



# Chemometric analysis of GC-MS chemical profiles and biological activities of three *citrus* essential oils in Indonesia

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## ARTICLE HISTORY

Received on: 28/07/2024

Accepted on: 16/10/2024

Available Online: 05/11/2024

## Key words:

*Citrus species*, principal component analysis, essential oils, antioxidant activity, phytotoxic and cytotoxic effects, chemometric analysis.

## ABSTRACT

*Citrus* species essential oils (EOs) are significant sources of bioactive compounds and demonstrate diverse therapeutic effects. However, limited documentation exists regarding the phytochemicals associated with their biological activities. This study aims to assess the antioxidant activity, plant inhibitory properties, and brine shrimp cytotoxic effects of EOs derived from three *Citrus* species: *C. sinensis*, *C. limon*, and *C. hystrix*. Utilizing chemometric analysis and gas chromatography-mass spectrometry (GC-MS) fingerprints, the volatile components contributing to antioxidant activity were elucidated. The peels of the Citrus samples were hydro-distilled to obtain EOs, and subsequently subjected to antioxidant, plant inhibitory, and brine shrimp cytotoxic assays. The results indicated that *C. limon* EO exhibited the highest antioxidant activity, while *C. hystrix* and *C. sinensis* EOs demonstrated pronounced inhibitory effects against *Artemia salina* and *Lactuca sativa*, respectively. GC-MS analysis facilitated the identification of key compounds in each EO. Principal component analysis and hierarchical cluster analysis effectively categorized Citrus EOs based on their antioxidant properties, highlighting the proximity of *C. limon* and *C. sinensis*. Among the identified compounds, D-limonene,  $\alpha$ -terpineol, caryophyllene, (+)-3-carene,  $\beta$ -pinene, (-)-spathulenol, trans-p-mentha-1(7),8-dien-2-ol, and trans-verbenol were the most discriminating compounds affected the antioxidant activity of *C. limon* and *C. sinensis* EOs.

## INTRODUCTION

*Citrus* species, part of the Rutaceae family, are globally renowned for their popular fruits and diverse health benefits. Among them, sweet oranges (*Citrus sinensis*) play a significant role, constituting approximately 70% of the world's citrus production and consumption. Alongside sweet oranges, other widely cultivated and consumed *Citrus* species include tangerines

or mandarins (*Citrus reticulata*), grapefruits (*Citrus vitis*), limes (*Citrus aurantifolia*), and lemons (*Citrus limon*) [1].

Indonesia has a substantial *Citrus* production of approximately 2.6 million tons per year, encompassing 255 different varieties, including sweet (*C. sinensis*), mandarin (*C. reticulata*), sour (*Citrus aurantium*), pomelo oranges (*Citrus maxima*), tangerines (*Citrus nobilis*), lemons, limes, and so on [2,3]. *Citrus*, beyond its application as a condiment, is utilized in sweet delicacies in European nations, enhancing dishes such as pan-seared chicken with orange-brandy sauce or pork tenderloin with blood oranges [4]. Notably, *C. limon* and *Citrus hystrix* are extensively used as spices in Asian countries due to their distinctive scents and natural oils [5,6].

Citrus essential oils (EOs), obtained through hydro distillation of citrus peels, are rich sources of bioactive compounds

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with applications in the pharmaceutical and food industries [7]. Comprising a complex mixture of aldehydes, esters, alcohols, ketones, acids, monoterpenes, and sesquiterpenes [8], these EOs exhibit diverse biological activities, including antibacterial, antiviral, fungicidal, and antioxidant properties [9,10]. Beyond their antimicrobial effects, Citrus EOs also serve a crucial role as antioxidative agents, protecting organisms and tissues from damage caused by reactive oxygen species (ROS) [11].

Previous studies have unveiled the phytochemical makeup of EOs derived from *Citrus species*. Among these components, the pivotal active compound found in Citrus EOs is D-limonene. However, its concentration significantly fluctuates across *Citrus species*, spanning from 34.2% to 81.9% of the total compounds. Lemon EO exhibits stronger antioxidant activity, as assessed by (2,2- azinobis-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and (2,2-diphenyl-1-picrylhydrazyl) (DPPH) free radicals, compared to other *Citrus* EOs [12]. The major components of this EO are Limonene (67.1%),  $\alpha$ -terpinene (8.0%), and  $\alpha$ -pinene (11.0%) [13]. In another study, *C. limon* EO also demonstrated significant inhibition in the growth of *Solanum lycopersicum* and *Lepidium sativum* germinating seeds at a level of 100  $\mu\text{g/mL}$  in comparison with *C. myrtifolia* and *C. bergamia* EOs [14]. Additionally, this EO was reported to exhibit moderate toxicity in animal gavage with 500 and 100 mg/kg doses at the sub-chronic stage [15].

Nevertheless, the precise phytochemical constituents responsible for the biological properties of *Citrus* EOs, especially across diverse *Citrus* species in Indonesia, remain unknown. This study aims to determine these associations by examining the chemical composition of EOs and their corresponding antioxidant, allelopathic, and cytotoxic characteristics, utilizing principal component analysis (PCA) and hierarchical cluster analysis (HCA). The significance of this study extends beyond uncovering the therapeutic potential inherent in *Citrus* EOs; it also delves into exploring their promising applications across agricultural, medicinal, and interdisciplinary domains.

