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Fractionation of Phenolic and Flavonoid Compounds from Kaffir Lime (*Citrus hystrix*) Peel Extract and Evaluation of Antioxidant Activity

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Abstract

The observation of side effects during drug consumption results a numerous research to search natural antioxidant such as kaffir lime which has not been utilized. Fruit peel has been reported to exhibit higher antioxidant content than the edible part and therefore, kaffir lime peel was selected in this study. This work was aimed to investigate the effect of solvent polarity (hexane, ethyl acetate and n-butanol) employed during fractionation of ethanolic crude extract and assess its antioxidative activity to neutralize DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. The results show the employment of solvents possessing different polarity resulted fractions of hexane, ethyl acetate and n-butanol with different phenolic and flavonoid amount in each fraction. Semi polar solvent of ethyl acetate performed as the best solvent with total phenolic and flavonoid content was detected 0.1157 mg Gallic Acid Equivalent/mg and 0.1147 mg Rutin Equivalent/mg, respectively. Accordingly, each fraction exhibited different antioxidant activity against DPPH. N-butanol fraction demonstrated the strongest antioxidant activity. Phenolics and flavonoids identification by HPLC (High Performance Liquid Chromatography) indicate the presence different phenolics and flavonoid compounds in each fraction that contribute to antioxidant activity to different extents.

Keywords: *antioxidant; fractionation; flavonoid; kaffir lime (Citrus hystrix); peel; phenolic*

Abstrak

FRAKSINASI SENYAWA FENOLIK DAN FLAVONOID DARI EKSTRAK KULIT JERUK PURUT (*Citrus hystrix*) DAN EVALUASI AKTIVITAS ANTIOKSIDAN. *Efek negatif dari konsumsi obat-obatan menyebabkan banyaknya penelitian untuk mencari antioksidan alami seperti jeruk purut yang selama ini belum dimanfaatkan secara maksimal. Kandungan antioksidan kulit buah dilaporkan lebih tinggi daripada bagian buah sehingga kulit jeruk purut dipilih pada penelitian ini. Tujuan penelitian ini adalah mempelajari pengaruh polaritas pelarut (heksana, etil asetat dan n-butanol) pada proses fraksinasi ekstrak kulit jeruk purut terhadap perolehan total fenolik dan flavonoid serta mempelajari kemampuan antioksidannya dalam menetralkan radikal bebas DPPH (2,2-diphenyl-1-picrylhydrazyl). Hasil penelitian menunjukkan bahwa penggunaan pelarut dengan polaritas berbeda menghasilkan fraksi heksana, etil asetat dan n-butanol dengan kandungan fenolik dan flavonoid tertentu di setiap fraksinya. Pelarut dengan polaritas sedang yaitu etil asetat merupakan pelarut terbaik dimana kandungan total fenolik dan flavonoidnya masing-masing 0,1157 mg Gallic Acid Equivalent/mg dan 0,1147 mg Rutin Equivalent/mg. Akibatnya, tiap fraksi mempunyai aktivitas antioksidan yang berbeda di dalam menetralkan DPPH. Fraksi n-butanol mempunyai aktivitas antioksidan tertinggi.*

Identifikasi senyawa fenolik dan flavonoid dengan menggunakan HPLC (High Performance Liquid Chromatography) menunjukkan bahwa di dalam masing-masing fraksi terdapat senyawa fenolik dan flavonoid yang berbeda yang menyebabkan kemampuan antioksidan dari masing-masing fraksi juga berbeda.

Kata kunci: *antioksidan; fraksinasi; flavonoid; jeruk purut (Citrus hystrix); kulit; fenolik*

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INTRODUCTION

Drugs consumption during disease treatment may promote side effects and therefore, the requirement to design alternative treatment from natural product such as herbal medicine or botanicals is essential. Plants contain phenolic compounds that experimentally exhibited properties of anti inflammatory (Menichini *et al.*, 2011), anti microbial (Yi *et al.*, 2008), anti adipogenesis (Kim *et al.*, 2012a), and anti diabetic (Kim *et al.*, 2009). All parts of plant such as fruit, seed, leave and root contain phenolic compounds to different extents (Kondo *et al.*, 2002; Olabinri *et al.*, 2009). In fruit itself, part of fruit such as skin, flesh and seed have been reported to have different amount of phenolic compounds. Accordingly, they have different antioxidant activity.

