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Exploration of the biochemical composition of *Citrus* L. seeds for industrial applications

B. Budianto[a,✉] and A. Suparmi[b]

[a]Institute Sains and Technology Al-Kamal, Jakarta, Indonesia

[b]SMAN 4 Tarakan, Kalimantan Utara, Indonesia.
✉Corresponding author: budianto_delta@yahoo.com

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SUMMARY: This study aimed to assess the biochemical profiles of *Citrus* L. seeds and elucidate the correlation patterns among varieties based on their biochemical compositions. Samples of *Citrus* L. seeds were subjected to extraction, and the resulting oils were analyzed for their biochemical levels. Principal Component Analysis (PCA) was usedto unveil the relationship patterns among *Citrus* L. The research findings revealed that several citrus seeds, including C. *Paradise*, *C. limon* (L.) Burm.f., *Citrus reticulate*, *C. maxima* (Burm.) Merr., and *Citrus sinensis*, exhibited similarities in their compositions of flavonoid compounds, phenolic acids, and carotenoids. However, aromatic volatile compounds displayed variations among varieties without discernible patterns of similarity. The N-serotonin compound exhibited significant variations among varieties; whereas fatty acid compounds demonstrated similarities between *Citrus latifolia* and *C. limon* (L.) Burm.f. The eight samples showed no similarity in their biochemical variation patterns.

KEYWORDS: *Acyl-Nω-methylserotonins; Antioxidant; Bioactive compound; Bioflavonoid; Phytochemical compound.*

RESUMEN: ***Exploración de la composición bioquímica de semillas de* Citrus *L. para aplicaciones industriales.*** El objetivo de este estudio era evaluar los perfiles bioquímicos de las semillas de *Citrus* L. y dilucidar los patrones de correlación entre variedades en función de sus composiciones bioquímicas. Se extrajeron muestras de semillas de *Citrus* L. y se analizaron los niveles bioquímicos de los aceites resultantes. Se utilizó el Análisis de Componentes Principales (ACP) para desvelar los patrones de relación entre las variedades de *Citrus* L. Los resultados de la investigación revelaron que varias semillas de cítricos, incluyendo *C. paradise*, *C. limon* (L.) Burm.f., *Citrus reticulate*, *C. maxima* (Burm.) Merr. y *Citrus sinensis*, presentaban similitudes en la composición de compuestos flavonoides, ácidos fenólicos y pigmentos. Sin embargo, los compuestos aromáticos volátiles mostraron variaciones entre variedades sin patrones discernibles de similitud. Mientras tanto, el compuesto N-serotonina mostró variaciones significativas entre variedades, mientras que los ácidos grasos demostraron similitudes entre *Citrus latifolia* y *C. limon* (L.) Burm.f. Las ocho muestras no mostraron similitudes en sus patrones de variación bioquímica.

PALABRAS CLAVE: *Acil-Nω-metilserotoninas; Antioxidante; Bioflavonoide; Compuesto bioactivo; Compuesto fitoquímico.*

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1. INTRODUCTION

Citrus plants boast a diverse array of varieties, each characterized by significant differences in their biochemical composition. Citrus seeds, often overlooked, contain bioactive compounds such as flavonoids, phenolic acids, carotenoids, N-serotonin, volatile compounds, and fatty acids. Despite extensive prior research on these compounds, their potential for further exploration remains untapped.

The flavonoids, phenolic acids, and carotenoids identified in citrus seeds exhibit robust antioxidant properties (Tundis *et al*., 2014) and hold promise as crucial components in pharmaceutical, culinary, and cosmetic applications (Maqbool *et al*., 2023). However, focused research on distinct citrus seed varieties and their biochemical compounds is limited, particularly in comprehending compositional variations among citrus varieties.

Serotonin, a seldom-discussed component, presents intriguing potential. Citrus amblycarpa and other citrus species' seed inner shells house numerous previously unidentified Acyl-Nω-methylserotonins and Branched-chain Acylserotonins (Kruk *et al*., 2022). This acyl derivative of N-methylserotonin is exceptionally rare in other plants (Servillo *et al*., 2015). Initial research indicates an association with antioxidant potential and other neurological functions, warranting further exploration.

Continuing exploration of volatile compounds and fatty acids in citrus seeds is also of interest. These seeds contain undiscovered volatile compounds, encompassing a variety of aromatic and organic elements (Park *et al*., 2021), with potential applications in pharmaceuticals (Mahmoud *et al*., 2014), cosmetics (Burnett *et al*., 2021), and the culinary domain (Kim *et al*., 2018). Despite their significance, the specific roles and broad uses of these volatile compounds in citrus seeds remain largely unexplored and necessitate further investigation.

Our research mapping from 2010 to 2023 revealed a progression in focus. The initial stage (2010-2016) emphasized psychochemical properties and variations in types of *citrus sinensis*. The production of citrus seed oil began in 2017, and its impact on pharmaceuticals, biodiesel, and other sectors was evaluated. However, from 2019 onwards, there has been a notable lack of increased research focus on the impact of citrus seeds. In 2020, heightened interest emerged in biochemical components associated with microbiological effects. Nevertheless, psychochemical comparisons of citrus seed oils and their effects have not yet achieved prominence. This suggests a potential research gap, necessitating further exploration into other overlooked biochemicals.

Furthermore, this research offers a broader and more comprehensive understanding of the biochemical composition of citrus seeds, focusing specifically on flavonoids, phenolic acids, carotenoids, volatile compounds, fatty acids, and serotonin. The anticipated outcome of this research is to pave the way for harnessing the potential applications of these compounds across various industries. Moreover, the findings from this study serve as a catalyst for the development of innovative products based on the collection of bioactive compounds identified in citrus seeds.

The main objective of this study was to examine the biochemical profile of citrus seeds and uncover patterns of relationships among different citrus based on their biochemical compounds. The overarching goal is to unlock the untapped potential inherent in citrus seeds and promote their utilization across diverse industrial sectors.

2. MATERIALS AND METHODS

2.1. Material

This study used citrus seeds that are abundant in Indonesia, including *citrus latifolia*, *C. limon* (L) Burm f., *citrus sinensis*, *C. paradise*, *citrus amblycarpa*, *C. maxima* (Burm.) Merr., *citrus reticulata*, and *citrus maxima*. The eight citrus seeds were obtained from waste from the beverage industry, and some were purchased from farmers on March 20, 2023.

Among the eight samples, four of them could be identified as original Indonesian varieties, namely *citrus maxima*, *C. maxima* (Burm.) Merr., *citrus paradise*, and *citrus amblycarpa*. Meanwhile, *citrus reticulata* comes from China, *citrus latifolia* from Persia, and *C. Limon* (L.) Burm f. from India.

2.2. Oil extraction

Oil extraction followed the method adapted from Aydeniz *et al*. (2014). The extraction process began with manual separation, washing, air drying, and freezing of citrus seeds at -20 °C. The seeds were

then roasted in an oven at 30-35 °C for 30 minutes. Afterward, the seeds were cooled to room temperature, and the water content was measured using an Ohaus MB45 water content meter (OHAUS Instruments; Shanghai, Co., Ltd.). Cold pressing of the seeds was conducted using a laboratory-scale pressing machine. Fine particles and any remaining water in the pressed oil were separated through filtration. The centrifugation (Magal M16R, Shanghai.China) process operated at a speed of 6800 x g for 10 minutes. Oil samples were transferred into colored glass bottles and stored at −18 °C.