## MATERIALS AND METHODS

### Plant materials and EO extraction

The fruits of *C. sinensis*, *C. limon*, and *C. hystrix* were purchased commercially (Jogjakarta market). A total of 5 kg of each *Citrus* species was peeled manually and the peels were air dried for 12 hours before being extracted by a Clevenger hydro distillation system. *Lactuca sativa* and *Raphanus sativus* seeds were obtained commercially (PT. Panah Merah, Purwakarta, Indonesia). These seeds were tested for germination and more than 90% were alive before being used in a plant inhibitory assay.

### DPPH radical scavenging activity

The method described by Andriana *et al.* [16] was employed to assess the DPPH free radical scavenging activity of *Citrus* EOs. The absorbances of samples were measured at 517 nm and expressed as radical scavenging activity percentage.

### Plant inhibitory potentials

Plant inhibitory assay was conducted using moist filter paper placed in a 12 well-plate (22.1 mm in diameter and

35 mm in depth) following the method reported by Minh *et al.* [17]. The seeds of the indicator plants were lettuce (*L. sativa*) and radish (*R. sativus*). Plant inhibitory potentials on *L. sativa* and *R. sativus* were expressed in inhibitory percentage of germination, shoot, and root size over control.

### Evaluation of cytotoxicity by the brine shrimp lethality assay

Cytotoxic activity of Citrus EOs was assessed using the brine shrimp (*Artemia salina*) lethality bioassay, with 5 mg of each extract dissolved in DMSO and serial dilutions in simulated seawater. After 24 hours, surviving brine shrimp nauplii were counted, and mortality was determined based on the absence of regulated forward movement within 30 seconds [18,19].

### Total phenolic contents

Total phenolic contents in Citrus EOs were determined using the Folin Ciocalteu (FC) reagent as reported previously [16] and expressed as milligrams of gallic acid equivalent (GAE) per gram of the sample.

### Total flavonoid contents

Total flavonoid contents were evaluated based on the method reported previously and expressed as quantified and reported in milligrams of quercetin (QE) per gram of the sample [20].

### Identification of functional groups by Fourier-transform infrared spectroscopy (FTIR)

An FTIR analysis was performed to find the chemical functional groups in EO samples following the procedure described previously by Indrianiingsih *et al.* [21]. The spectra were measured by the attenuated total reflection method and displayed as the percentage of transmittance.

### Identification of chemical constituents by gas chromatography-mass spectrometry (GC-MS)

The phytochemical constituents of Citrus EOs were identified by a GC-MS system (Agilent 7890B/MSD 5977 A, Agilent Technology, Inc., Santa Clara, CA) following the previous method [22]. Data peak processing was managed by the Agilent Chem Station software, incorporating the National Institute of Standards and Technology mass spectral library (Agilent Technology, USA).

### Statistical analysis

The data analysis was conducted using MetaboAnalyst 5.0 software (<https://metaboanalyst.ca/>) with the one-way analysis of variance method and Tukey's test with a 95% confidence interval ( $p < 0.05$ ) to determine the significance level among samples. A multivariate analysis was employed to reduce dimensional parameters, utilizing a correlation matrix consisting of 9 samples and their replicates as well as 103 variables (GC/MS peak area %) and antioxidant activities. HCA was applied to illustrate clusters and interrelationships between samples, forming the basis for the hierarchical clustering algorithms [23].

## RESULTS

### Assessment of DPPH scavenging activity, total phenolic and flavonoid contents

The antioxidant activities, total flavonoid, and phenolic contents of EOs from three different types of citrus are shown in Table 1. *Citrus sinensis* EO demonstrated the strongest

**Table 1.** DPPH scavenging activity, total flavonoid, and phenolic contents.

EO samples	DPPH scavenging activity (%)	Total phenolic contents (mg GAE/ g extract)	Total flavonoid contents (mg QE/ g extract)
<i>C. sinensis</i>	36.50 ± 8.45 <sup>b</sup>	19.30 ± 1.78 <sup>a</sup>	3.88 ± 0.13 <sup>b</sup>
<i>C. hystrix</i>	4.08 ± 0.21 <sup>c</sup>	15.09 ± 3.20 <sup>a</sup>	5.25 ± 0.50 <sup>b</sup>
<i>C. limon</i>	66.11 ± 2.91 <sup>a</sup>	18.14 ± 2.00 <sup>a</sup>	30.56 ± 5.31 <sup>a</sup>

Data were presented as mean ± SD. The mean value, indicated by distinct letters, exhibits a statistically significant difference according to Tukey's test ( $p < 0.05$ ).