Citrus has been well known for health benefits since the fruit contains a lot of vitamin, antioxidant compounds and others. Accordingly, citrus is potential to be further developed as natural antioxidant, especially for degenerative diseases which has been focused in recent years. Kaffir lime (*Citrus hystrix*) is a type of citrus fruits that possesses high content of phenolic compounds. In kaffir lime tree, only leaves have been used as spiced-citrus flavor. In several studies, fruit peel has been reported to exhibit higher antioxidant activity compared to the edible part (Gorinstein *et al.*, 2002). Thus, the investigation of peel as natural antioxidant is another challenge for treating degenerative diseases (Garg *et al.*, 2001; Irawaty *et al.*, 2014).

Even though investigations on kaffir lime peel has been performed previously to obtain optimum extraction conditions (Chan *et al.*, 2009; Irawaty *et al.*, 2014) and the extract has been explored for several applications (Irawaty *et al.*, 2014; Jamilah *et al.*, 2007; Putri *et al.*, 2013), but it is still limited to the usage of crude extract. Further treatment on crude extract such as fractionation step will provide deep insight of the ability of fractions to neutralize free radicals which the latter is responsible for degenerative diseases (diabetes, organ inflammation, etc.) and this has not been reported previously. Solvents possess different polarity will be employed to separate phenolic compounds based on like dissolves like principle. As a result, each fraction will exhibit radical antioxidative activity that allows its application to treat certain disease. The antioxidant capability of extract from peel/leaves/seed/flesh is different due to various factors, namely climate, variety, soil, degree of maturation and environment conditions which in turn influence secondary metabolites and phenolic compounds (Baniasadi *et al.*, 2014; Gebruers *et al.*, 2010; Shewry *et al.*, 2010). Hence, the present study attempts to separate phenolic and flavonoid compounds from crude extract of kaffir lime peel with solvents possess different polarity, specifically hexane, ethyl acetate and n-butanol with relative polarity values of 0.009, 0.228, and 0.586, respectively (Reichardt, 2003). Fractions were prepared using successive solvents of varying polarity, followed by assessment of antioxidant activity towards the neutralization of free radical compound.

MATERIALS AND METHODS

Sample Preparation

Kaffir lime was purchased at Keputran market (Surabaya, Indonesia) with no obvious physical or microbial damage was observed. Kaffir lime fruit was washed with tap water, peeled off sharply to collect the peel which further cut into small pieces with a size of approximately 0.5 cm. The kaffir lime peel were dried in an oven at 35^{o}C and kept in tight container for future use.

Extraction and Fractionation

Kaffir lime peel was extracted with ethanol using maceration method with a ratio of 1:40 ($^{w}/_{v}$) for 8 h at room temperature in dark condition. The solid part was then separated and filtered through a Whatman No. 1 filter paper. The filtrate was concentrated with a rotary evaporator (IKA RV 10) at 45°C. The crude extract was further used in fractionation step. The crude extract obtained was fractionated with solvents as shown in Figure 1. Volume ratio of crude extract and solvent was 1:1. The fractions of hexane, ethyl acetate, and n-butanol were collected individually and dried under vacuum below 45°C, while the residue was kept without any further treatment. The three fractions and residue were subjected for analysis to determine total phenolic content, total flavonoid content, antioxidant activity and identification of phenolic and flavonoid compounds using HLPC (High Performance Liquid Chromatography).

Determination of Total Phenolic Content

The amount of total phenolics was determined according to the Folin-Ciocalteu colorimetric method (Anagnostopoulou *et al.*, 2006) with slight modification. Briefly, 0.2 mL sample (crude extract, fraction, or residue) was mixed with 1 mL Folin-

Ciocalteu's reagent (1:1) and 3 mL Na_2CO_3 solution (7.5%). The mixture was incubated at room temperature for 30 min in dark condition. The absorbance was subsequently measured at 730 nm using a spectrophotometer (Shimadzu, UVmini-1240). Total phenolic content was expressed as Gallic Acid Equivalent (GAE)/mg sample, which is a common reference compound.