2.3. Biochemical analysis

The extraction of polyphenolic compounds from citrus seed oil followed the procedure outlined by García-Villalba *et al*. (2010). Initially, all oil samples underwent sep pak C-18 cartridge filtration, assisted by a vacuum in the manifold. To initiate the process, 3 grams of seed oil were dissolved in 3 mL of hexane (ChemStationAsia, Malaysia), applied to the cartridge, and rinsed with 5 mL of hexane to eliminate non-polar components. The residual hexane in the cartridge was removed using a nitrogen stream. The remaining phenolic fraction was subsequently extracted with 10 mL of methanol, and the resulting solution was filtered through a 0.45 µm pore size PTFE (polytetrafluoroethylene) membrane from Fisher Scientific before inclusion in the HPLC chromatogram. All samples were stored at -18 °C and analyzed within 24 hours. The flow through each cartridge was consistently managed concurrently on the manifold.

The identification process for specific phenolic acids and flavonoids adhered to the methodology outlined by Yilmaz and Karaman (2017) with minor adjustments. A C18 reversed-phase column (Zorbax Eclipse Plus, 250 mm x 4.6 mm i.d. x 5 µm) facilitated separation, and the analysis was conducted at 25 °C at a flow rate of 0.55 mL/min, and 25-µL injection volume. The chromatographic analysis involved the concurrent monitoring of phenolic extracts at 280 nm. Mobile phase A consisted of 100% acetonitrile (Merck, Darmstadt, Germany), and mobile phase B was ultra-pure water with 0.2% (v/v) sulfuric acid (Merck, Darmstadt, Germany) 95-97%, analytical grade) following the guidelines of the National Institute of Standards and Technology (NIST, 2014). Separation was performed using the following gradient program: 80% A / 20% B for 0-20 min, 70% A / 30% B for 20-26 min, 60% A / 40% B for 26-32 min, 50% A / 50% B for 32-38 min, 40% A / 60% B for 38-42 min, 45% A /55% B for 42-47 min, 35% A / 65% B for 47-52 minutes, 20% A / 80% B for 52-54 minutes, 10% A / 90% B for 54-56 minutes, 100% B for 56-72 minutes, 45% A /55% B for 72-76 minutes, and 80% A/ 20% B for 76-80 minutes. The identification of individual phenolic compounds depended on their retention time, and quantification was performed by measuring the peak area at 280 nm, using a standard curve prepared from the corresponding standard, according to NIST guidelines. All analyses were performed in triplicate.

The assessment of carotenoid content, encompassing total carotenoids, β-carotene, and lutein followed the methodology proposed by Franke *et al*. (2010). For the extraction process, 0.5 g of each oil sample was combined with 2 mL of petroleum ether: acetone (1:1, v/v) until complete dissolution. Absorbance was measured at 445 nm using a spectrophotometer (Infitek, SP-IUV7; Shandong, China), with petroleum ether (MilliporeSigma, WGK Germany): acetone (Merck, Darmstadt, Germany) serving as a blank. The determination of carotenoid content employed specific equations, utilizing absorption coefficients determined in petroleum ether (Güneşer and Yilmaz, 2019)

$$\mathbf{TC}\ (\mu g/g) = \frac{\mathbf{A} \times \mathbf{V} \times \mathbf{10^4}}{\mathbf{A}^{\%}_{\mathbf{1}\,cm} \times \mathbf{P}}$$

TC= Total Carotenoid content (µg/g); **A**= Absorbance value at 445 nm; $A^{\%}_{1\,cm}$ = Specific absorption coefficients for carotenoids ($A^{\%}_{1\,cm}$ = 2592, β-carotene extinction coefficient in petroleum ether); **V** = Volume of extraction solution (mL). **P**= sample weight (g).

In this study, TC was presented in units of mg/kg. To verify chlorophyll carotenoids as pheophytin-a, the AOCS Cc 13i-96 method (AOAC, 1997) was employed. This involved measuring absorbance at 630, 670, and 710 nm.

The determination of serotonin compounds in this study was made using HPLC and referring to Kruk *et al*. (2022). The mobile phase, composed of elution solvent A = 0.1% formic acid (BASF, China) in water and B (0.1% formic acid in acetonitrile), was employed for elution based on a scheme of 85-60% A and 15-40% B from 6-15 minutes, with a post-run

phase of 5 minutes involving 60-20% A and 40-80% B. The detection of serotonin derivatives took place at 324 nm, using a C18 column (1.8 µm, 2.1 × 50 mm). Parameters such as injection volume (2 µL), flow rate (0.4 mL/min), and column temperature (25 °C) were standardized. The contents of CS and FS were determined by referencing standard curves developed from standard solutions.

Fatty Acid Methyl Esters (FAMEs) were synthesized following the Ce 2-66 protocol (AOAC, 1997) and subsequently quantified using a Gas Chromatograph (Shimadzu's Nexis GC-2030; Maryland, USA) equipped with an HP 88 capillary column (100 m x 0.25 mm ID x 0.2 µm film thickness). The Gas Chromatograph was operated at various temperature settings: initially set at 120 °C for 1 minute, followed by an increase to 175 °C (10 °C/min) for 10 minutes, then to 210 °C (5 °C/min) for 5 minutes, and finally to 230 °C (5 °C/min) for an additional 5 minutes. For the analysis, a 1-µL injection volume was used with an injector split ratio of 1:50 and a flow rate of 2 mL/min, with hydrogen as the carrier gas. The injector and detector temperatures were maintained at 250 and 280 °C, respectively. Fatty acid identification was made through chromatography, employing a standard mixture of FAMEs for reference.

The volatile compound identification procedure was based on Guneser and Yilmaz (2017). Volatile compounds were gathered through headspace solid-phase microextraction (SPME) with specific fibers (2 cm to 50/30 µm DVB/Carboxen/PDMS; Supelco, Bellafonte). 2 grams of the oil sample, 1 gram of NaCl, and 20 µl of the internal standard (IS) (1 µl of 2-methyl-3-heptanone dissolved in 10 ml of methanol) were combined n a 40-ml SPME bottle, and stirred for 2 minutes. This mixture was then placed in a water bath at 45 °C for 15 minutes to stabilize the volatiles into the headspace. Subsequently, a needle was inserted into the bottle, and the needle fiber was introduced into the headspace at a depth of 2 cm for 10 minutes in a water bath. The volatiles collected on the needle fibers were then injected into a GC/MS equipped with an HP5 MS column (30-m x 0.25-mm i.d. x 0.25-µm). For the identification of volatiles, databases such as the National Institute of Standards and Technology (NIST, 2014) and The Wiley Registry of Mass Spectral Data (Wiley, 2006) were consulted, along with the Retention index (Kovats).

