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ANTIDIABETIC POTENTIAL OF KAFFIR LIME PEEL EXTRACT

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ABSTRACT

In Indonesia, kaffir lime fruit is commonly discharged as waste. On the other hand, the fruit peel contains phenolics and flavonoids compounds that can be used as one of natural antioxidant souces. In the present work, we investigated the possibility to extract phenolics and flavonoids compounds from kaffir lime peel. Several solvents possesses different polarity were employed. Following the extraction step, the extracts have been tested to be used as antidiabetic agent by retarding the conversion of starch to sugars. Several steps have been performed to achieve the aims. Firstly, kaffir lime peel was prepared by drying, grinding and sieving to obtain certain particle size. Secondly, kaffir lime peel extracts were prepared by soaking the kaffir lime peel with solvents possessing different polarity (water, ethanol, ethyl acetate and hexane). Thirdly, the extracts were subjected to analyses of total phenolic content, total flavonoid content and antidiabetic activity. Fourthly, phenolics and flavonoids compounds in the extracts were detected by using High Performance Liquid Chromatography. The results show extracts of kaffir lime peel, i.e. water, ethanol and ethyl acetate demonstrated antidiabetic activity to different extents. Ethanolic extract exhibited the strongest antidiabetic activity with 34.2% inhibition, whilst the phenolic and flavonoid contents in the extract were detected lower than the water extract. Ethyl acetate and water extract possessed the ability to retard starch conversion by 12.3 and 5.7 %, respectively. Several phenolics and flavonoids were identified in the three extracts. Gallic acid content was found higher in the ethanolic extract which can be up to 22.5 and 10 times higher compared to water and ethyl acetate, respectively. Different amounts of flavonoids of rutin, quercetin, naringin, hesperidin and naringenin have been detected present in the extracts. The ability to kaffir lime peel extracts, particularly the ethanol extract, to inhibit starch conversion promises to be used as nutraceutical supplements especially as antidiabetic agent.

Keywords: kaffir lime, peel, antidiabetic activity.

INTRODUCTION

Oxidative stress is the formation of excess reactive oxygen species (ROS) such as hydroxyl radical (•OH), superoxide ($O_2^{\bullet-}$) and peroxyl radical ($RO_2^{\bullet}$). These ROS involve in developing diseases such as heart disease, aging, diabetes and other degenerative problems. Natural antioxidants offer an alternative source of dietary ingredients to promote healthy life. The presence of phenolics compounds in plants is believed as an important contributor to the radical compound activities. Accordingly, they showed variety of pharmaceutical effects such as antimicrobial (Ammer *et al.*, 2016), anti-tumor (Sufian *et al.*, 2013), antioxidant (Irawaty *et al.*, 2014) and anti-inflammation (Putri *et al.*, 2013).

Diabetes is one of major health problems in both developed and developing countries that arises from imbalance modern life style. Whilst the amount of diabetes patient is around 382 millions in 2013, the number is predicted to increase by 55% in 2035 (Cho, 2013). Diabetes is a condition when the pancreas produces insufficient amount of insulin and/or the body unable use the insulin. There is a trend that diabetes patients toward younger and younger people that lead to problems in the future (Cho, 2013).

Medicinal plants for treating diabetes have been widely investigated. They included fruits (Abirami *et al.*, 2014), leaves (Ammar *et al.*, 2009), roots (Chen *et al.*, 2008) and agricultural by-products (Zhao *et al.*, 2012). Citrus becomes focus in the present study. Citrus is an excellent source of many nutrients and phytochemicals. Kaffir lime is one type of citrus fruit that commonly growth in Indonesia and other Southeast Asia countries. Kaffir lime leaves have been widely used in cooking, however, the fruit is usually discarded. The fruit peels were reported contain a variety of phenolics compounds (Kim *et al.*, 2005; Chan *et al.*, 2009). Phenolic compounds itself have been claimed to their ability to scavenge free radicals (Choi *et al.*, 2007; Choi *et al.*, 2014).