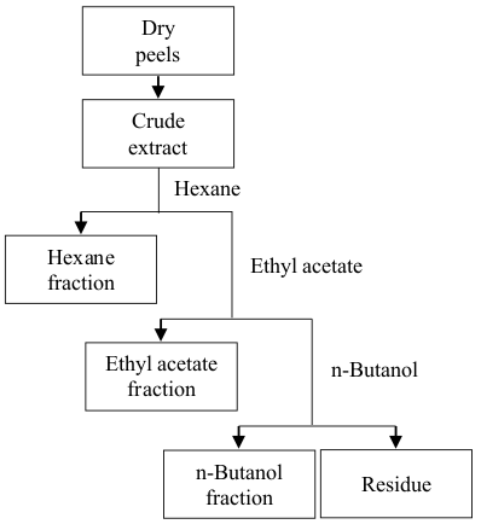

Figure 1. Schematic diagram for fractionation of crude extract

Determination of Total Flavonoid Content

Aluminum chloride colorimetric method was selected for determining total flavonoid content (Wu *et al.*, 2009). 0.4 mL of sample (crude extract, fraction, or residue) was added to 1.8 mL methanol, 2 mL 0.01 M aluminum chloride solution and the mixture was well mixed. After 30 min of incubation period in dark condition, the absorbance was measured at 431 nm against a blank of methanol using a spectrophotometer (Shimadzu, UVmini-1240). Total flavonoid content was expressed as Rutin Equivalent (RE)/ mg sample.

Antioxidant Activity by DPPH Method

Radical scavenging activity of sample to neutralize DPPH was measured according to (Liu *et al.*, 2011) with modification. Basically, 1.25 mL DPPH solution and 1 mL sample were mixed and incubated at room temperature for 30 min. Afterward, the absorbance of the mixture was measured using a spectrophotometer at 510 nm. For control, the procedure was repeated by using DPPH solution only without any sample. The antioxidant activity of the sample was measured as a decrease in the absorbance and calculated by using equation (1).

$$Radicals\ cavenging\ activity(\%) = \left(1 - \frac{A_1}{A_0}\right) x100\% \quad (1)$$

where A_1: absorbance of sample; A_0: absorbance of control.

In this study, antioxidant activity was expressed as IC_{50} which is defined as the concentration of sample required to scavenge the DPPH free radicals by 50%. IC_{50} was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations.

Phenolics and Flavonoids Identification by HPLC

The presence of phenolic compounds were detected using a Jasco HPLC (UV-2077 Plus) coupled with a UV detector. Phenolics separation was achieved on a C18 column and a mixture of acetic acid solution (3%) and methanol was employed as the mobile phase to facilitate the separation with total flow rate was 1 mL min^{-1}. By using the same column, the mobile phase for flavonoids separation were acetonitrile, water and acetic run with a rate of 1 $mL.min^{-1}$. Identification of phenolics and flavonoids was accomplished by comparing the retention times of peaks in samples to those of standards at 280 nm. Several standard compounds employed to identify phenolic compounds were gallic acid, catechin, caffeic acid, epicatechin, p-coumaric acid, ferulic acid, rutin and quercetin. Naringin, hesperidin, naringenin, hesperetin and nobiletin were used for flavonoids identification. Those compounds are generally found in citrus fruits.

RESULTS AND DISCUSSION

Total Phenolic Content

Phenolics are a major class of bioactive compounds in plants that has been known as ideal chemistry for free radical scavenging activity due to the presence of high activity as hydrogen or electron donor. Total Phenolic Content (TPC) in crude extract, fractions (hexane, ethyl acetate, n-butanol) and residue are displayed in Figure 2.

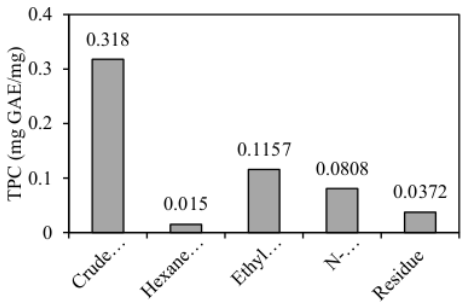

Figure 2. Total phenolic content of crude extract, fractions and residue

As seen, phenolic content detected in each sample was varied according to type of solvent

employed during fractionation. Whilst hexane fraction possessed the least amount of phenolics with TPC value is 0.015 mg GAE/mg, ethyl acetate exhibited the highest fraction with TPC value was observed 0.12 mg GAE/mg. N-butanol solvent gave lower TPC with approximately 0.08 mg GAE was detected in each mg of N-butanol fraction. Residue contains phenolic compounds which were not taken by hexane, ethyl acetate and n-butanol with TPC value was observed approximately 0.4 mg GAE/mg. Crude extract, however, shown the highest amount of TPC (0.32 mg GAE/mg) since the phenolic compounds have not been fractioned by solvents.