2.4. Statistical test

The statistical approach employed was analysis of variance (ANOVA), complemented by a post-hoc

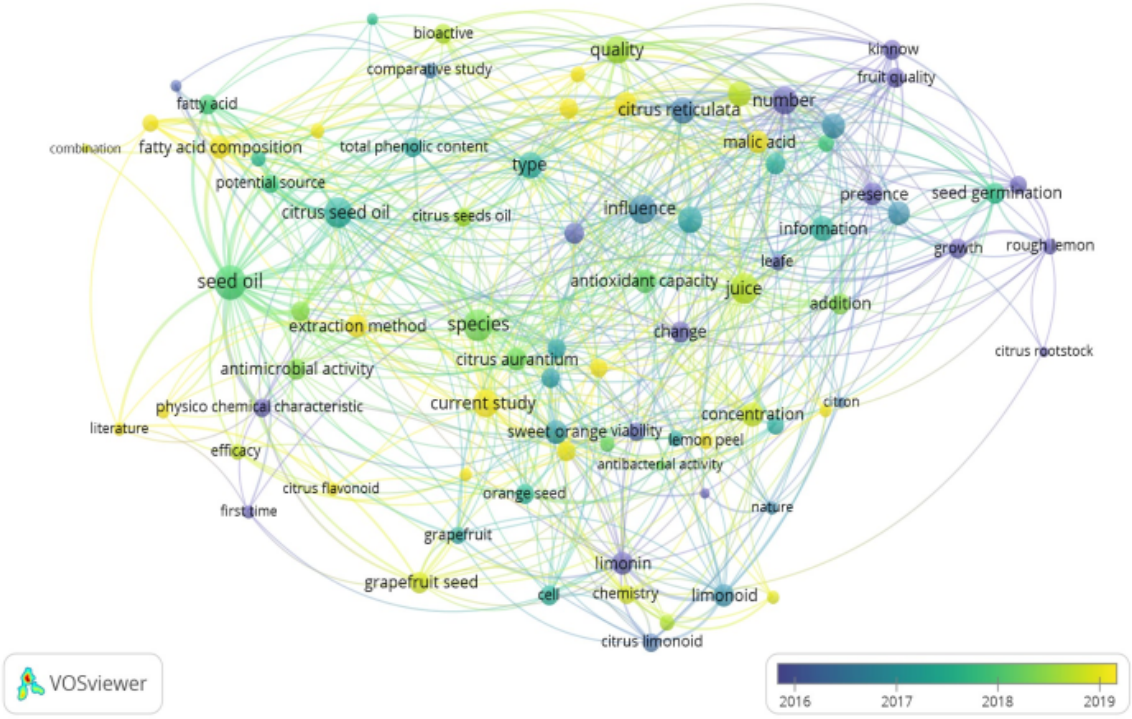

Figure 1. Overlay visualization of citrus seed research from 2010 to 2023

Tukey's Honestly Significant Difference (HSD) test to discern variations in biochemical composition among different *Citrus* L seeds. The significance threshold was set at $p < 0.05$. The analysis was conducted in three replicates, and the results were presented as Mean ± Standard Deviation. This analytical framework facilitated the identification of noteworthy differences in the biochemical composition of citrus seeds. Additionally, we conducted a Principal Component Analysis (PCA) using Minitab 21 software. This facilitated a comprehensive exploration of both similarities and distinctions in the biochemical composition among various citrus varieties, providing a more detailed understanding of their profiles.

3. RESULTS

3.1. Flavonoids, phenolic acids, and carotenoids

The flavonoid content, specifically catechin, in *citrus latifolia* seeds did not exhibit significant differences ($p > 0.05$) compared to *C. maxima* (Burm.) Merr. and citrus seeds. Similar findings were observed for *citrus amblycarpa* seeds against *citrus reticulate* and the *C. paradise* variety against *citrus maxima*. Notably, the variety *C. limon* (L.) Burm.f. displayed significantly different seeds ($p < 0.05$) across the seven samples.

Concerning eriocitrin compounds, no significant differences were observed in *citrus sinensis*, *C. paradise*, *citrus reticulate*, or *citrus maxima* seeds ($p > 0.05$). *C. maxima* (Burm.) Merr. seeds, however, exhibited the highest eriocitrin levels and were significantly different from the seven samples.

Citrus reticulate seeds demonstrated the highest rutin compound levels, significantly differing ($p < 0.05$) from the seven samples. Similar distinctions were observed for *citrus maxima* seeds in the naringin compound, *C. limon* (L.) Burm.f. (naringenin and neohesperidin), *citrus latifolia* (hesperidin), and *citrus amblycarpa* (kaempferol).

The phenolic acid compounds exhibited varying levels across the eight seeds, with *citrus latifolia* containing both the highest and lowest levels of gallic acid (tr-ferulic acid, rosmarinic acid, and tr-2-hydrocinnamic acid), showing significant differences from the other seven samples.

Additionally, *citrus latifolia* seeds demonstrated the highest levels of carotenoids, including total carotenoids, β-carotene, and lutein, and the lowest levels of total chlorophyll (pheophytin a). These differences were found to be significant ($p < 0.05$) across the seven samples. Detailed results regarding the levels of flavonoids, phenolic acids, and carotenoids are provided in Table 1.

The categorization of citrus seed varieties based on flavonoids, phenolic acids, and carotenoids is illustrated in Figure 2. Among the seed varieties, including *C. paradise*, *C. limon* (L.) Burm.f., *citrus reticulate*, *C. maxima* (Burm.) Merr., and *citrus sinensis*, there was a prominent influence on component 1, indicating their similarity in terms of the tested compounds. Conversely, the *citrus maxima*, *citrus amblycarpa*, and *citrus latifolia* varieties displayed dissimilarities, positioned distinctly apart in different quadrants (Figure 2A).

The grouping of flavonoids, phenolic acids, and carotenoids in citrus varieties is further depicted in Figure 2B. A narrow angle, indicative of similarity, was observed for compounds such as naringin, naringenin, total chlorophyll (pheophytin a), rosmarinic acid, rutin, tr-2-hydrocinnamic acid, neohesperidin, tr-ferulic acid, and eriocitrin. These compounds demonstrated a strong correlation in component 1.

Total carotenoid compounds, lutein, β-carotene content, and hesperidin exhibited similarities in component 2, albeit with a weak correlation. In contrast, syringic acid and catechin were positioned closely, indicating a strong correlation.

3.2. Acylserotonin

All seed varieties exhibited N-Acylserotonin compounds that did not display significant differences ($p > 0.05$) for < C21, ai-C21, Me-C20, n-C21, n-C25, and ai-C27. The prevalence of N-Acylserotonin compounds was found in the C22 to C24 homologs for all varieties, and the distribution of values was consistent across all samples. Notably, there was a substantial difference for Me-C22, with the *citrus maxima* variety recording the highest level (12 ± 0.3 mg/kg oil), which was found to be significantly different ($p < 0.05$) when compared among the seven samples. Comprehensive results regarding the composition of N-Acylserotonin for each variety are presented in Table 2.

The grouping of citrus seed varieties based on the N-Acylserotonins compound did not reveal a strong correlation between varieties. Each variety displayed

Table 1. Composition of flavonoid, phenolic acid, and carotenoid compounds in citrus seed varieties.

