Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 1, 2023, 21
https://doi.org/10.33263/BRIAC131.021

Lemon (*Citrus limon* L.): Antioxidative Activity and Its Marker Compound

Defri Rizaldy [1], Muhamad Insanu [1,*], Nurma Sabila [1], Ariranur Haniffadli [1], Aliya Azkia Zahra [1], Sausan Nabila Ednoliani Pratiwi [1], Siti Nurul Mudrika [1], Rika Hartati [1], Irda Fidrianny [1]

[1] Department of Pharmaceutical Biology, School of Pharmacy, Bandung Institute of Technology, Bandung – 40132, Indonesia
* Correspondence: insanu@fa.itb.ac.id (M.I.);

Scopus Author ID 55479820400
Received: 17.10.2021; Accepted: 15.12.2021; Published: 22.01.2022

Abstract: This study aimed to investigate the antioxidative activity of the lemon peel and flesh, analyze the relationship of total phenolic content (TPC) and total flavonoid content (TFC) also their correlation with the antioxidative activity, along with the identification and quantification of the marker. The TPC and TFC were evaluated by the colorimetric method. The antioxidative activity was determined using DPPH (2,2-diphenyl-1picrylhydrazil) and CUPRAC (Cupric ion Reducing Antioxidant Capacity). The correlation between TPC and TFC with antioxidative activity and a correlation between two measures was analyzed using Pearson's method. HPLC performed the identification and quantification of the marker (high-performance liquid chromatography). The AAI (Antioxidant activity index) in the DPPH method had a range of 1.388–14.923 and the CUPRAC method 0.112-0.784. The highest TPC and TFC were given by the peel's ethanolic extract (1.52 ± 0.02 g GAE/100 g) and n-hexane extract oh the flesh (3.02 ± 0.20 g QE/100 g), respectively. A positive and significant correlation was found between the TPC in lemon peel extract and the AAI using DPPH. The DPPH and CUPRAC methods did not give linear results to the antioxidative activity of lemon peel and flesh. Luteolin 7-O-glucoside was confirmed as a marker compound, and its content was 0.0238% in ethanolic extract of lemon flesh.

Keywords: antioxidant; lemon; *Citrus limon*; DPPH; CUPRAC.

1. Introduction

Reactive oxygen species (ROS) are known to be involved in various cellular processes, such as cell signaling and defense systems. Cellular ROS can react with various components, such as carbohydrates, lipids, proteins, and DNA. Excessive ROS cause cell damage and various chronic diseases for human [1]. The balance between ROS and antioxidants is very important to maintain health and reduce the risk of chronic disease. Our body naturally relies on the antioxidants to maintain permissible levels of ROS, which includes: (i) endogenous antioxidants, such as albumin, bilirubin, glutathione (GSH), and uric acid; (ii) antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, heme oxygenase, and NADPH; and (iii) antioxidants from food, such as vitamins C and E, carotenoids, and various polyphenolic compounds [2].

Lemon (*Citrus limon* L.) is the third most important crop in citrus species after oranges and mandarins, with production reaching 4.200.000 tons in 2007, and is a high demand plant

among Indonesian in pursuance of a healthy lifestyle [3]. Lemons contain multiple important natural components for health, such as phenolic compounds (mainly flavonoids) and other important nutrients such as vitamins, minerals, fiber, essential oils, and carotenoids [4,5]. Compounds with health-promoting effects and properties are vitamin C and flavonoids, which provide natural antioxidative activity [6,7]. In general, the fruit is mainly used for promoting health since it contains the most antioxidant compounds, especially in the skin part of the fruit [8].

Various methods can be used to determine the antioxidative activity of plant specimens, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the cupric ion reducing antioxidant capacity (CUPRAC). Many studies have been carried out previously to determine the antioxidative activity of lemon plants using the DPPH and CUPRAC methods [9-13]. Lemon has a variety of phytochemical compounds with diverse polarity indexes, including nonpolar, semipolar, and polar compounds. The various polarities of these compounds will give different results of the antioxidative activity. There have been no studies reporting the antioxidative activity of the flesh and skin of lemons extracted using solvents with different polarities.

The objective of this research was to determine the antioxidative activity of various extracts (n-hexane, ethyl acetate, and ethanol) from the lemon flesh and peel using DPPH and CUPRAC assays and their correlation with the total phenol and flavonoids content in each extract. This research also identifies and determines the marker compounds in the ethanol extract of lemon flesh.

2. Materials and Methods

2.1. Sample preparation.

Lemon peel and flesh were collected from Lembang, West Java, Indonesia. They were sorted, washed, dried, and ground into powder.

2.2. Extraction.

Each powdered sample (300 g) was extracted by reflux using three solvents with different polarities. The solvents used were n-hexane, ethyl acetate, and ethanol. The extraction for each solvent was repeated three times to get the maximum amount of the extract. The total was six extracts consisting of n-hexane peel extract (P1), n-hexane flesh extract (F1), ethyl acetate peel extract (P2), ethyl acetate flesh extract (F2) and ethanol peel extract (P3), and ethanol flesh extract (F3).