**Table 2.** Brine shrimp lethality property of EOs from three species of *Citrus*.

EOs	Conc. (ppm)	Mortality (%)	LC <sub>50</sub> (ppm)
<i>C. sinensis</i>	6,000	36.67 ± 15.28 <sup>b</sup>	> 6,000
	2,000	13.33 ± 5.77 <sup>cd</sup>	
	1,000	13.33 ± 5.77 <sup>cd</sup>	
<i>C. hystrix</i>	6,000	100.00 ± 0.00 <sup>a</sup>	3,188.23 ± 71.89
	2,000	26.67 ± 5.77 <sup>bc</sup>	
	1,000	16.67 ± 5.77 <sup>cd</sup>	
<i>C. limon</i>	6,000	10.00 ± 0.00 <sup>cd</sup>	> 6,000
	2,000	6.67 ± 5.77 <sup>d</sup>	
	1,000	3.33 ± 5.77 <sup>d</sup>	

Data were presented as mean ± SD. The mean value followed by the different letters showed a significant difference by Tukey's test ( $p < 0.05$ ).

antioxidant activity compared to the other samples. Conversely, the total flavonoid contents of *C. limon* EO exhibited the highest value among all samples. There is a relationship between the total phenolic contents and the antioxidant activities of the samples.

### Brine shrimp lethality assay

Table 2 shows the brine shrimp lethality test of EO samples. The *C. hystrix* EO demonstrated the highest death rate, with an LC<sub>50</sub> value of 3188.23 ± 71.89 ppm. On the contrary, *C. sinensis* and *C. limon* EOs exhibit a requirement exceeding 6,000 ppm to induce mortality in brine shrimp nauplii.

### Plant inhibitory activity

Table 3 illustrates the inhibitory activity of *Citrus* EOs on the germination and growth of *R. sativus* and *L. sativa*. The *C. limon* EO exhibited a significant inhibitory effect on *R. sativus* germination and shoot growth. At a concentration of 10,000 ppm, the observed inhibition rates on *R. sativus* germination and shoot length were the highest, measuring at 53.33% ± 11.55% and 97.41% ± 2.80%, respectively. On the other hand, *C. hystrix* EO demonstrated a strong inhibitory activity on the root length of *R. sativus* (95.73% ± 3.65%). The *C. sinensis* EO completely inhibited the germination and growth of *L. sativa* at a concentration of 10,000 ppm followed by the *C. limon* and *C. hystrix* EOs, respectively.

### Identification of the functional group of Citrus EOs

Figure 1 shows the FTIR spectra of EOs from three species of *Citrus*. According to this figure, there are transmittances in wave numbers 3445, 2902, 2692, 2293, 1713, 1421, and 770 cm<sup>-1</sup>. The wavenumber range of 3650–3250 cm<sup>-1</sup> indicated the presence of hydrogen bond (OH) presence in EO samples. *C. hystrix* EO showed a higher intensity in hydrogen bonds. Furthermore, all samples have a very low transmittance in wavenumber of 2,902 cm<sup>-1</sup>, indicating the

**Table 3.** Plant inhibitory potentials of EOs from three species of *Citrus* species.

EOs	Conc. (ppm)	Inhibition					
		<i>R. sativus</i>			<i>L. sativa</i>		
		Germination	Root	Shoot	Germination	Root	Shoot
<i>C. sinensis</i>	10000	23.33 ± 11.55 <sup>abcd</sup>	86.02 ± 9.85 <sup>bcd</sup>	77.87 ± 6.90 <sup>def</sup>	100.00 ± 0.00 <sup>a</sup>	100.00 ± 0.00 <sup>e</sup>	100.00 ± 0.00 <sup>d</sup>
	5000	6.67 ± 5.77 <sup>ab</sup>	83.86 ± 5.97 <sup>abcd</sup>	57.31 ± 3.57 <sup>cd</sup>	30.00 ± 0.00 <sup>ab</sup>	89.26 ± 4.83 <sup>de</sup>	92.22 ± 6.09 <sup>cd</sup>
	2500	6.67 ± 11.55 <sup>ab</sup>	81.52 ± 5.64 <sup>abc</sup>	57.41 ± 29.22 <sup>cd</sup>	26.67 ± 5.77 <sup>ab</sup>	32.96 ± 44.27 <sup>ab</sup>	61.02 ± 23.89 <sup>bc</sup>
	500	0.00 ± 0.00 <sup>a</sup>	71.93 ± 8.36 <sup>a</sup>	30.65 ± 4.94 <sup>a</sup>	23.33 ± 11.55 <sup>ab</sup>	17.98 ± 11.25 <sup>a</sup>	23.54 ± 14.01 <sup>a</sup>
<i>C. hystrix</i>	10000	40.00 ± 26.46 <sup>abcde</sup>	95.73 ± 3.65 <sup>d</sup>	96.3 ± 6.42 <sup>f</sup>	76.67 ± 15.28 <sup>cde</sup>	100.00 ± 0.00 <sup>e</sup>	81.34 ± 22.87 <sup>bcd</sup>
	5000	30.00 ± 0.00 <sup>abcde</sup>	93.51 ± 1.69 <sup>cd</sup>	91.02 ± 3.83 <sup>ef</sup>	66.67 ± 23.09 <sup>cde</sup>	90.39 ± 12.76 <sup>de</sup>	80.87 ± 20.34 <sup>bcd</sup>
	2500	13.33 ± 11.55 <sup>abc</sup>	82.69 ± 2.96 <sup>abcd</sup>	70.46 ± 7.46 <sup>cde</sup>	46.67 ± 11.55 <sup>bcd</sup>	38.45 ± 29.18 <sup>abc</sup>	73.32 ± 8.77 <sup>bcd</sup>
	500	6.67 ± 11.55 <sup>ab</sup>	84.50 ± 4.06 <sup>abcd</sup>	58.7 ± 6.76 <sup>cd</sup>	43.44 ± 20.82 <sup>bc</sup>	39.20 ± 11.99 <sup>abcd</sup>	20.22 ± 13.16 <sup>a</sup>
<i>C. limon</i>	10000	53.33 ± 11.55 <sup>e</sup>	91.05 ± 3.22 <sup>cd</sup>	97.41 ± 2.80 <sup>f</sup>	83.33 ± 11.55 <sup>e</sup>	87.02 ± 12.11 <sup>cde</sup>	100.00 ± 0.00 <sup>d</sup>
	5000	43.33 ± 11.55 <sup>de</sup>	86.20 ± 5.04 <sup>bcd</sup>	84.17 ± 4.11 <sup>ef</sup>	83.33 ± 11.55 <sup>e</sup>	67.92 ± 39.84 <sup>bcd</sup>	93.54 ± 8.28 <sup>d</sup>
	2500	16.67 ± 5.77 <sup>abcd</sup>	77.60 ± 11.82 <sup>ab</sup>	52.69 ± 10.34 <sup>bc</sup>	80.00 ± 26.46 <sup>de</sup>	52.68 ± 15.93 <sup>abcde</sup>	76.34 ± 6.65 <sup>bcd</sup>
	500	16.67 ± 5.77 <sup>abcd</sup>	77.08 ± 3.90 <sup>ab</sup>	31.85 ± 7.09 <sup>ab</sup>	76.67 ± 15.28 <sup>cde</sup>	49.44 ± 21.51 <sup>abcde</sup>	47.54 ± 4.42 <sup>ab</sup>