Biochemical	*Citrus latifolia*	C. *limon* Burm f.	*Citrus sinensis*	C. *paradise*	*Citrus amblycarpa*	*C. maxima* (Burm.) Merr.	*Citrus reticulate*	*Citrus maxima*
Flavonoids (mg/kg oil)								
Catechin	14.87±0.42ab	15.25±0.31c	14.01±0.31ab	15.00±0.31bc	13.00±0.41a	14.02±0.22ab	13.01±0.32a	15.03±0.33bc
Eriocitrin	31.01±0.51a	85.78±0.41bc	84.00±0.72b	85.01±0.32b	86.02±0.42bc	87.00±0.61c	84.00±0.31b	84.01±0.41b
Rutin	52.59±1.22a	76.48±0.21b	78.03±0.42c	77.00±0.33bc	76.01±0.32b	78.02±0.61c	80.01±0.52d	79.02±0.71cd
Naringin	234.28±31a	299.80±1.72b	300.01±1.01 bc	302.00±1.21bc	303.01±1.42bc	300.02±1.12bc	298.00±1.31b	321.03±2.12d
Naringenin	10.38±0.51a	13.23±0.32d	12.01±0.22bc	11.00±0.41ab	12.01±0.42bc	11.00±0.33ab	12.01±0.52bc	11.01±0.22ab
Hesperidin	909.67±1.32e	903.40±1.43cd	900.01±1.62b	901.01±1.12bc	902.02±1.43bcd	903.01±1.32cd	900.02±1.52b	890.01±1.51a
Neohesperidin	100.99±1.61a	125.91±1.71d	123.01±1.22bc	121.02±1.72bc	120.01±1.71bc	119.02±1.22b	121.02±1.43bc	118.01±23b
Kaempherol	8.64±0.61bc	9.56±0.42bc	8.02±0.53bc	9.01±0.61bc	10.01±0.52c	7.01±0.42ab	9.01±0.83bc	6.00±0.21a
Phenolic acids (mg/kg oil)								
Gallic acid	42.43±1.22d	29.41±1.22ab	30.01±1.32bc	29.01±1.53ab	28.01±1.23a	29.01±1.43ab	31.02±1.22bc	30.01±1.42bc
Syringic acid	6.93±0.12bc	7.13±0.21c	6.02±0.22ab	7.01±0.23c	5.02±0.52a	5.01±0.62a	6.00±0.53ab	7.01±0.12c
tr-Ferulic acid	222.97±2.22a	364.30±2.42f	340.01±2.41c	328.00±2.11b	356.01±1.21d	356.02±2.12d	340.01±2.61b	360.00±2.33e
Rosmaniric acid	58.08±1.51a	77.91±1.12d	78.01±1.31d	77.00±1.22cd	76.01±1.23bc	78.01±1.22d	75.01±1.12b	76.41±1.22bc
tr-2-Hydrocinnamic acid	41.65±1.32a	47.22±1.12bc	47.01±1.12bc	46.01±1.23b	48.01±1.42c	48.01±1.32c	46.01±1.32b	48.01±1.12c
Carotenoids (mg/kg oil)								
Total carotenoid	7.64±0.41d	5.49±0.62bc	6.01±0.32c	5.01±0.23b	5.02±0.33b	6.01±0.41c	5.02±0.32b	4.01±0.12a
b-Carotene	7.37±0.32c	5.30±0.42ab	6.01±0.33bc	5.01±0.42ab	6.02±0.41bc	5.01±0.32ab	5.02±0.23ab	4.01±0.32a
Lutein	7.35±0.21c	5.29±0.41bc	6.01±0.32cd	5.02±0.31ab	7.01±0.32c	6.01±0.32bc	4.01±0.21a	5.01±0.33ab
Total chlorophyll	0.21±0.04a	0.34±0.03ab	1.02±0.21c	1.02±0.31c	1.03±0.31c	1.04±0.41c	1.05±0.42c	1.02±0.42c

ANOVA Tukey's HSD Posthoc with significance threshold at $p < 0.05$. Results in Mean ± STD deviation, with 3 repetitions.

Table 2. Composition of N-Acylserotonin compounds in citrus seed varieties.

Acylserotonin (mg/Kg oil)	*Citrus latifolia*	*C. limón* Burm f.	*Citrus sinensis*	*C. paradise*	*Citrus amblycarpa*	*C. maxima* (Burm.) Merr.	*Citrus reticulate*	*Citrus maxima*
<C21	2.70±0.32a	2.80±0.31a	2.10-±0.22a	2.21±0.23a	2.31±0.33a	2.11±0.33a	2.40±0.21a	2.10±0.21a
ai-C21	1.31±0.42a	1.31±0.41a	1.50±0.22a	1.10±0.22a	1.21±0.11a	1.21±0.32a	1.12±0.22a	1.02±0.21a
Me-C20	1.12±0.10a	1.02±0.21a	1.03±0.22a	1.02±0.32a	1.01±0.21a	1.02±0.11a	1.02±0.32a	1.02±0.22a
n-C21	0.12±0.02a	0.11±0.03a	0.12±0.02a	0.12±0.03a	0.12±0.01a	0.13±0.02a	0.12±0.03a	0.21±0.02a
Me-C21	0.60±0.02ab	0.71±0.01b	0.31±0.03a	0.60±0.02ab	0.70±0.01b	0.31±0.02a	0.21±0.02a	0.50±0.02ab
n-C22	3.31±0.52ab	4.11±0.22ab	3.02±0.51ab	4.01±0.51b	3.02±0.61ab	3.01±0.52ab	2.02±0.41a	3.02±0.32ab
ai-C23	3.61±0.31b	3.11±0.62ab	3.02±0.32ab	2.02±0.32a	3.01±0.51ab	3.01±0.62ab	3.01±0.52ab	3.00±0.62ab
Me-C22	9.01±0.23b	9.00±0.32b	10.02±0.22c	11.01±0.32d	8.02±0.22a	9.03±0.22b	8.01±0.42a	12.01±0.32e
n-C23	5.02±0.31a	6.03±0.32b	6.81±0.32bc	7.02±0.32c	5.01±0.33a	6.00±0.12b	5.00±0.23a	6.01±0.41b
Me-C23	9.71±0.22d	9.81±0.33d	6.02±0.21a	7.01±0.32b	8.02±0.23c	9.02±0.24d	11.02±0.32e	8.01±0.33c
n-C24	4.31±0.42b	3.71±0.63ab	3.02±0.52ab	2.02±0.42a	2.01±0.32a	2.00±0.53a	3.01±0.43ab	2.01±0.52a
ai-C25	1.00±0.42a	0.90±0.32a	1.00±0.31a	1.01±0.42a	2.01±0.32b	1.00±0.53a	1.01±0.31a	1.01±0.41a
Me-C24	11.00±0.21a	12.01±0.32b	11.02±0.32a	12.02±0.33b	14.02±0.31d	15.01±0.42e	14.01±0.22d	13.02±0.21c
n-C25	1.12±0.12a	1.01±0.22a	1.02±0.23a	1.02±0.21a	1.01±0.24a	1.02±0.12a	1.02±0.32a	1.02±0.22a
iso-C26	1.31±0.43ab	1.31±0.31ab	1.50±0.21b	1.10±0.21a	1.21±0.12a	1.20±0.32a	1.10±0.22a	1.01±0.31a
Me-C25	3.10±0.23c	2.60±0.32bc	1.31±0.31a	2.01±0.22b	1.01±0.21a	2.01±0.13b	ND	2.01±0.23b
n-C26	0.51±0.02a	ND	1.01±0.03b	1.02±0.02b	1.02±0.03b	1.02±0.02b	1.01±0.04b	1.01±0.02b
ai-C27	1.01±0.21a	0.81±0.21a	1.31±0.32a	1.02±0.21a	1.02±0.31a	1.01±0.41a	1.02±0.32a	1.02±0.22a

ANOVA Tukey HSD Posthoc with significance threshold at $p < 0.05$. Results in Mean ± STD deviation, with 3 repetitions.
ND: Not Detected; **ai-C21** (18-Methyleicosanoic); **ai-C23** (20-Methyldocosanoicacid); **ai-C25** (22-Methyltetracosanoic acid); **iso-C26** (24-Methyleicosanoicacid); **ai-C27** (25-Methylhexacosanoic acid); **iso-C28** (26-Methyleicosanoicacid).