2.3. Antioxidative activity by DPPH assay.

Bloid's method [14] with slight modification was carried out to determine DPPH scavenging activity. This method is begun by preparing a DPPH solution 50 µg/ml. Ascorbic acid was used as standard. DPPH 50 µg/ml was used as control, and methanol was used as the blank. Each extract was prepared in various concentrations and mixed with DPPH 50 µg/ml (1:1). The absorbance was measured at 517 nm by a UV-visible spectrophotometer after 30 minutes of incubation. Analysis was performed in triplicate. Inhibitory concentration 50% (IC_{50}) was determined by calibration curve between % of DPPH scavenging activity and concentration. Dividing the final concentration of DPPH with IC_{50} of DPPH was used to evaluate the antioxidant activity index (AAI).

2.4. Total phenolic content.

The determination of phenolic content was using Folin-Ciocalteu reagent) [15]. Gallic acid was used as standard with different concentrations (40-160 µg/ml) to obtain the calibration curve. The absorbance was observed at 765 nm using a UV-visible spectrophotometer. Analysis for the standard and each extract were done in triplicate. The total phenolic content of each extract was determined by calibration curve and expressed as gram gallic acid equivalent per 100 grams extract (g GAE/100 g).

2.5. Total flavonoid content.

Total flavonoid content is determined by Chang's method [16]. Quercetin was used as standard with different concentrations (30-120 µg/ml) to obtain a calibration curve. The absorbance was measured at 415 nm using a UV-visible spectrophotometer. The analysis was performed in triplicate for the standard and each extract. The total flavonoid content of each extract was calculated using a calibration curve and reported as gram quercetin equivalent per 100 grams extracts (g QE/100 g).

2.6. Statistical analysis.

Statistical analysis was determined using one-way ANOVA followed by post hoc Tukey performed by SPSS 20 for Window. Each sample measurement was conducted in triplicate. All results were presented as means (standard deviation). Correlation between the total phenolic, flavonoid, and antioxidative activity and the correlation between antioxidative activity testing methods was determined as Pearson's correlation coefficient.

2.7. Identification and quantification of marker compound.

Marker compound identification and assessment of their content were conducted using high-performance liquid chromatography. LiChrospher® 100 RP-C18 5 µm was used as stationary phase. The mobile phases were 0.01% H3PO4 (A) and methanol (B) with a flow rate of 1 ml/min (Pump CTO-20A, Shimadzu, Japan). The elution gradient started with 40% until 60% A for 5 min, increased to 70% A until 10 min, followed by 40% B until 15 min. The injection volume was 20 µm, and the column temperature was 30ºC. UV/Vis SPD-20A (Shimadzu, Japan) was used to detect at 360 nm. Luteolin 7-O-glucoside used as a standard was 5 µg/ml, and 10000 µg/ml of extract was used. Marker content of the compound calculated using this formula:

$$\frac{AUC\ compound\ in\ extract}{AUC\ standard} \text{x} \frac{\mu g/ml\ standard}{\mu g/ml\ extract} \text{x}\ 100\%$$

3. Results and Discussion

Previous studies [9-13, 17] have reported that *C. limon* L. has antioxidative activities. No studies have focused on the antioxidative activity of different extracts (n-hexane, ethyl acetate, and ethanol) from the peel and flesh of lemons grown in Lembang, West Java – Indonesia, using DPPH and CUPRAC methods.

3.1. AAI of peel and flesh extracts from the lemon.

DPPH and CUPRAC assays have been widely used to determine the antioxidative activity of various extracts or pure compounds. DPPH is a free radical with a purple color and absorbance at a wavelength of 517 nm. Antioxidants will transfer hydrogen to DPPH, thus forming a stable compound and turning yellow [18]. The CUPRAC assay uses neocuproine reagent combined with cupric chloride in ammonium acetate buffer pH 7. If the sample is an antioxidant, then Cu^{2+} is reduced to Cu^{+} and forms a yellow complex with neocuproine, which can be read at a wavelength of 450 nm [19].

Before starting the test using both methods, it is necessary to carry out a verification process using ascorbic acid as the standard in this study. Ascorbic acid using the DPPH method had AAI 64.141 ± 2.537, and the CUPRAC method had AAI 9.295 ± 0.083. Subsequently, the sample was tested using both methods. The AAI of DPPH and CUPRAC from lemon peel and lemon flesh in various extracts were presented in Table 1.

In the DPPH method, IC_{50} is the concentration of the sample or standard that can reduce about 50% absorbance of DPPH. In the CUPRAC method, EC_{50} is the concentration of the sample or standard that can increase 50% absorbance of the CUPRAC. The lower the IC_{50} or EC_{50} value, the stronger the antioxidative activity. However, if the concentration of the radical solution were different, the result of IC_{50} and EC_{50} values would also be different. Thus, the AAI is used to produce an equivalent value. AAI was divided into 4 groups, poor (AAI < 0.5), moderate ($0.5 \leq AAI \leq 1$), strong ($1 \leq AAI \leq 2$), and very strong (AAI > 2) [20].

Extraction was carried out using three solvents with increasing polarity; n-hexane, ethyl acetate, and ethanol, respectively. n-Hexane is a nonpolar solvent that selectively extracts nonpolar compounds. After n-hexane extracts nonpolar compounds, then ethyl acetate will extract semipolar compounds. Finally, ethanol is a polar solvent that will extract most polar compounds and a small number of semipolar and nonpolar compounds. Using this method, the maximum yield of nonpolar, semipolar, and polar compounds were obtained and the antioxidative activity was compared. The AAI of DPPH and CUPRAC of lemon peel and lemon flesh affected by increasing polarity solvent were given in Table 1. There were significant differences between the AAI of the three different extracts in lemon peel and lemon flesh.

