lime (Citrus hystrix DC) fruit peel oil and leaf oil and their activities against respiratory tract pathogens. Journal of Traditional and Complementary Medicine. 10(6). 2020;594-598.

[6] Fair RJ, Tor Y. Antibiotics and Bacterial Resistance in the 21st Century.Perspect Medicin Chem.2014 Jan;6:PMC.S144 59

[7] Li X, Shen D, Zang Q, Qiu Y, Yang X. Chemical Components and Antimicrobial Activities of Tea Tree Hydrosol and Their Correlation with Tea Tree Oil. Natural Product Communications [Internet]. 16(9). 2021; Available from: DOI:10.1177/1934578X211038390

[8] Gnatta JR, De Brito Poveda V, Padoveze MC, Graziano KU, Turrini RNT, Da Silva MJP. Melaleuca alternifolia essential oil soap: a potential alternative for hand hygiene. Eur J Clin Microbiol Infect Dis. 2021 Jul;40(7):1517–20.

[9] Siti HN, Mohamed S., Kamisah Y. Potential Therapeutic Effects of Citrus hystrix DC and Its Bioactive Compounds on Metabolic Disorders. Pharmaceuticals (Basel). 15(2). 2022 Jan 29;167.

[10] Waikedre J, DugayA,Barrachina I, Herrenknecht C,CabalionP,Fournet A.Chemical composition and antimicrobial activity of the essential oils from New Caledonian Citrus macroptera and Citrus hystrix. Chem Biodivers. 7(4). 2010 Apr;871.

[11] FAO report. Citrus fruit statistics 2015. Food and Agriculture Organization of the United Nations (FAO); 2016.

[12] Quyen NTC, Ngan TTK, Phong HX, Hien TT, Dung LT. Extraction of Kaffir Lime (Citrus hystrix DC.) essential oil by steam distillation and evaluation of chemical constituents. IOP Conf Ser: Mater Sci Eng. 2020 Dec 1;991(1):012015.

[13] Hoa DM, Chi NTD, Duy VH. Study on extraction, chemical component investigation and initial application of Melaleuca alternifolia (Tea tree) oil in mouthwash production. CTU Journal. 2016;45:90.

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

[15] Kamal FZ, Stanciu GD, Lefter R, Cotea VV, Niculaua M, Ababei DC, et al. Chemical Composition and Antioxidant Activity of Ammi visnaga L. Essential Oil. Antioxidants. 2022 Feb 10;11(2):347.

[16] Ibrahim GS, El-Shall FN, Arafa AA, Shalabi A, El Awady M. Bio-production and characterization of carotenoid yellow pigment from Kocuria sp. GMA and exploring its sustainable antioxidant, antimicrobial and antibiofilm properties. Egypt J Chem. 2024 Aug 1;67(8):57–68.

[17] Multiskan Microplate spectrophotometer [EN] User Manual book [Internet]. Available from: https://www.thermofisher.com / order/catalog/product/A51119500C

[18] Biotek Synergy HT multi-mode Microplate Reader Operator's Manual [Internet]. BioTek Instruments, Inc.; Available from: https://www.manualslib.com/manual/1416962/Biotek-Synergy-Ht.html

[19] Roesler R, Malta LG, Carrasco LC, Pastore G. Evaluation of the Antioxidant Properties of the Brazilian Cerrado Fruit Annona crassiflora (Araticum). Journal of Food Science [Internet]. 2006 Mar [cited 2023 Dec 19];71(2). Available from: https://ift.onlinelibrary.wiley.com/doi/10.1111/j.1365-2621.2006.tb08882.x

[20] Merck, Germany. Columbia Blood Agar Pharm. MSDS (material safety data sheet) or SDS, CoA and CoQ, dossiers, brochures and other available documents. [Internet]. Available from: https://www.sigmaaldrich.com/VN/en/product/mm/146559#product-documentation

[21] Merck, Germany. MUELLER-HINTON (MH) agar- overview [Internet]. Available from: https://www. merckmillipore. com/ INTL/en/product/MUELLER-HINTON-MH-agar,MDA_CHEM-103872

[22] Merck, Germany. ryptic Soy agar MSDS (material safety data sheet) or SDS, CoA and CoQ, dossiers, brochures and other available documents. [Internet]. Available from: https://www.merckmillipore.com /INTL/en/product/Tryptic-Soy-agar,MDA_CHEM-105458

[23] Merck, Germany. SABOURAUD dextrose agar- Overview. [Internet]. Available from: https://www. merckmillipore. com/INTL/en/product/SABOURAUD-40-0-dextrose-agar,MDA_CHEM-105438