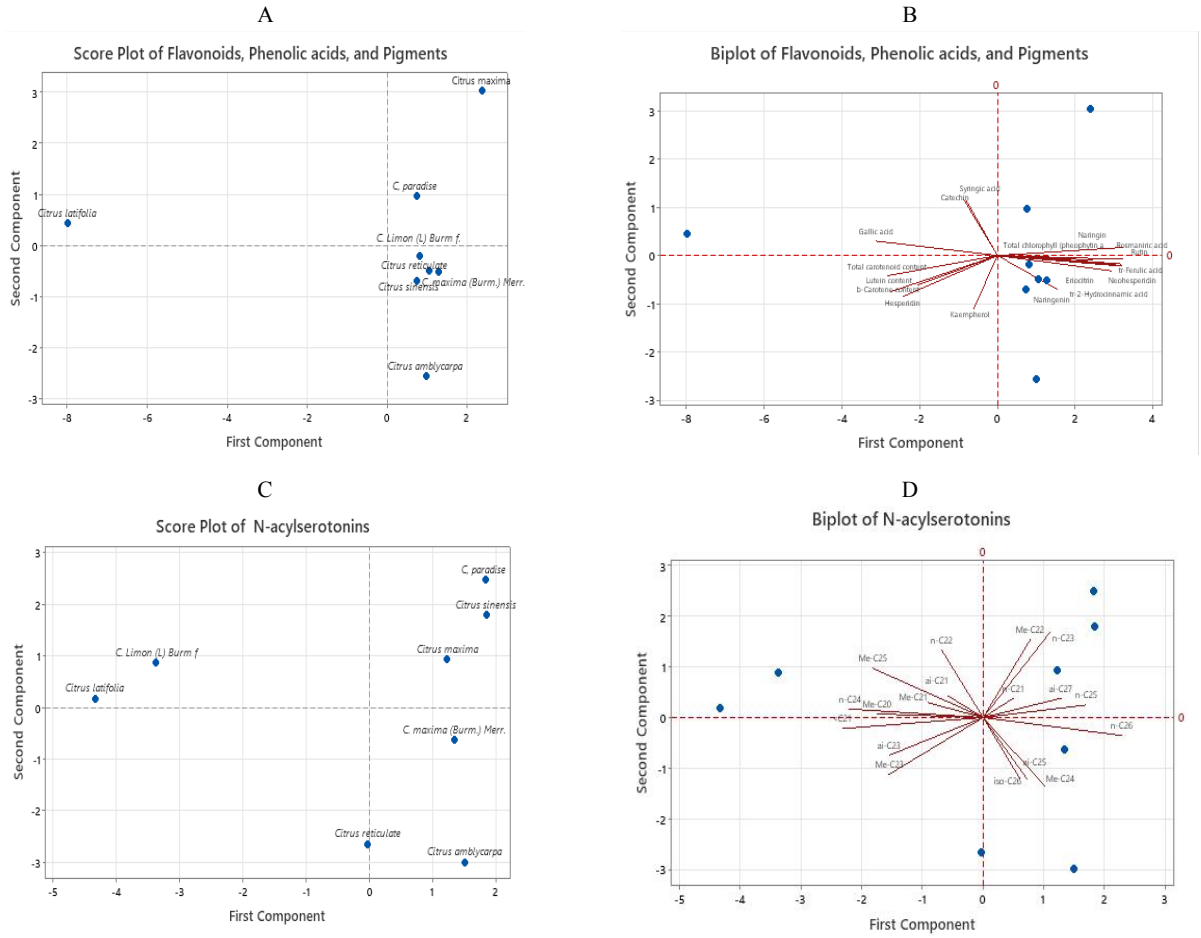

Figure 2. Grouping of citrus seed varieties. PCA score-plot based on flavonoids, phenolic acids, carotenoids (A), biplot (B). Score-plot based on N-Acylserotonins (C) and biplot (D), using the mean result of 3 repetitions.

a wide angle, but *C. paradise* and *citrus sinensis* varieties exhibited similarity compared to other varieties. As illustrated in Figure 2C, the even distribution of N-Acylserotonins compounds in seed varieties did not indicate any noticeable similarity among the samples.

Strong correlations with N-Acylserotonin compounds were observed, particularly in component 1, for ai-C25, Me-C24, and iso-C28. Additionally, in component 2, there were strong correlations for compounds < C21, Me-C20, n-C24, and Me-C21. The compounds Me-C23 and ai-C23 also exhibited similarities, albeit in the negative (-) area.

Furthermore, the n-C21 compound demonstrated similarity to ai-C27, n-C25, n-C23, and Me-C22. As illustrated in Figure 2D, the levels of N-Acylserotonins were evenly distributed into four quadrants, indicating similarity despite having distinct strong correlations for each N-Acylserotonin compound.

3.3. Volatile aromatic

None of the samples exhibited significant differences ($p > 0.05$) for the compounds 3-Methoxy-1-butanol, 3-Carene, α-Ocimene, and Phenylethyl alcohol. The D-Limonene compound predominated, with the highest level (5902.07 ± 62 ppm) observed in the *citrus latifolia* variety, significantly differing ($p < 0.05$) from other varieties (except *citrus sinensis*).

The second dominant compound was b-Myrcene, reaching its highest levels at 124.89 ± 0.4 ppm in the *citrus latifolia* variety, and it was significantly different ($p < 0.05$) from other varieties. Comprehensive results for the composition of volatile compounds are provided in Table 3

The grouping of citrus seed varieties based on aromatic volatile compounds did not reveal a strong correlation between varieties. All samples exhibited very wide angles between each other, and the distri-

Table 3. Composition of volatile aromatic compounds in citrus seed varieties.