Data were presented as mean ± SD. The mean value followed by the different letters showed a significant difference by Tukey's test ( $p < 0.05$ ).

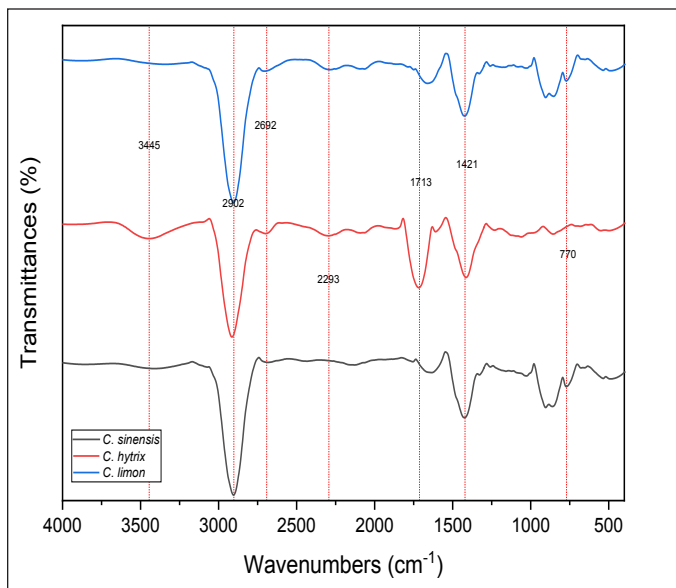


Figure 1. FTIR spectra of EOs from three species of *Citrus*.

presence of aliphatic compounds in all samples. A slight transmittance intensity was also shown at a wavenumber of  $2,293\text{ cm}^{-1}$ , indicating the presence of triple bond carbon because it was followed with spectra between  $1,600$  and  $1,300\text{ cm}^{-1}$ . Moreover, wavenumber  $1,713\text{ cm}^{-1}$  indicated the double bond carbon ( $\text{C}=\text{C}$ ), and all samples seem to possess a high-intensity transmittance at  $1,421\text{ cm}^{-1}$  that indicates the presence of Vinyl  $\text{C}-\text{H}$  in-plane bend. The final transmittance detected at  $770\text{ cm}^{-1}$  showed the content of the phenyl group in the EO samples [24].

#### Identification of phytochemical constituent of essential oils of *Citrus* by GC-MS

Supplementary Material 1 shows the phytochemical constituents of *Citrus* EOs, while Figure 2 illustrates their GC-MS chromatograms. A total of 103 compounds from various chemical classes were identified in EO samples by a GC-MS system. Of which, *D*-limonene (27.86%), *L*- $\alpha$ -Pinene (16.64%), *cis*-(-)-1,2-Epoxy-*p*-menth-8-ene (5.24%), and Carvone (4.65 %) were accounted as major components of *C. sinensis* EO.