In the present study, the AAI on DPPH method from peel and flesh extracts of lemon fruit had a range of 1.388–14.923. The highest AAI using the DPPH method was found in the ethyl acetate flesh extract (F2) 14.923, followed by the ethanol peel extract (P3) 11.636, and ethyl acetate peel extract (P2) 11.073. The standard ascorbic acid using the DPPH method had an AAI 64.141. In the CUPRAC method, AAI in the peel and flesh extracts of lemon fruit ranged from 0.112-0.784. The highest AAI using the CUPRAC method was found in n-hexane peel extract (P1) 0.784, followed by ethyl acetate peel extract (P2) 0.550, and ethanol peel extract (P3) 0.403. The standard ascorbic acid using the CUPRAC method had AAI 9.295.

Based on the results, it presented that by using the DPPH method, four extracts belong to the very strong AAI group, which were ethyl acetate and ethanol extracts of lemon peel and flesh. Meanwhile, both n-hexane peel and flesh extract were included in the moderate and strong AAI group. In the CUPRAC method, two extracts belong to the moderate AAI group, the n-hexane and ethyl acetate lemon peel extracts. The four other extracts belong to the poor AAI group.

Al-Sayyed [21] reported that in the DPPH method, methanol flesh extract had higher antioxidative activity than ethanol extract. On the other hand, in the CUPRAC method, methanol flesh extract had higher antioxidative activity than ethanol extract. Makni [17] reported that using the DPPH method, lemon peel's ethanol-water extract (7:3 v/v) had higher antioxidative activity than lemon flesh. This was related to this study that ethanolic extract of lemon peel had higher AAI than ethanolic extract of lemon flesh. Papoutsis [22] reported that methanol lemon pomace extract had higher antioxidative activity than ethanol in the DPPH and CUPRAC methods. In addition, the ethanolic extract of lemon pomace still had higher antioxidative activity than the acetone lemon pomace extract in the DPPH and CUPRAC methods. Lemon pomace was a residual waste from the lemon fruit, which contained the skin, flesh, and seeds. It was reported that the petroleum ether peel extract had the highest antioxidative activity compared to chloroform, methanol, and ethyl acetate extracts in the DPPH method, respectively [23].

Table 1. Antioxidative activities of lemon extracts by DPPH and CUPRAC assays.

Sample	AAI DPPH		
	Peel	**Flesh**	**Ascorbic acid**
n-Hexane extract	1.388 ± 0.100 ^{a}	0.760 ± 0.063 ^{a}	64.141 ± 2.537
Ethyl acetate extract	11.073 ± 0.920 ^{b}	14.923 ± 0.444 ^{b}	
Ethanol extract	11.636 ± 0.795 ^{b}	3.231 ± 0.138 ^{c}	
	AAI CUPRAC		
n-Hexane extract	0.784 ± 0.019 ^{a}	0.157 ± 0.003 ^{a}	9.295 ± 0.083
Ethyl acetate extract	0.550 ± 0.009 ^{b}	0.112 ± 0.004 ^{b}	
Ethanol extract	0.403 ± 0.006 ^{c}	0.193 ± 0.002 ^{c}	

a-c = different letters in the same column show the significant difference ($p < 0.05$)

3.2. Total phenolic content and total flavonoid content.

Total phenolic content (TPC) in each extract was calculated using a gallic acid calibration curve (y=0.0053x + 0.0368) and expressed as gallic acid equivalent (GAE). TPC in the various extract of lemon peel and flesh showed various values ranging from 0.14 to 1.52 g GAE/100 g. N-hexane extract of lemon flesh had the lowest TPC (0.14 g GAE/100 g), and the highest TPC was given by ethanol extract of lemon peel. Meanwhile, total flavonoid content (TFC) in various extracts were determined using linear regression equation of quercetin (y=0.00655x + 0.0232) and expressed as quercetin equivalent (QE). TFC in various extracts of peel and flesh from lemon showed results in the range of 0.85-2.89 g QE/100 g. The lowest TFC was given by ethyl acetate flesh extract, while ethanol peel extract showed the highest TFC (Table 2). A recent study stated that higher TPC and TFC were attained by increasing the solvent's polarity [24]. This statement was in accordance with the TPC result from this study.

Table 2. Total phenolic and flavonoid content in lemon extracts.

Sample	Phenolic content (g GAE/100 g)	Flavonoid content (g QE/100 g)
P1	0.89 ± 0.08^{b}	2.72 ± 0.03^{a}
F1	0.14 ± 0.02^{c}	3.02 ± 0.20^{a}
P2	1.38 ± 0.07^{a}	2.49 ± 0.40^{a}
F2	0.90 ±0.14^{b}	0.85 ± 0.10^{b}
P3	1.52 ± 0.02^{a}	2.89 ± 0.04^{a}
F3	1.47 ± 0.17^{a}	1.04 ± 0.07^{b}

Data: mean ± SD. a-c = different letter in the same column show significant difference ($p<0.05$), GAE: gallic acid equivalents; QE: quercetin equivalents, P: peel, F: flesh, 1: n-hexane extract, 2: ethyl acetate extract, 3: ethanol extract, n = 3.