The extraction of phenolics from kaffir lime peel using methanol, acetone and ethanol 60% have been reported in literature (Chan *et al.*, 2009). The present study use different solvents and the extracts were then subjected for antidiabetic activity.

MATERIAL AND METHODS

Material preparation

Kaffir lime was collected from Keputran market, Surabaya, Indonesia. Fresh kaffir lime was peeled and dried under sunlight for 48 h to obtain moisture of 5-6% $^{w}/_{w}$. Subsequently, the peel was ground and sieved. The material was then kept in a closed container at a temperature of ± 5 ^{o}C for further use.

Chemicals

Gallic acid (≥ 99%, Sigma®), rutin (≥ 94%, Sigma®), Folin-Ciocalteu reagent (Merck), sodium carbonate (≥ 99%, Merck), aluminum chloride (99%, Ferak), α-amylase from *Aspergillus niger* (Fluka), methanol (99%, Merck), ethanol, ethyl acetate, hexane, 3,5-dinitrosalicylic acid (85%, Sigma®), sodium hydroxide (99%, Merck), fenol (99.5%, Riedel de Häen),

glucose (Merck), rochelle salt (≥ 99%, Merck) and amylum (Merck) were used as it.

Procedures

Kaffir lime powder was soaked with solvents possessing different polarity (water, ethanol, ethyl acetate and hexane) with a ratio of 1:40 $^{w}/_{v}$ at room temperature. The mixture was then centrifuged (Hettich, EBA 20) at 3,000 rpm to remove the solid part. Crude extract obtained was further subjected for total phenolic compounds, total flavonoid content and *in-vitro* antidiabetic scavenging assay by using a spectrophotometer (Shimadzu, UVmini-1240). Total phenolics content is presented as gallic acid equivalent (GAE). Total flavonoids content is reported as rutin equivalent (RE). The α-amylase inhibitory effect was assessed to determine the antidiabetic activity of kaffir lime extract

Total phenolic content analysis

Total phenolic content (TPC) was determined by using colorimetric method (Anagnostopoulou *et al.*, 2006) with modification. Extract (0.2 mL) was prepared in a reaction tube and added water (1.7 mL) and Folin-Ciocalteu's reagent (1:1) (1 mL) and allowed to stand at room temperature for 1 min. Subsequently, Na_2CO_3 solution (3 mL) was added into the mixture. After incubating at room temperature for 30 min, the absorbance was measured at 730 nm using a spectrophotometer (Shimadzu, UVmini-1240).

Total flavonoid content analysis

Total flavonoid content (TFC) was determined by using aluminum chloride colorimetric method (Wu *et al.*, 2009) with modification. In brief, extract (0.4 mL) was mixed with methanol (1.8 mL) and 10% aluminum chloride solution (2 mL). The mixture was then incubated at room temperature for 30 min. After that, the absorbance was measured at 431 nm using a spectrophotometer (Shimadzu, UVmini-1240).

α-amylase activity assay

Prior to assess the antidiabetic activity, the extract was concentrated by using a rotavapor (IKA HB-10, IKA RV-10) to remove the solvent. 1 mL of extract (1% $^{w}/_{w}$) was added to 1% $^{w}/_{w}$ amylum (1 mL) sample and α-amylase solution (1 mL). The mixture was incubated at room temperature in dark conditions for 10 min. The reaction was stopped by adding dinitrosalicylic acid solution (3 mL) and heat the mixture to develop the red-brown color. After that, Rochelle salt solution (1 mL) was added to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance was recorded (Shimadzu, UVmini-1240). The assay was repeated without extract for control. The antidiabetic activity was measured as a decrease in the absorbance and was calculated by using the following equation:

$$\text{Percentage inhibition} = [(1 - A_S/A_C)] \times 100 \quad (1)$$

where A_S and A_C are the absorbances of sample and the control, respectively.