[24] Younis H, El Shalakany W, Amin S, Abdel-Reheem M, Ibrahima H. Biological activities and related phenolic compounds content of olive and plum stones ethanolic extract. Egypt J Chem. 2023 Jun 7;0(0):0–0.
[25] Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clinical Microbiology and Infection. 2014 Apr;20(4):O255–66.
[26] Wayne, NJ. M07- Method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. [Internet]. Clinical and Laboratory Standards Institute.; 2018. Available from: https://clsi.org/media /1928/m07ed11_ sample.pdf
[27] Sarker S., Nahar L., Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods. 2007 Aug;321.
[28] Gouda H, Morsy A, Youssef A, Tolba I, Hassan G. Phytochemical Profile and Antimicrobial Assessment of Abutilon fruticosum Guill. & Perr. Growing in Gebel Elba, Egypt. Egypt J Chem. 2022 Sep 20;0(0):0–0.
[29] Finland M, Garner C, Wilcox C, Sabath LD. Susceptibility of Pneumococci and *Haemophilus influenzae* to Antibacterial Agents. Antimicrob Agents Chemother. 1976 Feb;9(2):274–87.
[30] Phillips I, Eykyn S, King BA, Jenkins C, Warren CA, Shannon KP. The in vitro antibacterial activity of nine aminoglycosides and spectinomycin on clinical isolates of common Gram-negative bacteria. J Antimicrob Chemother. 1977;3(5):403–10.
[31] Moosa MYS, Sobel JD, Elhalis H, Du W, Akins RA. Fungicidal Activity of Fluconazole against *Candida albicans* in a Synthetic Vagina-Simulative Medium. Antimicrob Agents Chemother. 2004 Jan;48(1):161–7.
[32] Son L.Hoang, Nhi H.M.Nguyen. Antibacterial Synergism of Blended Essential Oils from Piper betle L. and Melaleuca alternifolia. South Asian Research Journal of Natural Products. 2023;6(2):88–100.
[33] Moody J., Garcia L., Ed. Synergism Testing: Broth Microdilution Checkerboard and Broth Macrodilution Methods. In: In Clinical Microbiology Procedures Handbook. ASM Press: Washington, DC, USA.; 2010.
[34] IBM SPSS Statistics 28 Brief Guide [Internet]. IBM Corp; Available from: https://www.ibm.com/docs/en/SSLVMB_28.0.0/pdf/IBM_SPSS_Statistics_Brief_Guide.pdf
[35] Li Y., Lv O., Zhou F., Li Q., Wu Z., Zheng Y. Linalool Inhibits LPS-Induced Inflammation in BV2 Microglia Cells by Activating Nrf2. Neurochem Res. 40th ed. 2015;1520–5.
[36] Brand C., Townley S. L., Finlay-Jones J. J., Hart P. H. Tea Tree Oil Reduces Histamine-Induced Oedema in Murine Ears. Inflamm Res. 51st ed. 2002;283–9.
[37] Veras H. N. H., Araruna M. K. A. R., Costa J. G. M. Topical Antiinflammatory Activity of Essential Oil of Lippia sidoides Cham: Possible Mechanism of Action. Phytother Res. 27th ed. 2013;179–85.
[38] Mei Z., Wu Q., Hu S., Lib X., Yang X. Triptolide Loaded Solid Lipid Nanoparticle Hydrogel for Topical Application. Drug Develop Ind Pharm. 31st ed. 2005;161–8.
[39] 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. IJMS. 2020 Jun 25;21(12):4531.
[40] 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. Appl Microbiol Biotechnol. 2016 Oct;100(20):8865–75.
[41] Yang SK, Tan NP, Chong CW, Abushelaibi A, Lim SHE, Lai KS. The Missing Piece: Recent Approaches Investigating the Antimicrobial Mode of Action of Essential Oils. Evol Bioinform Online. 2021 Jan;17:117693432093839.
[42] Bassolé IHN, Juliani HR. Essential Oils in Combination and Their Antimicrobial Properties. Molecules. 2012 Apr 2;17(4):3989–4006.
[43] Kim J, Marshall MR, Wei C i. Antibacterial activity of some essential oil components against five foodborne pathogens. J Agric Food Chem. 1995 Nov;43(11):2839–45.
[44] Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol. 2001 Sep 12;91(3):453–62.
[45] Mulyaningsih S, Sporer F, Zimmermann S, Reichling J, Wink M. Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of Eucalyptus globulus against antibiotic-susceptible and antibiotic-resistant pathogens. Phytomedicine. 2010 Nov;17(13):1061–6.
[46] Geda AK. Antibacterial Activity of Essential Oils and their Combinations. Fett/Lipid. 1995 Jan;97(12):458–60.