Another study reported that TPC of lemon peel (20.4 g GAE/100 g) was higher than the flesh (10.6 g GAE/100 g) [17]. It was similar as TPC of the peel was higher than the flesh for every extract in this study. Other research stated the TPC of ethyl acetate peel extract (0.72 g GAE/100 g) was higher than n-hexane extract (0.55 g GAE/100 g) [9]. This research also showed the TPC of ethyl acetate extract (1.38 g GAE/100 g) was higher than n-hexane extract (0.89 g GAE/100 g). The previous study showed the TPC and TFC of methanol extract of the leave of lemon were 9.81 g GAE/100 g and 3.87 g GAE/100 g [25]. TPC of methanol extract of pulp and seeds of lemon ranged from 0.24 to 0.35 g GAE/100 g and 0.21 to 0.34 g GAE/100 g. TFC of the pulp and seeds varied from 0.39 to 0.54 g GAE/100 g and from 1.86 to 2.53 g GAE/100 g, respectively [26]. TPC and TFC of lemon seed oil by different methods showed various results. Lemon seed oil's highest TPC and TFC were found in the supercritical fluid extraction method, 165.90 µg GAE/ml and 21.69 µg QE/ml [27].

Among various extracts (water, ethanol, acetone, 50% ethanol, and 50% methanol extract), the absolute methanol extract of lemon peel had the highest TFC (0.50 QE/100 g). Then followed by 50% ethanol and 50% methanol extracts (0.42 and 0.38 g QE/100 g, respectively) [22]. This result is in accordance with current research, which presented the highest TFC for ethanol extract (2.89 g QE/100 g). TFC of ripening and unripe peel of lemon in various extracts (ethyl acetate, acetone, ethanol, and methanol) also showed varying values. TFC of the ripen peel in the range of 27- 48.2 g QE/100 g. Methanol extract of ripening peel was the highest TFC of the ripen peel, and the lowest was ethyl acetate extract. Meanwhile, TFC of the unripe peel expressed result in the range of 24.5-42.5 g QE/100 g. Similar to the ripen peel, the highest TFC of the unripe peel was shown by methanol extract and the lowest ethyl acetate extract [28]. This research also showed the ripen peel of lemon has a higher result than the unripe peel.

The previous study showed the increase of TFC and TPC by irradiation pretreatment using UV-C, which is suspected by the increasing of phenylalanine ammonia-lyase activity that had an important role in the synthesis of the compound [29]. Pretreatment with microwave significantly affected TPC and TFC of lemon pomace. TPC increased from 1.07 to 2.21 g GAE/100 g as the sample was pre-treated with microwave (120-480 W for 2 minutes). However, TPC would decrease above 600 W. The increase of TPC can be explained due to the electromagnetic radiation from the microwave, which causes the cleavage of flavonoids. However, irradiation power above 600 W might cause total degradation of flavonoids so that the result would decrease. This condition also applied to TFC [30].

3.3. Correlation between the TPC and TFC with AAI DPPH and CUPRAC in lemon extracts.

The strong antioxidative activity was indicated by the high AAI value in each method. This is explained by TPC and TFC values that contribute to antioxidative activity when the correlation is positive and significant (Table 3).

Table 3. Pearson's correlation coefficient of TPC and TFC of lemon extracts with AAI DPPH and AAI CUPRAC.

Antioxidative parameter	Pearson's correlation coefficient (r)	
	TPC	TFC
AAI DPPH Peel	0.977^{**}	0.019^{ns}
AAI DPPH Flesh	0.249^{ns}	-0.689^{*}
AAI CUPRAC Peel	-0.952^{**}	-0.168^{ns}
AAI CUPRAC Flesh	0.309^{ns}	0.207^{ns}

ns = not significant, * = significant at $p < 0.05$, ** = significant at $p < 0.01$.

In this study, the final results used AAI as antioxidative activity, where the higher AAI had the higher antioxidative activity. As shown in Table 3, the TPC gave a significant positive correlation with AAI DPPH only for lemon peel extract ($r = 0.977$, $p < 0.01$). The higher TPC in lemon peel extract is related to the higher antioxidative activity. It indicated that phenolic compounds in lemon peel extract contributed to antioxidative activity by the DPPH method. The TPC showed a positive but not significant correlation in lemon flesh extract by DPPH and CUPRAC methods ($0.249 \leq r \leq 0.309$).

Meanwhile, the TFC did not provide a significant positive correlation in any extract. The TPC gave a negative correlation with AAI CUPRAC of lemon peel extract. Many studies used IC_{50} and EC_{50} in the final results, so the higher TPC and TFC will be related to the lower IC_{50} and EC_{50} thus, the correlation will be negative and significant.