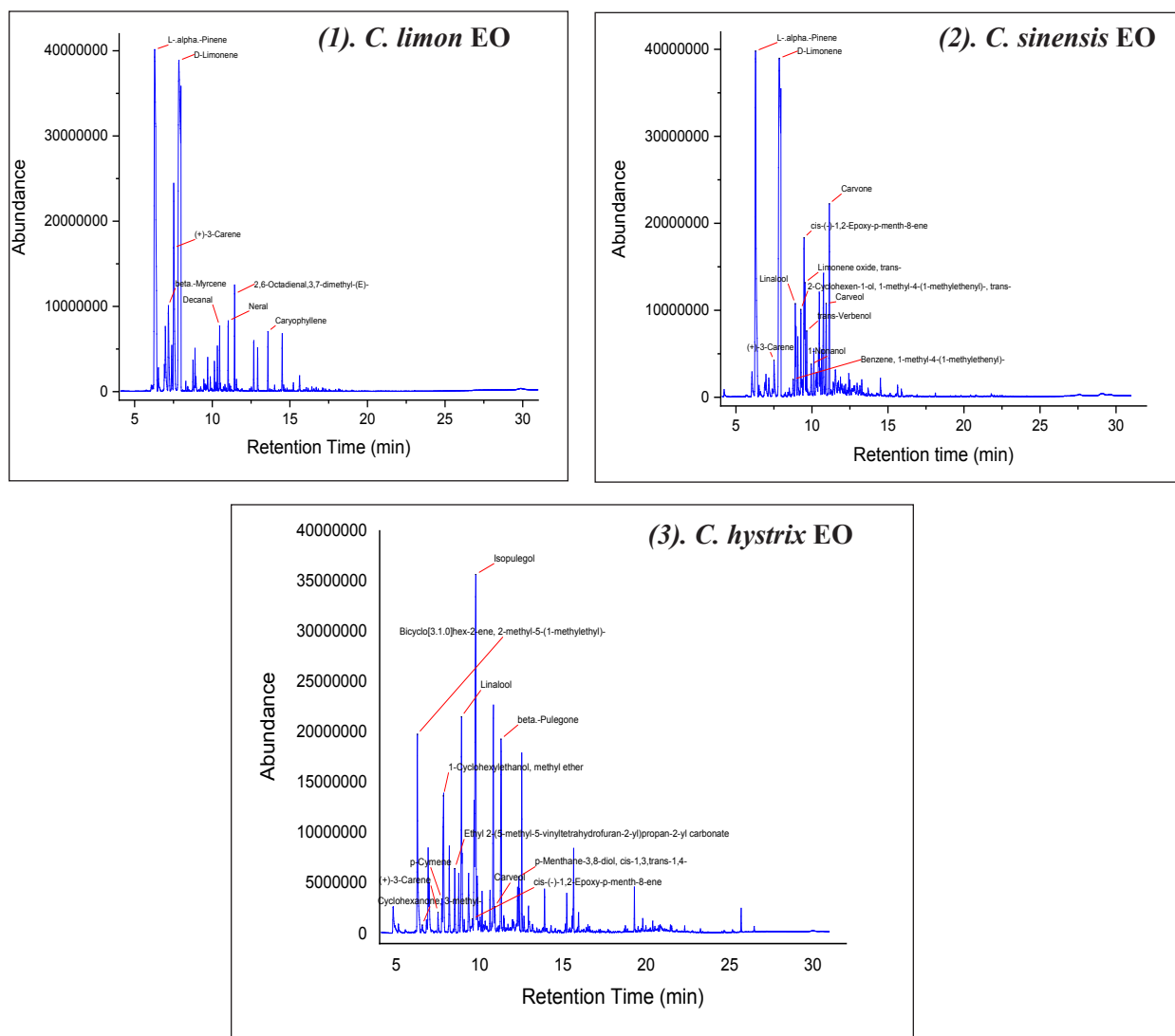


Figure 2. GC-MS chromatograms of EO samples.

On the other hand, p-Cymene (5.96%), Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate (7.02%), Isopulegol (15.95%), trans-Carveol (6.79), and  $\beta$ -Pulegone (6.91) were the dominant compounds containing in *C. hystrix* EO. While for *C. limon* EO, L- $\alpha$ -Pinene (27.43%), D-limonene (36.41%), and p-Menthatriene (9.26%) were significant contents. These various chemical components might affect the biological activity of *Citrus* EO. However, which components contribute to their antioxidant, plant inhibitory, and cytotoxic properties require more investigation.

**Chemometric analysis based on GC-MS chemical profiles concerning antioxidant activity**

Figure 3 shows PCA score plots of GC-MS chemical profiles and antioxidant activities as the target cell of classification. PCA successfully reduced variables measured from all samples into two principal components, PC1 of 58.8% and PC2 of 34.7%, which explained about 93.1% of the variation in the dataset. Notably, *C. limon* and *C. sinensis* EOs exhibited similar antioxidant activity, so they are placed in the same negative ordinate (quadrants II and III).

Figure 4 displays the PCA loading plot based on the chemical profiles and antioxidant activities of EO samples determined by the GC-MS system. The *Citrus* EO samples were clearly dispersed along the PCs in the PCA plot. D-limonene and  $\alpha$ -terpineol were among the most discriminating chemicals on negative PC1, while for negative PC2, they were caryophyllene and (+)-3-carene. Conversely,  $\beta$ -pinene and (-)-spatulene exhibited high selectivity for positive PC1; however, trans-p-mentha-1(7),8-dien-2-ol and trans-verbenol affected the positive PC2.

Figure 5 illustrates the HCA dendrogram, which reflects the relationship between EOs' chemical components and their antioxidant activities. This dendrogram categorized the EOs from *Citrus* species into two primary clusters. Cluster II grouped EOs from *C. sinensis* and *C. limon*,

indicating their closeness in phytochemical components and antioxidant activities. In contrast, *C. hystrix* EO was situated in Cluster I, suggesting distinct properties compared to the other samples.