Biochemical (mg/Kg oil)	*Citrus latifolia*	*C. limón* Burm f.	*Citrus sinensis*	*C. paradise*	*Citrus amblycarpa*	*C.maxima* (Burm.) Merr.	*Citrus reticulate*	*Citrus maxima*
3-Methylbutanal	40.00±0.31d	39.05±0.42c	38.02±0.21b	37.07±0.31a	36.82±0.42a	37.01±0.22a	40.10±0.23d	41.00±0.21e
Acetoin	24.23±0.61bc	18.11±0.52a	24.08±0.82bc	25.12±0.41cd	26.08±0.51d	24.09±0.71bc	23.12±0.41b	25.06±0.62c
Hexanal	8.91±0.41a	16.30±0.41d	8.09±0.12a	9.04±0.61ab	10.05±0.42b	11.05±0.62bc	9.08±0.71ab	12.08±0.71c
Furfural	24.07±0.41c	29.36±0.52f	24.04±0.31bc	24.00±0.31bc	21.01±0.51a	23.02±0.41b	26.02±0.32d	27.05±0.22e
Methyl pyrazine	23.02±0.31d	20.06±0.34a	21.01±0.31b	23.04±0.52d	23.02±0.32d	22.02±0.42c	20.03±0.42a	21.01±0.22b
2-Furan menthol	4.07±0.21a	7.96±0.51c	5.00±0.61ab	6.02±0.52bc	7.05±0.72c	5.06±0.52ab	7.07±0.62c	6.08±0.52bc
Isoamyl acetate	2.81±0.31bc	2.52±0.51bc	3.02±0.42c	1.00±0.02a	2.01±0.41b	3.00±0.21c	1.01±0.03a	1.01±0.03a
Butrylactone	0.38±0.03bc	0.41±0.04c	0.32±0.04ab	0.23±0.04a	0.41±0.03c	0.42±0.03c	0.38±0.02bc	0.28±0.03a
2.5-Dimethlypyrazine	5.00±0.61ab	4.11±0.52a	4.67±0.71ab	4.71±0.71ab	4.81±0.51ab	3.89±0.41a	4.89±0.82ab	6.01±0.62b
Butyl isobutyrate	0.89±0.02a	1.01±0.06a	2.01±0.41ab	2.01±0.62ab	3.02±0.21b	1.02±0.06a	1.02±0.08a	2.00±0.51ab
a-Thujene	2.86±0.04bc	2.38±0.21bc	3.01±0.12c	4.00±0.21d	2.02±0.02b	1.01±0.03a	3.01±0.11c	1.02±0.06a
a-Pinene	24.96±0.81d	17.68±0.72b	20.01±0.82c	21.02±0.81c	15.02±0.61a	15.03±0.71a	16.02±0.71ab	17.03±0.61b
Isopropyl pentanoate	11.52±0.32ab	11.59±0.51ab	12.00±0.22b	13.02±0.22c	11.03±0.21a	12.02±0.33b	13.04±0.33c	11.05±0.34a
Benzaldehyde	5.24±0.62ab	6.51±0.31b	8.01±0.52c	7.02±0.71bc	5.02±0.12a	6.03±0.21b	6.03±0.32b	8.03±0.61c
b-Pinene	47.59±0.61d	31.34±0.51a	35.06±0.72b	37.02±0.82c	36.04±0.83bc	35.03±0.41b	31.02±0.41a	38.07±0.61c
b-Myrecene	124.89±0.41e	87.33±0.61a	90.02±0.42b	91.04±0.52b	92.05±0.52b	98.04±0.51d	94.03±0.51c	91.00±0.61b
a-Phellandrene	3.99±0.32bc	3.57±0.51bc	3.01±0.32b	4.00±0.42c	3.58±0.52bc	4.02±0.51c	2.01±0.21a	3.01±0.31b
Octanal	3.51±0.53c	2.03±0.32b	3.01±0.62bc	2.03±0.22b	2.04±0.21b	3.05±0.73bc	2.01±0.33b	1.01±0.21a
3-Carene	2.89±0.51a	2.33±0.61a	3.01±0.51a	3.02±0.61a	3.00±0.42a	3.01±0.62a	3.51±0.72a	3.01±0.51a
3-Methoxy-1-butanol	2.61±0.52a	2.71±0.71a	3.01±0.81a	2.03±0.72a	2.04±0.62a	3.01±0.43a	2.01±0.42a	3.01±0.62a
Hexyl acetate	4.15±0.51a	6.51±0.52b	5.01±0.42ab	4.00±0.72a	6.00±0.72b	6.01±0.82b	5.01±0.62ab	4.01±0.82a
b-Cymene	22.27±0.32e	14.38±0.71a	21.03±0.42d	20.02±0.21c	19.02±0.21b	23.02±0.41f	21.02±0.21d	20.02±0.31c
D-Limonene	5902.07±62.01e	4568.84±40.02a	5900.10±52.11e	5800.21±40.11d	5700.21±30.02c	6700.32±45.10f	5400.23±38.23b	5860.21±40.01d
a-Ocimene	1.71±0.51a	1.89±0.61a	2.01±0.52a	2.01±0.52a	2.01±0.63a	2.00±0.61a	1.02±0.71a	2.01±0.62a
g-Terpinene	32.11±0.71c	27.97±0.52ab	30.01±0.62b	28.01±0.43b	27.01±0.73a	28.02±0.42b	28.02±0.52b	32.01±0.63c
1-Octenol	1.04±0.06ab	0.55±0.03a	1.01±0.21ab	1.00±0.52ab	2.01±0.05b	1.02±0.42ab	1.01±0.51ab	2.01±0.51b
(*Z*)-Linalooloxide	1.88±0.12ab	1.91±0.12ab	2.01±0.61b	1.02±0.22a	2.04±0.53b	1.02±0.31a	2.02±0.51b	1.01±0.31a
a-Terpinolene	12.19±0.31c	10.14±0.22a	11.02±0.43b	12.03±0.11c	11.03±0.32b	12.02±0.42c	10.02±0.52a	12.02±0.32c
Phenylethyl alcohol	ND	0.58±0.03a	1.01±0.04a	1.02±0.05a	1.00±0.06a	0.50±0.08a	0.40±0.05a	1.02±0.06a
(*E*)-Limonene oxide	0.93±0.02a	1.32±0.05a	1.00±0.21a	2.01±0.11b	1.02±0.08a	1.02±0.07a	1.01±0.07a	2.01±0.22b
4-Carvomenthol	0.85±0.03a	ND	1.01±0.12b	1.02±0.31b	2.02±0.22c	1.03±0.32b	1.02±0.51b	2.01±0.33c
a-Terpineol	49.32±0.52bc	47.17±0.32a	50.09±0.33c	49.05±0.63bc	48.07±0.63ab	47.08±0.54a	51.08±0.73d	50.07±0.43cd
Decyl acetate	0.87±0.04a	0.71±0.06a	1.02±0.13a	2.02±0.23b	1.01±0.22a	1.01±0.13a	2.01±0.32b	1.01±0.12a

ANOVA Tukey's HSD Posthoc with significance threshold at $p < 0.05$. Results in Mean ± STD deviation, with 3 repetitions.

bution of aromatic volatile compounds was uniform across all varieties, indicating a lack of similarity among them (see Figure 3A).

A strong correlation, indicated by a narrow angle, was observed for the compounds 4-carnomenthol, butyl iso butyrate, 2,5 dimethylpyrazine, 1-octenol, and phenylethyl alcohol. Similar properties were also noted in the compound group 3-Methylbutanol, benzaldehyde, and (E)-limonene oxide in component 1 (see Figure 3B)

In component 2, a narrow angle was observed for the compounds butyrolactone, isopropyl pentanoate, (z)-linalooxide, and α-thujene. A negative correlation was seen for the furfural, hexanal, and 2-furan menthol compounds.