High antioxidative activity in the extract was influenced by the presence of phenolic compounds with more hydroxyl groups or conjugated double bonds. Flavonoids belong to a group of phenolic compounds with OH in ring A and or ring B. High antioxidative activity is recorded in flavonoids with a hydroxyl group at C-3'-C-4', OH at C-3, oxo function at C-4, double bonds at C-2 and C-3. The hydroxyl group with ortho position at C-3'-C-4' provides high antioxidative activity for flavonoids. Aglycone flavonoids will contribute to higher antioxidative activity than flavonoid glycosides [31]. The TFC in P1 (2.72 g GAE/100 g) was higher than P2 (2.49 g GAE/100 g), but the AAI DPPH of P1 (1.388) was lower than P2 (11.073). These data suggested that most of the flavonoid compounds in P1 had OH at other positions, while in P2 had a hydroxyl group at C-3'-C-4', OH at C-3, oxo function at C-4, and double bonds at C-2 and C-3, hence the antioxidative activity is different among two extracts. The TPC in F2 (0.90 g GAE/100 g) was lower than F3 (1.47 g GAE/100 g), but AAI DPPH of F2 (14.923) was greater than from F3 (3.231). From these data, it can be predicted that the number of phenolic compounds in F2 had a higher ability to transfer hydrogen than F3.

Table 4. Pearson's correlation coefficient between DPPH and CUPRAC assays.

Antioxidative parameter	Pearson's correlation coefficient (r)	
	AAI CUPRAC Peel	AAI CUPRAC Flesh
AAI DPPH Peel	-0.928**	
AAI DPPH Flesh		-0.840**

** = significant at $p < 0.01$.

The correlation between two antioxidative activity methods in this study was analyzed using Pearson's method (Table 4). Pearson's method can analyze whether the AAI in each method presents linear results or not. Both methods showed linear results when their correlation expressed a positive and significant correlation, which means that one method with another presented different results, but both methods exposed linear results. The data in Table 3 figured that the two methods did not give linear results for the lemon peel and flesh extracts ($r = -0.928$, $p < 0.01$ and $r = -0.840$, $p < 0.01$, respectively).

3.4. Identification and Marker Content.

Marker compound of ethanol lemon flesh extract was identified using HPLC. Ethanol lemon flesh extract had the highest yield compared to the other sample. Luteolin 7-O-glucoside, rutin, quercetin, kaempferol and apigenin was used as standard. Figure 1 demonstrated that peaks number 1, 2, and 3 had similar retention time with standard, 5.062 with 4.958 (luteolin 7-O-glucoside), 5.475 with 5.466 (rutin), and 8.350 with 8.345 (quercetin), which means the

ethanol lemon flesh extract contained luteolin 7-O-glucoside, rutin, and quercetin. Kaempferol and apigenin were not detected in the chromatogram. The highest AUC in extract chromatogram was represented by luteolin 7-O-glucoside, so it was stated as a marker of ethanol lemon flesh extract. After calculation of the AUC in extract and standard, the content of luteolin- 7-O-glucoside as a marker in lemon flesh extract was 0.0238%.

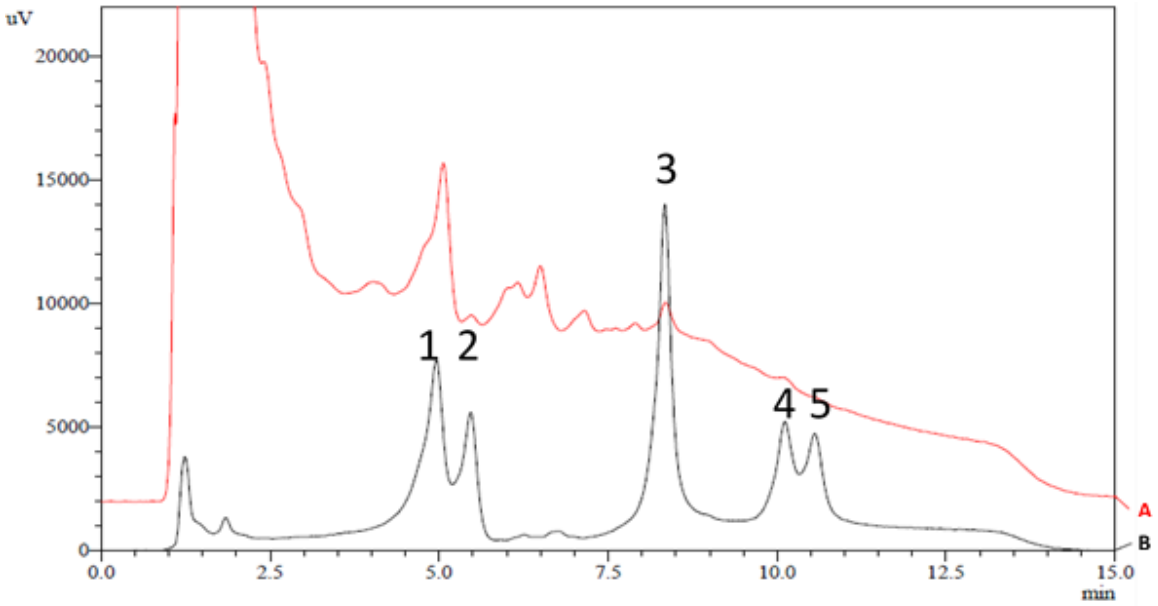

Figure 1. Marker content in ethanol lemon flesh extract. A: ethanol lemon flesh extract, B: standards. 1: Luteolin 7-O-glucoside, 2: Rutin, 3: Quercetin, 4: Kaempferol, 5: Apigenin.