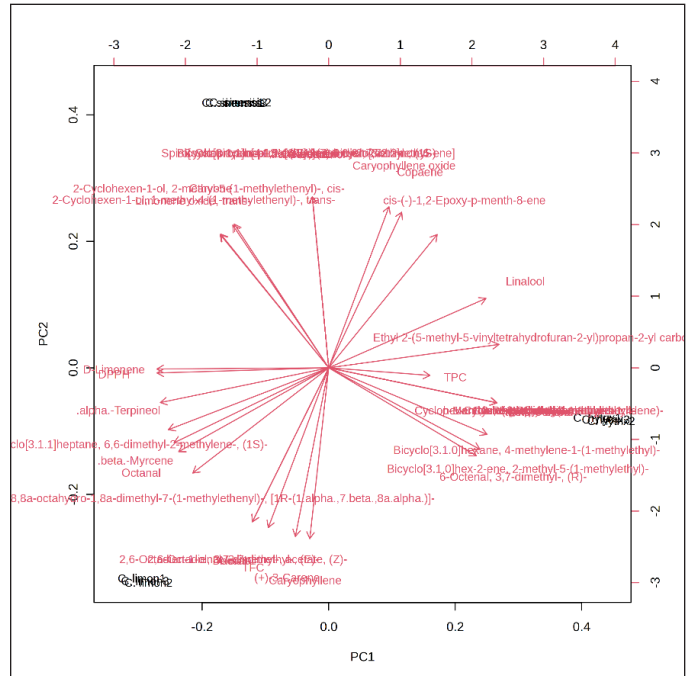


Figure 4. PCA loading plot based on GC-MS chemical profiles and biological activities of EO.

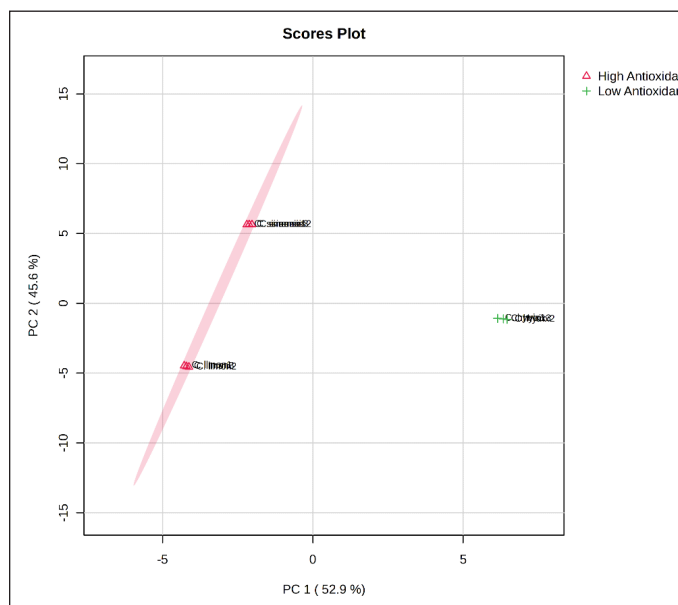


Figure 3. Score plot from PCA analysis on antioxidant activity citrus EOs.

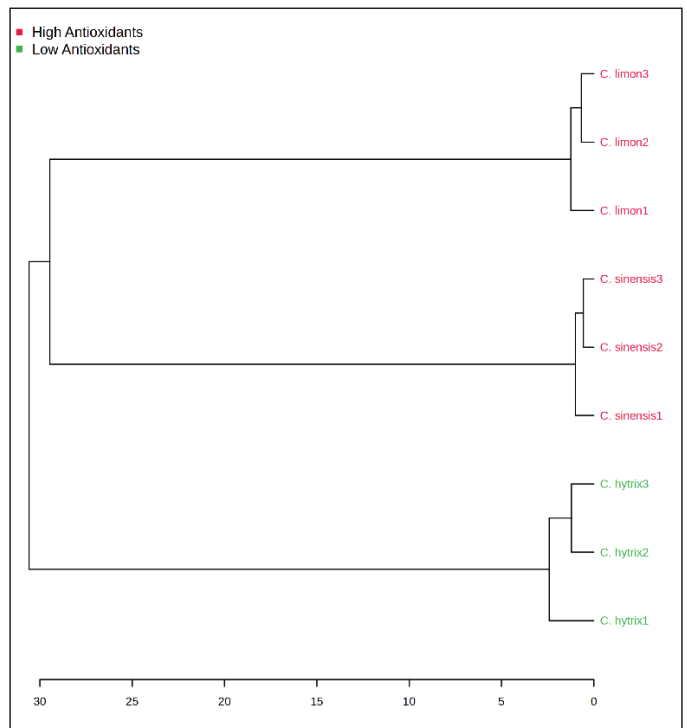
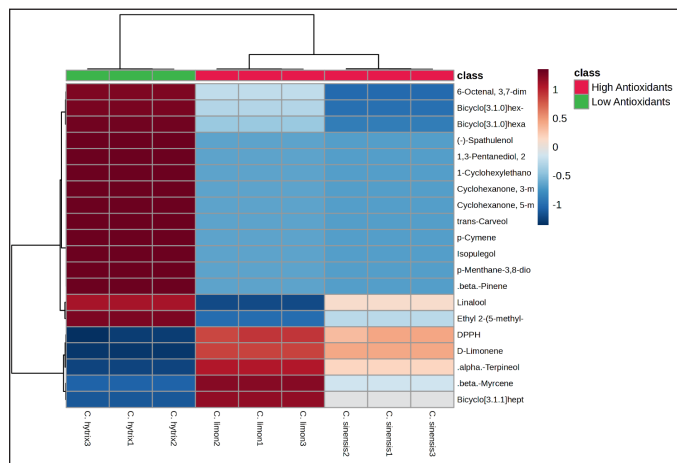