3.4. Fatty acids

None of the varieties exhibited significant differences ($p > 0.05$) for the compounds lauric acid, ara-

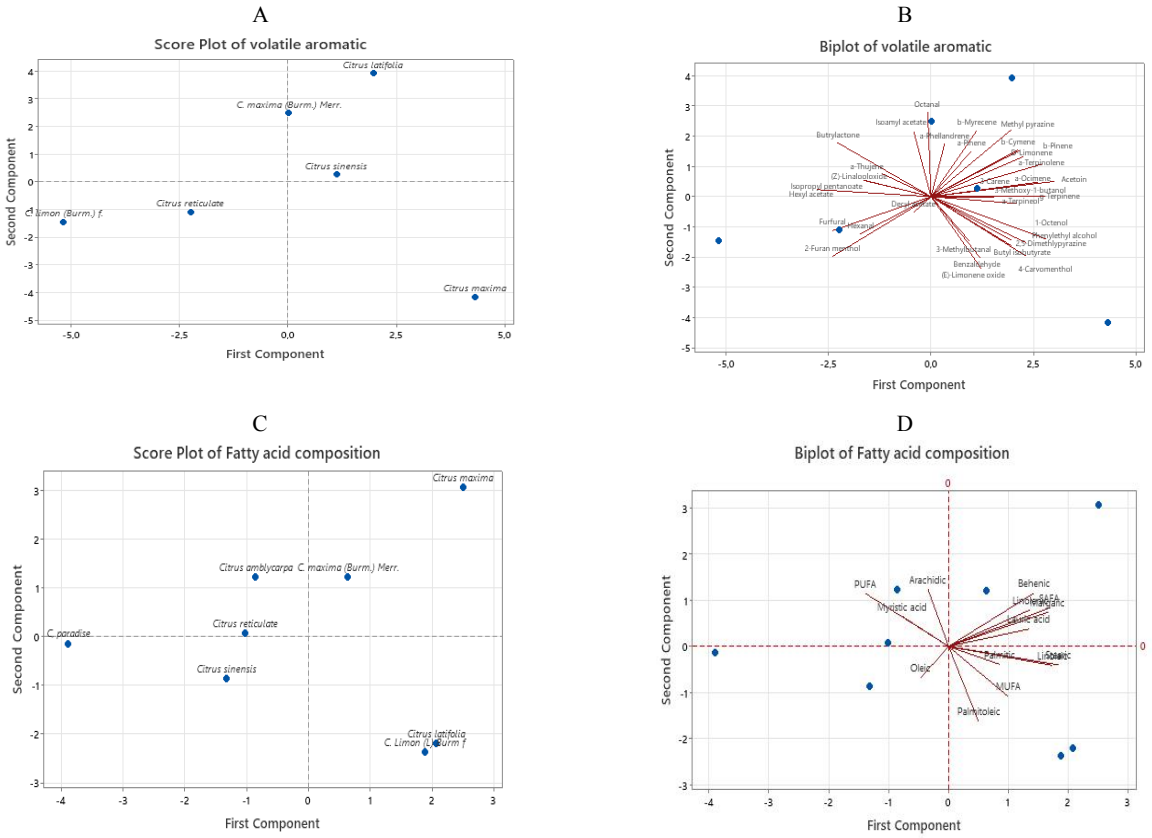

Figure 3. Grouping of citrus seed varieties. based on the composition of volatile aromatic compounds: PCA score plot based on volatile aromatic compounds (A), biplot (B). Score-plot based on fatty acids (C) and biplot (D), using the mean result of 3 repetitions.

Table 4. Composition of fatty acid compounds in citrus seed varieties.

Biochemical (mg/Kg oil)	*Citrus latifolia*	*C. limón* Burm f.	Citrus sinensis	*C. paradise*	*Citrus amblycarpa*	*C.maxima* (Burm.) Merr.	*Citrus reticulate*	*Citrus maxima*
Lauric acid	2.81±0.41a	2.21±0.60a	2.80±0.71a	1.90±0.50a	2.10±0.61a	2.70±0.60a	2.50±0.71a	2.95±0.61a
Myristic acid	0.30±0.03b	0.24±0.04b	1.02±0.06c	1.01±0.10c	1.02±0.06c	0.039±0.01a	ND	1.02±0.03c
Palmitic	28.36±1.51g	27.09±1.41fg	21.03±1.32cd	24.73±1.32e	20.85±1.81c	15.74±1.61b	11.68±1.21a	28.15±1.71g
Palmitoleic	0.66±0.05b	0.72±0.04c	0.65±0.05b	0.4±0.04ab	0.27±0.05a	0.38±0.04a	0.30±0.04a	0.26±0.04a
Margaric	4.45±0.05b	4.59±0.04b	3.80±0.06a	3.40±0.02a	3.60±0.07a	4.70±0.06b	4.80±0.06b	5.95±0.06c
Stearic	26.01±0.91e	26.62±0.91e	23.67±0.42cd	14.90±1.22a	24.01±0.81d	23.06±0.71c	20.03±1.21b	24.04±0.71d
Oleic	21.48±0.91de	20.72±1.32d	10.81±0.92c	28.44±1.22f	20.85±1.41d	6.03±0.82b	1.10±0.91a	10.74±1.21c
Linoleic	26.18±1.31f	26.98±1.30f	24.31±1.41e	3.92±0.81a	23.75±0.91b	16.05±1.60c	17.02±1.20c	20.53±1.41d
Linolenic	8.01±0.31b	9.01±0.32c	7.04±0.32a	8.07±0.42b	8.08±0.22b	9.03±0.42c	7.03±0.31a	10.03±0.21d
Arachidic	ND	0.22±0.041a	0.31±0.04a	0.40±0.01a	0.30±0.03a	0.20±0.03a	0.40±0.04a	0.70±0.04a
Behenic	0.28±0.03a	0.28±0.05a	0.10±0.03a	0.20±0.03a	0.30±0.02a	0.40±0.02a	0.20±0.05a	0.50±0.11a
SAFA	34.42±0.81ab	33.35±0.81ab	32.00±0.81a	31.70±0.71a	35.00±0.81bc	34.02±0.91ab	32.01±0.61a	36.02±0.51c
MUFA	27.09±0.61de	27.81±0.41e	21.45±0.81a	22.01±0.72ab	24.01±0.81c	21.01±0.90a	26.01±0.90d	23.03±0.82b
PUFA	37.66±0.80a	37.70±0.71a	53.27±0.81d	51.00±0.62c	49.01±0.71b	50.05±0.82bc	51.08±0.82c	49.04±0.62b

ANOVA Tukey's HSD Posthoc with significance threshold at $p < 0.05$. Results in Mean ± STD deviation, with 3 repetitions.
Saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA)
ND: Not Detected

chidic, and behenic. However, palmitic compounds showed significant differences ($p < 0.05$) among varieties, except for *citrus latifolia* and *citrus maxima*.

Polyunsaturated fatty acids (PUFA) dominated, reaching 53.27 ± 0.8 ppm in the citrus seed variety, and were significantly different from other varieties. The second dominant group was saturated fatty acids (SAFA), which exhibited significant differences in Citrus maxima varieties. The highest levels of monounsaturated fatty acids (MUFA) were observed in the *C. limon* (L.) Burm.f. variety (27.8±0.4 mg/kg oil), and it was significantly different from other varieties. The complete composition of fatty acid compounds is detailed in Table 4.

Based on the composition of fatty acid compounds, the *citrus latifolia* and *C. limon* (L.) Burm.f. varieties exhibited similarity in the component 1 area, characterized by very narrow angles. In contrast, other varieties demonstrated different conditions, showing no strong correlation with each other (see Figure 3C).

In the composition of fatty acid compounds, palmitic, linoleic, and stearic exhibited a narrow angle or strong correlation in component 1. Another group displaying a strong correlation consisted of behenic, linolenic, lauric acid, and margaric. In component 2, the myristic acid compound was very strongly correlated (narrow angle) with PUFA, while this condition did not apply to oleic or arachidic. Complete results can be observed in the biplot of the fatty acid composition test (Figure 3D).