Chromatographic analyses

Phenolics/flavonoids compounds were analyzed using High Performances Liquid Chromatography (Jasco, UV-2077 Plus). Briefly, analysis was carried out on HPLC coupled with UV detector at 280 nm using a C_{18} column (250 mm x 4.6 mm, 5 microm) (Phenomenex). Mobile phase employed for phenolics separation was a mixture of acetic acid and methanol with a flow rate of 1 mL min^{-1}. For flavonoids separation, acetonitrile, water and acetic acid were used as mobile phase with a flow rate of mL min^{-1}. All separation was performed at 25 ± 1 ^{o}C.

RESULTS AND DISCUSSIONS

Solvents possessing different polarities such as water, ethanol, ethyl acetate and hexane have been employed to extract phenolic compounds from kaffir lime peel. The extracts were then tested for their activity to inhibit starch conversion to glucose. Figure-1 shows the ability of kaffir lime peel extract obtained from different extraction solvents to inhibit starch conversion.

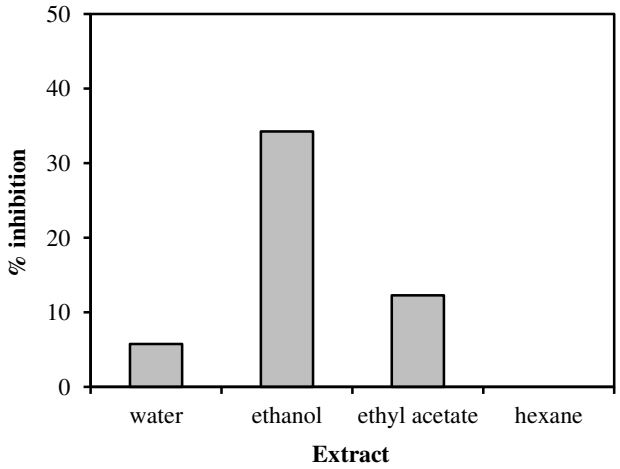

Figure-1. Inhibitory effect of extracts on α-amylase activity.

As seen in Figure-1, the extracts demonstrated different activities toward starch hydrolysis. The % inhibition of ethanol extract is 34.2, making it amongst the most potent kaffir lime peel extract to inhibit starch conversion to glucose. Extracts of ethyl acetate and water showed lower activity with the % inhibition values of 12.2 and 5.7, respectively. On the other hand, the employment of hexane as solvent did not show any activity toward starch inhibition. It seems that hexane did not facilitate the diffusion of antidiabetic compounds from kaffir lime peel. Therefore, when the extract was further assessed for its antidiabetic activity, it did not demonstrate any performance.

The inhibition of starch conversion by enzyme has been shown to have relationship to phenolics content (Patel *et al.*, 2011). Accordingly, the higher the total phenolics content in the extract, the higher the inhibition

factor of the extract to retard glucose formation. The total phenolics content of the four extracts determined in this study are shown in Figure-2. As seen, the amount of TPC is varied with time and type of solvent used during extraction. Generally, the longer contact between kaffir lime peel and the solvent, more phenolics compounds extracted from the peel. This has been observed when ethanol and ethyl acetate were employed as the solvent. After three minutes of extraction period, the TPC values are 16.1 and 2.5 mg GAE/g for ethanol and ethyl acetate, respectively. When the contact time is increased to 40 min, the TPC values of ethanol and ethyl acetate extracts were 28.1 and 5.4 mg GAE/g, respectively. However, this trend did not exhibited by water since the increase of extraction time did not facilitate the diffusion of phenolics compounds from kaffir lime peel. As seen in Figure-2, at t = 3 min, the TPC value is 25.4 mg GAE/g and the addition of 6 min to the extraction time increased the TPC value by a factor of 1.1. Prolonged the extraction time up to 50 min did not assist the release of phenolics compounds from the peel. In contrast with other solvents, there is no TPC observed in the hexane extract. The contact time observed in this study is in agreement with the extraction time required to extract phenolics compounds from plants reported in literature (Dent *et al.*, 2013; Maran *et al.*, 2013; Thoo *et al.*, 2013).