Figure 5. Dendrogram of HCA to determine the closeness of EO samples.





**Figure 6.** Heatmap of top 20 phytochemical components of EOs from three Citrus species in correlation with antioxidant activity.

A heatmap analysis was conducted to assess the relationship between the chemical compounds of EO samples and their biological properties. This visualization, presented in Figure 6, showcased the top 20 compounds present in the EO samples derived from the percentage of peak area in GC-MS analysis, utilizing color intensity to represent their abundance.

In the assessment of antioxidant activity, the EO derived from *C. limon* exhibited notably high potential as an antioxidative agent, as evidenced by the percentage of DPPH scavenging activity. This observation indicated a dominant presence of antioxidant compounds in *C. limon* EO compared to the other EOs studied. Among the 20 identified compounds, D-limonene, Linalool,  $\alpha$ -Terpineol, and  $\beta$ -Myrcene were the most abundant in both *C. limon* and *C. sinensis* EOs. These compounds likely contribute significantly to the antioxidant activity observed in these EOs. Nonetheless, further investigations are essential to precisely determine the specific roles of these compounds.

## DISCUSSION

In the current study, we evaluated the antioxidant, plant inhibitory, and cytotoxic properties of three distinct *Citrus* EOs originating from Indonesia as well as their phytochemical components. Utilizing chemometric techniques, HCA, and PCA, we discriminated between EO samples to elucidate their relationship in terms of antioxidant activity and phytochemical compositions.

In terms of antioxidant properties, the EO from *C. limon* exhibited the highest potency at 66.11% DPPH scavenging activity, surpassing the activity levels of *C. sinensis* and *C. hystrix* EOs, which recorded values of 36.50% and 4.08%, respectively. This result was similarly reflected in the total flavonoid contents of the EO samples, with *C. limon* EO showing the highest concentration at 30.56 mg QE/g extract. These observations might be attributed to the notably elevated concentration of antioxidative agents in *C. limon* EO such as D-limonene compared to the other samples. This result aligned with prior studies that identified D-limonene as the predominant constituent in *C. limon* peel [12,25] and leaf EO [26]. Notably, D-Limonene stands out as the primary volatile

compound in lemon EO, at levels usually ranging between 70% and 48% [27]. Subsequent investigations have elucidated the diverse antioxidant properties of D-limonene through in vitro assays, including DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging, and superoxide radical scavenging assays, showcasing its efficacy in reducing ROSs through varied mechanisms [28]. Additionally, in vivo assessments have revealed that D-limonene enhances antioxidant levels and augments the protein expression of inducible cyclooxygenase-2 (COX-2) and in nitric oxide synthase (iNOS) UC rats [29].

In terms of plant growth inhibition, the *C. hystrix* EO demonstrated the strongest inhibitory activity. Prior research has shown that a methanol extract derived from *C. hystrix*, at a concentration of 10 mg/ml, effectively inhibited the germination of lettuce seeds and impeded the growth of their roots. This finding suggests the potential use of the extract as a bioherbicide in the future for weed management [30]. In this study, the main phytochemical compounds *C. hystrix* EO were Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate (7.02%) and Isopulegol (15.95%). The scientific literature documents the phytotoxic activity of EOs, which manifests in varying degrees of inhibition on seed germination and radical elongation. This activity appears to be caused by monoterpenes, specifically oxygenated compounds, especially ketones, alcohols, aldehydes, and phenols [31].

Furthermore, in the field of cytotoxicity assays, which evaluates the capacity of cytotoxic substances to induce cellular harm or cell mortality, the EO derived from *C. hystrix* exhibited a greater mortality rate in comparison to other citrus oils. This higher cytotoxicity aligns with prior studies that showed *C. hystrix* EO had cytotoxic activity on *A. salina* and demonstrated a greater cytotoxicity effect on different cell lines including human cervix carcinoma, murine melanoma, and human lung fibroblast (MRC-5) [32]. Moreover, the current investigation demonstrates that the EO of *C. hystrix* possessed a notably elevated level of Isopulegol (15.95%). Isopulegol is a monoterpene found in different plant species that have been scientifically proven to possess pharmacological properties [33].