A previous study revealed that rutin, hesperidin, eriocitrin, and neohesperidin were detected in lemon juice by HPLC analysis. The mobile phase was H2O/formic acid (0.1%) (A), and acetonitrile (B), C18 RP column was used as stationary phase. The juice's two abundant compounds were eriocitrin and hesperidin, with concentrations of 16.7 and 14.1 mg/100 ml juice [32]. Caffeoyl N-tryptophan, vicenin 2, hydroxycinnamoyl-O-glucoside acid, kaempferol-3-O-rutinoside, eriocitrin, and quercetin-3-O-rutinoside were found in the ethanol-water extract of lemon peel. Six phenolic compounds were identified using HPLC coupled with mass spectrometry (LC-MS/MS). Formic acid solvent 0.1% (A) and acetonitrile (B) were used as mobile phases and C18 300A as stationary phases [17]. Seven phenolic compounds, naringin, hesperidin, erocitrin, kaempferol, kempferol-7-O-glucuronide, *p*-coumaric acid, and gallic acid, were detected in lemon through HPLC-MS analysis. Eriocitrin showed the highest concentration (3.35 mg/g) and naringin showed the lowest concentration (0.11 mg/g) [33].

Sixteen phenolic compounds were detected from methanol extract of lemon peel using the HPLC system. Gallic acid (2.39 mg/100 ml) and 1,2-dihydroxybenzene (2.30 mg/100 ml) were detected as two abundant compounds. The lowest phenolic compound was p-coumaric (0.01 mg/100 ml) [34]. Other research reported eleven volatile compounds discovered from previous research served as biomarkers. The eleven biomarker compounds were trans-α-bergamotene, β-bisabolene, limettin, cis-α-bergamotene, citronellal, β-pinene, caryophyllene, α-cubebene, champene, α-terpineol, and (Z)-β-farnesene. These eleven compounds of lemon peel were higher than other citrus species [35]. Coumarin and psoralen also displayed protonated molecules in the ESI(+)MS spectra from lemon's flavedo [36]. Twenty-six volatile compounds were identified by GC-MS in the lemon peel oil. These compounds consisted of four main chemical families, monoterpenes, sesquiterpenes, aldehyde, and esters [37].

4. Conclusion

Various antioxidant test methods can be used parallel to determine antioxidant activity because each method will give different results. All ethyl acetate and ethanol extracts of lemon peel and flesh have very strong antioxidant activity using the DPPH method. All lemon peel and flesh extracts had moderate to poor antioxidant activity using the CUPRAC method. The phenolic compound in lemon peel extract contributed to antioxidative activity by the DPPH method. The DPPH and CUPRAC methods did not give linear results in the antioxidant activity of lemon peel and flesh. The marker compound of lemon flesh extract was luteolin 7-O-glucoside, and its content was 0.0238%. Lemon peel can be used as a natural antioxidant in the food and nutrition industry.

Funding

This research was funded by the PPMI program, grant number 18/IT1.C10/SK-KP/2021.at Bandung Institute of Technology.

Acknowledgments

The authors are grateful to the School of Pharmacy - Bandung Institute of Technology authorities for accommodating the necessary facilities to conduct this research.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Sarmiento-Salinas, F.L.; Perez-Gonzalez, A.; Acosta-Casique, A.; Ix-Ballote, A.; Diaz, A.; Treviño, S.; Rosas-Murrieta, N.H.; Millán-Perez-Peña, L.; Maycotte, P. Reactive oxygen species: Role in carcinogenesis, cancer cell signaling and tumor progression. *Life Sci* **2021**, *284*, 119942, https://doi.org/10.1016/j.lfs.2021.119942.
2. Chedea, V.S.; Pop, R.M. Total polyphenols content and antioxidant DPPH assays on biological samples, 2nd ed. Elsevier Inc. **2019**, https://doi.org/10.1016/b978-0-12-813768-0.00011-6V.
3. Zhuo, L.C.; Chen, C.F.; Lin, Y.H. Dietary supplementation of fermented lemon peel enhances lysozyme activity and susceptibility to *Photobacterium damselae* for orange-spotted grouper, *Epinephelus coioides*. *Fish Shellfish Immunol* **2021**, 117, 248–252, https://doi.org/10.1016/j.fsi.2021.08.015.
4. Harikrishnan, R.; Thamizharasan, S.; Devi, G.; Van Doan, H.; Ajith Kumar, T.T.; Hoseinifar, S.H.; Balasundaram, C. Dried lemon peel enriched diet improves antioxidant activity, immune response and modulates immuno-antioxidant genes in *Labeo rohita* against *Aeromonas sorbia*. *Fish Shellfish Immunol* **2020**, *106*, 675–684, https://doi.org/10.1016/j.fsi.2020.07.040.
5. Serna-Escolano, V.; Martínez-Romero, D.; Giménez, M.J.; Serrano, M.; García-Martínez, S.; Valero, D.; Valverde, J.M.; Zapata, P.J. Enhancing antioxidant systems by preharvest treatments with methyl jasmonate and salicylic acid leads to maintain lemon quality during cold storage. *Food Chem* **2021**, *338*, 128044, https://doi.org/10.1016/j.foodchem.2020.128044.
6. Haida, Z.; Ab Ghani, S.; Juju Nakasha, J.; Hakiman, M. Determination of experimental domain factors of polyphenols, phenolic acids and flavonoids of lemon (*Citrus limon*) peel using two-level factorial design. *Saudi J. Biol. Sci* **2021**, https://doi.org/10.1016/j.sjbs.2021.09.022.
7. González-Molina, E.; Domínguez-Perles, R.; Moreno, D.A.; García-Viguera, C. Natural bioactive compounds of *Citrus limon* for food and healty. *J. Pharm. Biomed. Anal* **2010**, *51*, 327–345, https://doi.org/10.1016/j.jpba.2009.07.027.
8. Ledesma-Escobar, C.A.; Priego-Capote, F.; Luque de Castro, M.D. Relevance and analysis of Citrus flavonoids. *Polyphenols in Plants* **2019**, 133–150, https://doi.org/10.1016/b978-0-12-813768-0.00009-8.
9. Al-Qassabi, J.S.A.; Weli, A.M.; Hossain, M.A. Comparison of total phenols content and antioxidant potential of peel extracts of local and imported lemons samples. *Sustain Chem Pharm* **2018**, *8*, 71–75, https://doi.org/10.1016/j.scp.2018.03.001.
10. Dong, X.; Hu, Y.; Li, Y.; Zhou, Z. The maturity degree, phenolic compounds and antioxidant activity of