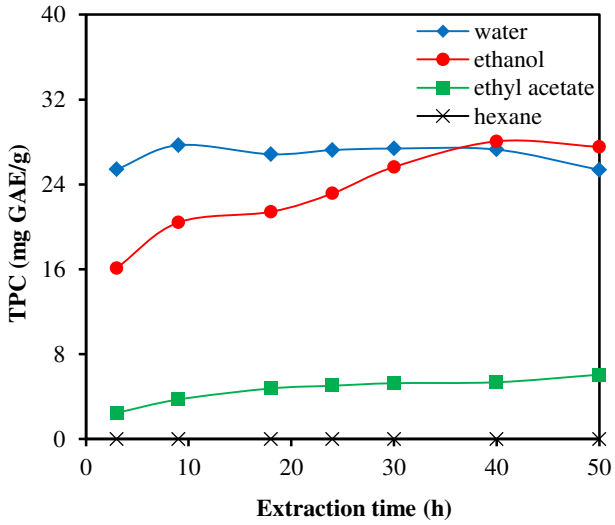

Figure-2. Profiles of phenolics compounds extracted from the peel.

Flavonoids content is another important parameter investigated to assess the antioxidant activity of natural extract. Figure-3 shows the profiles of total flavonoids detected in kaffir lime peel extracts. The amount of flavonoids extracted from kaffir lime peel is increased with extraction time. This has been observed for the three solvents, i.e. water, ethanol dan ethyl acetate. For example for water-based extract, 11.3 mg RE/g was detected after 3 min of extraction time and prolonged the system to 40 min, the TFC was increased to 13.8 mg RE/g. Extending the extraction for another 10 min did not improve the extraction, indicating the maximum release of the compounds to the solvent. Similar to the TPC profiles (Figure-2), the TFC profile of hexane extract was steady since the beginning of the extraction stage, indicating no flavonoids compounds detected in the hexane extract. This result is in agreement with antidiabetic property of the hexane extract which did not show any activity towards starch hydrolysis to sugars (Figure-1).

As seen in Figure-3, the TFC values of the four extracts are as follows: water > ethanol > ethyl acetate > hexane with TFC values were 13.8, 10.3, 2.7 and 0 mg RE/g after the system was aging for 40 min. Among four solvents employed in this study, water demonstrated the best solvent to extract flavonoids from kaffir lime peel, similar to the case of phenolics extraction (Figure-2). The result indicates major phenolics and flavonoids present in kaffir lime peel tend to be polar compounds. This finding supports previous work that polar solvents are required to extract antioxidant compounds from plants (Chan *et al.*, 2009; Sultana *et al.*, 2009; Dent *et al.*, 2013; Thoo *et al.*, 2013).

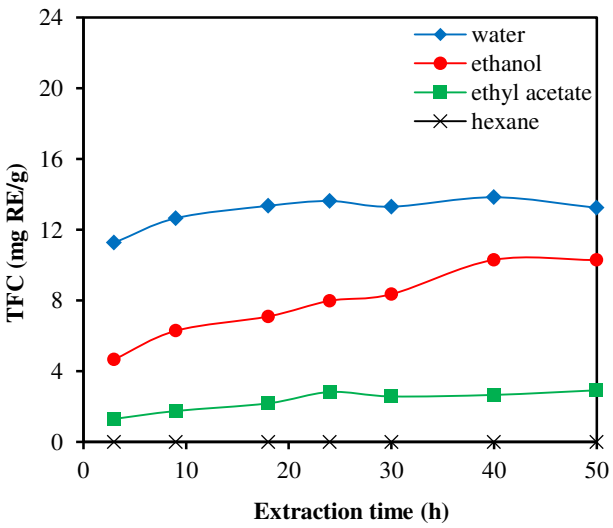

Figure-3. Profiles of flavonoid compounds extracted from the peel.