In the realm of biological activity, citrus EOs have demonstrated inhibitory effects against several microorganisms. For instance, the EO from *C. limon* has been shown to inhibit bacteria including *Lactobacillus plantarum*, *Lactobacillus mesenteroides*, and *Escherichia coli* [34]. Similarly, *C. sinensis* EO exhibits inhibitory activity against gram-positive bacteria such as *Staphylococcus aureus*, *Lactobacillus monocytogenes*, and *Enterococcus faecium* along with some gram-negative bacteria including *Salmonella enteritidis* and *Pseudomonas aeruginosa* [35]. Furthermore, *C. hystrix* EO has demonstrated an inhibitory effect against various bacteria such as *S. aureus*, *Enterococcus faecalis*, *E. coli*, *Staphylococcus epidermidis*, and *Proteus vulgaris* [36]. The observed inhibitory effects of Citrus EOs against these bacteria are attributed to specific volatile components such as D-limonene and isopulegol, which are believed to contribute significantly to their antimicrobial properties [37,38].

The present study found the isopulegol and ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate as the major component present in *C. hystrix* EO. In

contrast, a previous study reported different major compounds in the Citrus peel EO are sabinene,  $\beta$ -pinene, citronellal, limonene, terpinen-4-ol, and  $\alpha$ -pinene [39]. Limonene was identified as the predominant constituent in nearly all Citrus peel EO samples, except *C. hystrix* and *C. micrantha* EOs, which were dominated by  $\beta$ -pinene. The most abundant compounds were monoterpenes, followed by sesquiterpenes and ester. According to another study, the kaffir lime peel EO has three primary chemical components: D-limonene (17.10%), 3-carene (13.77%), and  $\gamma$ -terpinene (12.56%). All three chemicals belong to the monoterpene hydrocarbon group and are characterized by the presence of double bonds, which are known as alkenes [40].

The variations in the primary chemical constituents of EOs between the current findings and previous studies can be attributed to disparities in the geographical regions where the plants are cultivated, resulting in distinct chemical compositions. The chemical composition of EOs within a plant is determined by various factors including the plant species, climate, geographical location, season, soil composition, extraction technique, and the specific plant portion used for oil extraction. These variations highlight the importance of considering multiple factors when studying and comparing EO compositions from different sources [41,42].

To understand the relation of antioxidant and phytochemical components of *Citrus* EOs as well as closeness among samples, PCA and hierarchy cluster analysis were employed. PCA and HCA analyses successfully discriminated EO samples into two groups, namely EO with high and low antioxidant activity. EOs of *C. limon* and *C. sinensis* were clustered in the same place showing the closeness of both samples. However, *C. hystrix* EO was put in a different cluster meaning the different properties of this sample to *C. limon* and *C. sinensis* EOs. PCA and HCA were widely used to discriminate many samples based on their similarity, e.g., to cluster EO of *Juniperus rigida* [43] and *Foeniculum vulgare* [44]. In line with the previous study, the present study highlights the power of chemometric analysis to classify and cluster as well as to determine the relationship among observed variables.

## CONCLUSION

In the present study, EOs extracted from the peels of three distinct *Citrus* species from Indonesia, namely *C. sinensis*, *C. limon*, and *C. hystrix*, were evaluated for their antioxidant potential, plant inhibitory effects, and lethality properties against brine shrimp. We employed HCA and PCA to gauge the proximity of these samples concerning their antioxidant activity. Among the EOs tested, *C. limon* EO exhibited the most robust antioxidant activity against the DPPH free radical, while *C. sinensis* EO demonstrated the highest inhibitory effect on the growth of *L. sativa*, a vegetable plant. GC-MS analysis unveiled D-limonene as the predominant component in *C. limon* and *C. sinensis* EOs, constituting 36.41% and 27.86%, respectively. Meanwhile, *C. hystrix* EO featured Isopulegol as the major volatile compound, accounting for 15.95% of its composition. PCA and HCA analyses effectively classified the *Citrus* EO samples into two distinct categories. Specifically, *C. limon*

and *C. sinensis* were grouped together as samples with high antioxidant activity, whereas *C. hystrix* EO exhibited lower activity. The clustering in HCA highlighted the proximity between *C. limon* and *C. sinensis* EOs. This study underscores the potent antioxidant, plant inhibitory, and brine shrimp cytotoxic effects exhibited by *Citrus* species EOs, suggesting their potential as sources for natural antioxidative, herbicidal agents, and pharmaceutical substances. However, further elucidation is necessary to ascertain the specific volatile components responsible for these observed biological activities.

## ACKNOWLEDGEMENTS

The authors extend their gratitude to LPPM Universitas Terbuka (UT) for their support through the applied research scheme project, under contract number B/551/UN31.LPPM/PT.01.03/2023. Furthermore, the authors express gratitude to the Advanced Characterization Laboratories Serpong, National Research and Innovation Agency, for providing facilities and valuable scientific and technical assistance through E-Layanan Sains (ELSA) and Badan Riset dan Inovasi Nasional (BRIN).

## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## ETHICAL APPROVALS

This study did not involve experiments on animals or human subjects.

## DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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## USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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**How to cite this article:**

Einstivina Nuryandani, Erliana Novitasari, Muhammad Fatih Abdurrahman, Nurul Habibie, Ardiba Rakhmi Sefrienda, Dedy Kurnianto, Jasmadi Jasmadi, Yusuf Andriana. Chemometric analysis of GC-MS chemical profiles and biological activities of three *citrus* essential oils in Indonesia. *J Appl Pharm Sci*. 2024;14(12):187–195.