Eureka lemon [*Citrus limon* (L.) Burm. f.]: A negative correlation between total phenolic content, antioxidant capacity and soluble solid content. *Sci Hortic (Amsterdam)* **2019**, *243*, 281–289, https://doi.org/10.1016/j.scienta.2018.08.036.
11. Ehiobu, J.M.; Idamokoro, M.E.; Afolayan, A.J. Phytochemical content and antioxidant potential of leaf extracts of *Citrus limon* (L.) Osbeck collected in the Eastern Cape Province, South Africa. *South African J Bot* **2021**, *141*, 480–486, https://doi.org/10.1016/j.sajb.2021.06.001.
12. Himed, L.; Merniz, S.; Monteagudo-Olivan, R.; Barkat, M.; Coronas, J. Antioxidant activity of the essential oil of *Citrus limon* before and after its encapsulation in amorphous SiO2. *Sci African* **2019**, *6*, https://doi.org/10.1016/j.sciaf.2019.e00181.
13. Zhang, P.; Zhou, Z. Postharvest ethephon degreening improves fruit color, flavor quality and increases antioxidant capacity in 'Eureka' lemon (*Citrus limon* (L.) Burm. f.). *Sci Hortic (Amsterdam)* **2019**, *248*, 70–80, https://doi.org/10.1016/j.scienta.2019.01.008.
14. Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature* **1958**, *181*, 1199–1200 https://doi.org/10.1038/1811199a0.
15. Pourmorad, F.; Hosseinimehr, S.J.; Shahabimajd, N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African J Biotechnol* **2006**, *5*, 1142–1145, https://doi.org/10.1055/s-2007-987042.
16. Chang, C.C.; Yang, M.H.; Wen, H.M.; Chern, J.C. Estimation of total flavonoid content in propolis by two complementary colometric methods. *J Food Drug Anal* **2002**, *10*, 178–182, https://doi.org/10.38212/2224-6614.2748.
17. Makni, M.; Jemai, R.; Kriaa, W.; Chtourou, Y.; Fetoui, H. *Citrus limon* from Tunisia: Phytochemical and physicochemical properties and biological activities. *Biomed Res Int* **2018**, *2018*, https://doi.org/10.1155/2018/6251546.
18. Alam, M.N.; Bristi, N.J.; Rafiquzzaman, M. Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharm J* **2013**, *21*, 143–152, https://doi.org/10.1016/j.jsps.2012.05.002.
19. Apak, R.; Güçlü, K.; Demirata, B.; Özyürek, M.; Çelik, S.E.; Bektaşoğlu, B.; Berker, K.I.; Özyurt, D. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules* **2007**, *12*, 1496–1547, https://doi.org/10.3390/12071496.
20. Scherer, R.; Godoy, H.T. Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. *Food Chem* **2009**, *112*, 654–658, https://doi.org/10.1016/j.foodchem.2008.06.026.
21. Al-Sayyed, H.F.; Al-Kurd, R.A.; Mwalla, M.M.; Abdel Qader, S.M. Determination of antioxidant content and capacity of four Jordanian fresh Citrus fruits. *J Agric Sci* **2019**, *11*, 605, https://doi.org/10.5539/jas.v11n3p605.
22. Papoutsis, K.; Pristijono, P.; Golding, J.B.; Stathopoulos, C.E.; Scarlett, C.J.; Bowyer, M.C.; Vuong, Q. Impact of different solvents on the recovery of bioactive compounds and antioxidant properties from lemon (*Citrus limon* L.) pomace waste. *Food Sci Biotechnol* **2016**, *25*, 971–977, https://doi.org/10.1007/s10068-016-0158-8.
23. Zhu, J.; Arsovska, B.; Kozovska, K. Acupuncture treatment in osteoarthritis. *Int J Recent Sci Res* **2020**, *11*, 37471–37472, https://doi.org/10.24327/IJRSR.
24. Sabu, S.; Ashita, T.; Stephy, S. Chitosan and lemon peel extract coating on quality and shelf life of yellowfin tuna (*Thunnus albacares*) meat stored under refrigerated condition. *Indian J Fish* **2020**, *67*, 114–122, https://doi.org/10.21077/ijf.2019.67.1.91361-15.
25. Khettal, B.; Kadri, N.; Tighilet, K.; Adjebli, A.; Dahmoune, F.; Maiza-Benabdeslam, F. Phenolic compounds from Citrus leaves: Antioxidant activity and enzymatic browning inhibition. *J Complement Integr Med* **2017**, *14*, https://doi.org/10.1515/jcim-2016-0030.
26. Xi, W.; Lu, J.; Qun, J.; Jiao, B. Characterization of phenolic profile and antioxidant capacity of different fruit part from lemon (*Citrus limon* Burm.) cultivars. *J Food Sci Technol* **2017**, *54*, 1108–1118, https://doi.org/10.1007/s13197-017-2544-5.
27. Park, Y.-S.; Kim, I.; Dhungana, S.K.; Park, E.-J.; Park, J.-J.; Kim, J.-H.; Shin, D.-H. Quality Characteristics and antioxidant potential of lemon (*Citrus limon* Burm. f.) seed oil extracted by different methods. *Front Nutr* **2021**, *8*, 1–10, https://doi.org/10.3389/fnut.2021.644406.
28. Khatiwora, E.; Adsul, V.B.; Torane, R.C.; Gaikwad, S.; Deshpande, N.R.,; Kashalkar, R. V. Spectroscopic determination of total phenol and flavonoid contents of *Citrus limon* peel from north eastern region of India. *J Drug Deliv Ther* **2017**, *7*, 21–24, https://doi.org/10.22270/jddt.v7i1.1368.
29. Papoutsis, K.; Vuong, Q. V.; Pristijono, P.; Golding, J.B.; Bowyer, M.C.; Scarlett, C.J.; Stathopoulos, C.E. Enhancing the total phenolic content and antioxidants of lemon pomace aqueous extracts by applying uv-c irradiation to the dried powder. *Foods* **2016**, *5*, 1–10, https://doi.org/10.3390/foods5030055.
30. Papoutsis, K.; Pristijono, P.; Golding, J.B.; Stathopoulos, C.E.; Bowyer, M.C.; Scarlett, C.J.; Vuong, Q. V. Enhancement of the total phenolic compounds and antioxidant activity of aqueous *Citrus limon* L. pomace extract using microwave pretreatment on the dry powder. *J Food Process Preserv* **2017**, *41*, 1–8, https://doi.org/10.1111/jfpp.13152.
31. Heim, K.E.; Tagliaferro, A.R.; Bobilya, D.J. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. *J Nutr Biochem* **2002**, *13*, 572–584, https://doi.org/10.1016/S0955-2863(02)00208-5.