4. DISCUSSION

The seed varieties of C. *paradise*, *C. limon* (L.) Burm.f., *Citrus reticulate*, *C. maxima* (Burm.) Merr., and *citrus sinensis* exhibited similarities in terms of flavonoid composition, phenolic acids, and carotenoids. In contrast, *citrus maxima*, *citrus amblycarpa*, and *citrus latifolia* varieties demonstrated no similarities. Notably, hesperidin was the predominant compound among flavonoids, with concentrations ranging from 890 to 930 ppm. Additionally, tr-Ferulic acid dominated among phenolic acid compounds. These results are in agreement with the findings of Güneşer *et al*. (2018).

Despite reported findings by various researchers, the utilization of flavonoids, phenolic acids, and carotenoids in citrus varieties on an industrial scale remains limited. Notably, bioflavonoid compounds have been shown to facilitate the apoptosis of hepatocellular carcinoma cells. Specifically, flavone glycosides such as neohesperidin, hesperidin, and naringin have demonstrated the ability to induce the death of liver cancer cells. The use of Annexin V-FITC/PI staining and flow cytometry has revealed the apoptosis of HepG2 cells in this context (Banjerdpongchai *et al*., 2016).

In human kidney cells (HEK 293T cells) subjected to H_2O_2-induced oxidative stress, flavonoids from *citrus amblycarpa* seeds (FLS) have demonstrated a protective effect. FLS was observed to decrease malondialdehyde levels, indicative of reduced oxidative damage, while concurrently elevating the levels of crucial antioxidant enzymes, including CAT, SOD, GSH, and GSH-Px (Yang *et al*., 2020). The key phenolic substances identified in Citrus amblycarpa seeds, namely 1,2-dihydroxybenzene, kaempferol, catechin, and isorhamnetin, have been recognized for their ability to safeguard against oxidative damage to cells (Yang *et al*., 2020). Citrus amblycarpa seed extract has been identified as a potential source of various phytochemical substances, encompassing alcaloids, flavonoids, saponins, tannins, steroids, and glycosides. The extract possesses the ability to significantly increase sleep duration while concurrently reducing sleep time (Rahman *et al*., 2022).

Aromatic volatile compounds were found uniformly across all varieties without exhibiting similarities. Notably, discrepancies in previous research (Güneşer *et al*., 2018) highlight variations in the *citrus sinensis* variety, where compounds such as 3-Methylbutanal and 3-Methoxy-1-butanol were absent under cold-pressing conditions.

Despite reports of the effectiveness of these compounds in cancer treatment by earlier researchers, their optimal utilization remains unexplored. Mahmoud *et al*. (2014) conducted research revealing that Limonene, administered at a dose of 100 mg/kg, was more effective in reducing bilirubin, while a dose of 50 mg/kg demonstrated greater efficacy in reducing oxidative stress and mitigating liver damage. Additionally, compounds such as α-pinene, 1,8-cineole, karyophyllene, and geraniol have been identified as having anticancer properties by inhibiting cancer cell proliferation (Tunjung *et al*., 2020)

The grouping of citrus seed varieties based on the N-Acylserotonins compound reveals dissimilarities, with an exception observed in the *C. paradise* and

citrus sinensis varieties. Our findings diverge from those of Kruk *et al*. (2022), particularly regarding the absence of the N-serotonin (ai-C25) compound in *citrus reticulate* and *citrus maxima* varieties. Similarly, iso-C26 was not detected in *citrus reticulate*, *C. maxima* (Burm.) Merr., and *citrus maxima* varieties, and ai-C27 were absent from *citrus reticulate* varieties according to Kruk *et al*. (2022)

The germination of citrus seeds has been shown to enhance antioxidant activity and increase the content of phenolic components. Despite this, a lack of correlation between the content of phenolic compounds and antioxidant activity suggests the potential presence of other antioxidants (Falcinelli *et al*., 2020). Addressing this, Kruk *et al*. (2022) highlighted that the inner skin of citrus seeds contains acylserotonin, an active antioxidant. This acyl derivative of N-methylserotonin, rarely found in plants, and citrus seeds also contains serotonin compounds with branched chains.

In the context of bone cancer therapy, the combination of gold nanoparticles (AuNPs) with *citrus reticulata* seed extract has proven effective in reducing gold-to-gold nanoparticles, as evidenced by FT-IR testing (Ahati *et al*., 2022).

Based on fatty acid composition*, citrus latifolia* and *C. limon* (L.) Burm.f. varieties exhibited similarities. Prior investigations indicated that *citrus latifolia* seed oil lacks lauric acid (C12:0), a trait shared with *C. maxima* (Burm.) Merr. seeds, which also lack lauric acid (C12:0) and myristic acid (C14:0) (Fathollahy *et al*., 2021). Similar findings were observed in *citrus amblycarpa* seed oils (Malacrida et al., 2012) and citrus seeds (Malacrida *et al.*, 2012). Our research reinforces these observations, highlighting the prevalence of lauric acid (C12:0) and myristic acid (C14:0) in *C. limon* (L.) Burm.f. seeds (Malacrida *et al*., 2012) and *C. paradise* (Burnett *et al*., 2021).

The utilization of citrus sinensis seed oil, coupled with alkaline catalytic transesterification, has successfully met biodiesel quality standards (ASTM6751 and EN14214) (Ezekoye *et al*., 2019). Biodiesel derived from citrus seed oil exhibits a higher density than petroleum-derived biodiesel at 15 °C (Agarry *et al*., 2013). *Citrus* sp. seeds with various catalysts such as green copper oxide nanoparticles, NaOH, and CaO yield comparable results, as observed in *citrus medica* (Dhanasekaran *et al*., 2016). The antibacterial properties of *citrus sinensis* seed oil, containing 36% linoleic acid and 27% oleic acid, have been harnessed in the production of medical soap (Atolani *et al*., 2020)

5. CONCLUSIONS

The seeds of *C. paradise*, *C. limon* (L.) Burm.f., *citrus reticulate*, *C. maxima* (Burm.) Merr., and *citrus sinensis* shared similarities in their flavonoid, phenolic acid, and carotenoid profiles. However, distinct differences were observed in other varieties. The uniformity in these chemical compositions offers promising opportunities for sustainable innovation, particularly in harnessing the positive effects of flavonoids on oxidative damage and antioxidant activities in *Citrus* L. seeds.

Although volatile aromatic compounds exhibit variances without discernible patterns across varieties, their reported potential in cancer treatment underscores their significance. Noteworthy diversity in N-serotonin compounds exists among varieties, while certain varieties, such as *citrus latifolia* and *C. limon* (L.) Burm.f., share similarities in fatty acid compounds.

The versatile applications of *Citrus* L. seed oil, including biodiesel production, medical soap formulation, cancer treatment, and the development of modern chemotherapy drugs, underscore the manifold variations and potential uses that warrant further exploration in the industry.

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DECLARATION OF COMPETING INTEREST

The authors of this article declare that they have no financial, professional or personal conflicts of interest that could have inappropriately influenced this work.

AUTHORSHIP CONTRIBUTION STATEMENT

B, Budianto: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing.

A. Suparmi: Formal analysis, Methodology, Writing – review & editing.

CONFLICT OF INTEREST

We (the authors) have no conflict of interest in the writing of this article.

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