The results (Figure 2) show how the solvent polarity influences type of phenolic compounds can be extracted based on 'like and dislike property' (Patel *et al.*, 2011; Widyawati *et al.*, 2014). The sequence of the fractions and residue based on TPC detected are ethyl asetate fraction > n-butanol fraction > residue > hexane fraction. This indicates that phenolic compounds in crude extract prefer to be extracted with solvent possessing moderate polarity degree (semi-polar) such as ethyl acetate and n-butanol than solvents with strong polarity (water) or low polarity (hexane) (Zhao and Hall, 2008). The highest TPC value observed in the fraction of ethyl acetate indicates that major phenolic compounds in kaffir lime peel were semi polar compounds. The capability of ethyl acetate to extract more phenolics compared to other solvents was also reported by other group (Anagnostopoulou *et al.*, 2006). Complex formation among phenolics may facilitate the extraction of the phenolic compounds in ethyl acetate medium has been suggested in literature (Zhu *et al.*, 2011).

Total Flavonoid Content

Flavonoids are a noticeable group of secondary metabolites in citrus fruits that may have beneficial effects on human health (Kim *et al.*, 2012b; Lee *et al.*, 2007; Yao *et al.*, 2004). Total flavonoid contents of crude extract, fractions and residue are shown in Figure 3. The results show the total flavonoid contents varied from 0.0011 to 0.206 mg RE/mg. Among the fractions, ethyl acetate contained the highest amount of flavonoid content, followed by n-butanol and hexane fractions. This finding indicated that higher flavonoid content is associated with higher total phenolic content of the fraction.

Free Radical Scavenging Activity

In this study, scavenging activity of crude extract and fractions (hexane, ethyl acetate, n-butanol and residue) were tested on free radical DPPH. The ability of the sample to neutralize DPPH was expressed as IC_{50}. IC_{50} is the sample concentration required to inhibit the DPPH neutralization by 50%. Accordingly, the lower IC_{50} value, the stronger ability of the sample to quench DPPH. Figure 4 shows the antioxidant activity of crude extract, fractions and residue performed on DPPH.

As seen in Figure 4, crude extract exhibited the lowest IC_{50} that around 0.09 mg of crude extract is necessary to inhibit DPPH neutralization by 50%. Following the crude extract, the n-butanol fraction showed the second lowest position to neutralize the same DPPH solution with IC_{50} is reported around 0.44 mg/mL. Ethyl acetate fraction, residue, and hexane fraction have IC_{50} values of 1.5443, 5.3644 and 53.8386 mg/mL, respectively. Ascorbic acid was selected as positive control. Since sample with lower IC_{50} value means that it has higher antioxidant activity to neutralize DPPH, thus the sequences of the four fractions based on their antioxidant activity are as follows: n-butanol fraction > ethyl acetate > residue > hexane. Looking at the TPC values of ethyl acetate and hexane fractions (Figure 2), higher TPC value exhibited by ethyl acetate fraction may promise lower IC_{50} since more phenolic compounds are available to neutralize DPPH. Our study, however, observed higher IC_{50} in the fraction of ethyl acetate. This finding leads to the assumption that whilst ethyl acetate was able to extract more phenolic compounds, not all compounds performed well against DPPH. A reason to explain this because each phenolic compound will exhibit particular activity or selectivity to react with specific target (Islam *et al.*, 2016; Lau *et al.*, 2015; Liu *et al.*, 2017). Further identification of phenolic compounds by instruments such as HPLC may explain this.

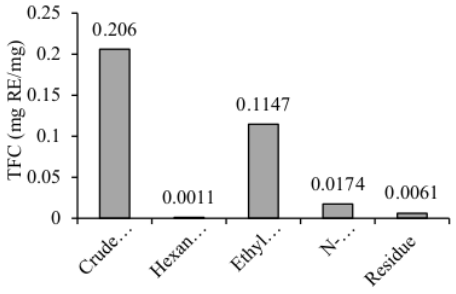

Figure 3. Total flavonoid content of crude extract, fractions and residue

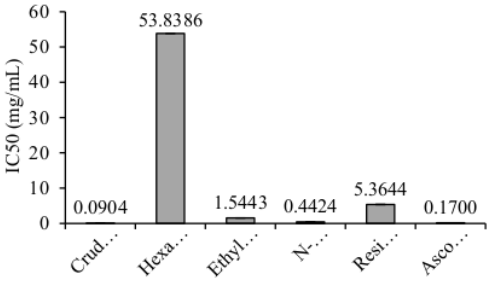

Figure 4. IC_{50} of crude extract, fractions and residue to neutralize DPPH. Ascorbic acid was selected as positive control