32. Loizzo, M.R.; Sicari, V.; Tundis, R.; Leporini, M.; Falco, T.; Calabrò, V. The influence of ultrafiltration of *Citrus limon* L. Burm. cv femminello comune juice on its chemical composition and antioxidant and hypoglycemic properties. *Antioxidants* **2019**, *8*, 2–11, https://doi.org/10.3390/antiox8010023.
33. Scurria, A.; Sciortino, M.; Albanese, L.; Nuzzo, D.; Zabini, F.; Meneguzzo, F.; Alduina, R.; Presentato, A.; Pagliaro, M.; Avellone, G.; Ciriminna, R. Flavonoids in lemon and grapefruit integropectin. *Preprints* **2021**, 2021020620, https://doi.org/10.20944/preprints202102.0620.v1.
34. Özcan, M.M.; Ghafoor, K.; Al Juhaimi, F.; Uslu, N.; Babiker, E.E.; Mohamed Ahmed, I.A.; Almusallam, I.A. Influence of drying techniques on bioactive properties, phenolic compounds and fatty acid compositions of dried lemon and orange peel powders. *J Food Sci Technol* **2021**, *58*, 147–158, https://doi.org/10.1007/s13197-020-04524-0.
35. Zhang, H.W.H.; Chen, J.; Peng, Z.; Shi, M.; Chen, M.; Yuan, Z.; Liu, Y.; Zhang, Hongyan, X.J. Volatile compounds in fruit peels as novel biomarkers for the identification of four Citrus species. *Molecules* **2019**, *24*, https://doi.org/10.3390/molecules24244550.
36. Jungen, M.; Lotz, P.; Patz, C.D.; Steingass, C.B.; Schweiggert, R. Coumarins, psoralens, and quantitative 1H-NMR spectroscopy for authentication of lemon (*Citrus limon* [L.] Burm.f.) and Persian lime (*Citrus* × *latifolia* [Yu.Tanaka] Tanaka) juices. *Food Chem* **2021**, *359*, 129804, https://doi.org/10.1016/j.foodchem.2021.129804.
37. Aguilar-Hernández, M.G.; Sánchez-Bravo, P.; Hernández, F.; Carbonell-Barrachina, Á.A.; Pastor-Pérez, J.J.; Legua, P. Determination of the volatile profile of lemon peel oils as affected by rootstock. *Foods* **2020**, *9*, 1–9, https://doi.org/10.3390/foods9020241.