Comparing the results of antidiabetic activities (Figure-1) and the amount of phenolics and flavonoids detected in the extracts (Figures-2 and 3), it was found that different trend observed between the two. Ethanol extract exhibited the highest antidiabetic activity to retard starch digestion to sugars, however, the amounts of TPC and TFC in the extract were lower than the water extract. On the other hand, even though the water extract had the highest TPC and TFC values, it showed the lowest antidiabetic activity. The reason for this discrepancy may can be explained by further treatment performed on the extracts prior to the antidiabetic activity assay. Heat treatment conducted to remove solvent may have degraded phenolics/flavonoids present in the extract. Thus, the extracts did not show antidiabetic activity as expected.

Phenolics/flavonoids compounds are important plant constituents since they have demonstrated their antioxidative properties; in this study we tested the

antidiabetic property. Each phenolics and/or flavonoids may exhibit specific performance as insulin promoter (Kannappan and Anuradha, 2010), anti-inflammation agent (Putri *et al.*, 2013), adipogenesis inhibitor (Kim *et al.*, 2012), chrotiromosomal stabilizer (Bakheet and Attia, 2011) and antifibrotic (Tsai *et al.*, 2012). Phenolics/flavonoids compounds in the extracts of water, ethanol and hexane were determined by using HPLC. Chromatograms obtained from HPLC are not shown. In this study we used eleven phenolic/flavonoid standards to compare with the chromatograms exhibited by the three extracts. Qualitative analysis performed on the chromatograms obtained show the presence of several standard compounds to certain extents (Table-1).

Table-1. Phenolics/flavonoids analysis in kaffir lime peel extracts.

Compounds	Extract		
	Water	Ethanol	Ethyl acetate
Gallic acid	+	+	+
Catechin	-	-	-
Caffeic acid	-	-	-
Epicatechin	-	-	-
p-Coumaric acid	-	-	-
Ferulic acid	-	-	-
Rutin	+	+	+
Quercetin	++	+++	+
Naringin	+	+	+
Hesperidin	++	+++	+
Naringenin	-	+	+

(+) indicates the presence of the target standard compound. More (+) signs show higher area observed in chromatogram of the selected extract.
(–) indicates the absence of the target standard compound.

As seen in Table-1, the three extracts contain gallic acid, rutin, quercetin, naringin, hesperidin and naringenin to different extents. Other standard compounds were not detected in our extracts. Other phenolic/flavonoid standards are required to identify peaks observed in the chromatograms. Further analysis to determine the concentration of each phenolics/flavonoids was also performed and the results are shown in Table-2. As seen, phenolic compound of gallic acid was detected mostly in the ethanol extract, followed by ethyl acetate and water extract. This could be the reason explaining the highest ability of the ethanolic extract to inhibit the digestion of starch to sugars. Our study also shows the presence of rutin and naringin in the three extracts in similar amount. Without detailed quantification of other flavonoids, i.e. quercetin and hesperidin, determination of compounds that are responsible for antidiabetic activity of extracts cannot be determined.

Table-2. Concentration of phenolics/flavonoids detected in kaffir lime peel extracts (ppm).

Compounds	Extract		
	Water	Ethanol	Ethyl acetate
Gallic acid	12.4	279.4	28.5
Rutin	59.6	57.6	59.1
Quercetin	n/a	n/a	n/a
Naringin	46.3	46.4	47.2
Hesperidin	n/a	n/a	n/a
Naringenin	-	54.2	47.7

n/a: not available

CONCLUSIONS

The antidiabetic potential of kaffir lime peel has been investigated by the α-amylase inhibition assay. The results obtained in the present study demonstrate the extracts of kaffir lime peel have an antidiabetic activity. The ethanol extract exhibited the best antidiabetic property against α-amylase inhibitory assay, while the water extract was originally contained the highest amount of phenolics and flavonoid contents. However, the lack of correlation observed between the antidiabetic activity and the identified phenolics/ flavonoids in the extracts shows that other compounds may responsible for the reason.

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