Table 1. Phenolic compounds detected in crude extract, fractions and residue

Sample	Gallic acid	Catechin	Caffeic acid	Epicatechin	p-Coumaric acid	Ferulic acid	Rutin	Quercetin
Crude extract	+	+	+	–	–	+	+	–
Hexane fraction	–	–	–	–	–	–	–	+
Ethyl acetate fraction	–	–	–	–	–	–	+	+
N-butanol fraction	–	–	+	+	+	+	–	–
Residue	+	–	+	+	+	+	+	+

+ detected, – not detected

Table 2. Flavonoid compounds detected in crude extract, fractions and residue

Sample	Naringin	Hesperidin	Naringenin	Hesperetin	Nobiletin
Crude extract	–	+	+	+	+
Hexane fraction	–	–	–	–	+
Ethyl acetate fraction	–	–	+	–	+
N-butanol fraction	+	–	–	–	–
Residue	–	–	–	–	–

+ detected, – not detected

Phenolics Identification by HPLC

Different phenolic contents in each sample (crude extract and fractions) resulted different total phenolic content observed in this study as well as its ability to quench DPPH as shown in Figures 2 and 3. Therefore, crude extract and the four fractions have been subjected for HPLC analysis to identify the phenolic compounds and the result is shown in Table 1.

It is shown in Table 1 that phenolic compounds distributed in crude extract and each fraction with different extents. The polarity of solvent used in the fractionation step influences the distribution of phenolic compounds (Irawaty *et al.*, 2014). Phenolic compound with relative low polarity degree such as quercetin was detected in low-medium solvents, *i.e.* hexane and ethyl acetate. However, the same compound was not detected in crude extract. Similar observation was also noted for epicatechin and p-coumaric acid that the two compounds were not detected in the crude extract but presented in n-butanol fraction and residue (Table 1). In addition to quercetin, rutin was detected in the fraction of n-butanol but not in ethyl acetate fraction. The presence of rutin in the n-butanol fraction may facilitate the quenching process of DPPH rather than quercetin since we observed that n-butanol fraction exhibited higher activity to neutralize DPPH. Without quantification analysis performed on the sample, however, this cannot be further explaining our observation. The stability of phenolic compounds against oxidation process may also contribute to the observation phenolic compounds in the four fractions as detected in this study. For example, catechin was initially detected in the crude extract and after fractionation, none of the fractions contained this compound. The poor oxidative stability of catechin reported in literature may explain this observation (Gadkari and Balaraman, 2015).

Flavonoids Identification by HPLC

The presence of flavonoid compounds was detected by HPLC and the results are tabulated in Table 2. Among five flavonoid standards employed, crude extract was found to have four compounds which were identified as hesperidin, naringenin, hesperetin and nobiletin. Fractionation step performed on the crude extract has distributed flavonoids into the four fractions with different extents. For example, nobiletin was detected in the fractions of hexane and ethyl acetate. Naringenin was only found in the fraction of ethyl acetate. On the other hand, both hesperidin and hesperetin were not noticed in the fractions which the compounds were originally present in the crude extract.

CONCLUSION

Solvent polarity employed during fractionation stage has facilitated the separation of phenolic and flavonoid compounds present in kaffir lime peel. Different classes of phenolic and/or flavonoid compounds are capable to supply scavenging activity against DPPH to different extents. Although higher total phenolic and flavonoid contents were detected in the fraction of ethyl acetate than n-butanol, our results showed, unexpectedly, the fraction of n-butanol exhibited higher scavenging activity toward DPPH. This inconsistency may be due that certain phenolic compound has the ability to react with particular radical compound (DPPH was selected in this study) and/or the concentration of each phenolic and flavonoid compound in the fractions will also important which the latter was not determined in this study. Accordingly, higher total phenolic and flavonoid content in a sample did not guarantee higher scavenging activity against the same radical compound. Further investigation is necessary to confirm this. Based on the amount of total phenolic and flavonoid compounds which can be extracted from crude extract of kaffir lime peel, ethyl acetate was observed as the best solvent.

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