

Assessment on antioxidant and in vitro antidiabetes activities of different fractions of Citrus hystrix peel

by Wenny Irawati

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Assessment on antioxidant and in vitro antidiabetes activities of different fractions of *Citrus hystrix* peel

*Irawaty, W. and Ayucitra, A.

Chemical Engineering Department, Faculty of Engineering, Widya Mandala Catholic University
Surabaya, Kalijudan 37 Surabaya 60114, East Java, Indonesia

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Abstract

The effect of solvent polarity during fractionation of ethanolic extract of *Citrus hystrix* peel for its antioxidative and antidiabetic activity was investigated. Dried *Citrus hystrix* peel was soaked in ethanol solution and the extract was subsequently fractionated with solvents possesses different polarity, followed by solvent evaporation to obtain the hexane fraction, ethyl acetate fraction and water residue. Antioxidative activity of each part was examined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and metal chelating activity. Two different assays to assess the antidiabetic potential have been performed by using α -amylase and α -glucosidase. Both the antioxidant and antidiabetic activities were presented as IC_{50} . The IC_{50} of DPPH scavenging ability observed in the hexane fraction, ethyl acetate fraction and water residue were found 2.368, 0.0252 and 1.080 mg/mL, respectively. The highest metal chelating activity was also exhibited by ethyl acetate fraction with the IC_{50} value of 0.117 mg/mL. The same fraction also demonstrated the highest activity against α -amylase and α -glucosidase during antidiabetes tests with the IC_{50} values were detected 0.087 and 0.49 mg/mL, respectively. Preliminary phytochemical screening performed on each fraction indicates the presence of some potent phytochemical constituents with different extents. The finding suggests ethyl acetate is the best solvent to extract antioxidant and antidiabetic compounds from the extract. More phytochemicals detected in ethyl acetate fractions compared to other fractions may contribute to both activities.

Keywords

Citrus hystrix peel

Extraction

Fractionation

Antioxidant

Antidiabetes

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Introduction

Free radicals are produced as a normal part of metabolism within the mitochondria, respiratory burst, enzyme reactions, and auto-oxidant reactions that can be prompted by aging. External factors such as environmental pollutants and ultraviolet light promote the production of free radicals which in turn increase oxidative stress followed by the occurrence of pathophysiological conditions such as neurodegenerative disorders, cardiovascular diseases, diabetes, and cancer (Lee *et al.*, 2004). Diabetes is a global disease that affects large proportion of world population due to absolute or relative lack of insulin, glucose intolerance during pregnancy, and other specific causes such as protein depletion in tissues, abnormal fat metabolism, and the decrease of body cells to utilize glucose (Fatima *et al.*, 2012). In general, a balanced diet of antioxidant-rich food of plant sources can significantly slow down the development of those free-radicals-caused pathophysiological disorders (Association, 1988). More specific for diabetes, several attempts have been employed to reduce hyperglycemia such as the consumption

of sulfonylureas products to stimulate the insulin secretion (Mandarino and Gerich, 1984; Henquin, 2000), the use of sensitizers to improve insulin action (Young *et al.*, 1995), and the employment of enzyme inhibitors to diminish insulin requirement (Cheatham *et al.*, 1994). Unfortunately, the therapeutic drugs are believed to come with several side effects (Prato and Pulizzi, 2006). Hence, the need to search benign alternatives with little or no side effects from natural resources gain much attention and exploration and this refers to the use of phytochemicals to augment endogenous antioxidants to assist biological cells against deleterious effects caused by oxidative stress (Cirico and Omaye, 2006; Ammar *et al.*, 2009; Kannappan and Anuradha, 2010; Bakheet and Attia, 2011; Landete, 2012; Abirami *et al.*, 2014; Sarepoua *et al.*, 2015).

Citrus, which is consumed worldwide, is an important source of vitamin C and phenolics compounds. Citrus fruits and/or their compounds have been claimed to protect liver (Putri *et al.*, 2013; Abirami *et al.*, 2015), decrease glucose production (Purushotham *et al.*, 2009; Nizam Uddin *et al.*, 2014), retard the transport of glucose through the

*Corresponding author.

Email: wenny_i_s@ukwms.ac.id

intestines and liver (Li *et al.*, 2009), inhibit intestinal glucose absorption (Mahmoud *et al.*, 2015), stimulate beta cells to increase insulin secretion (Nizam Uddin *et al.*, 2014; Mahmoud *et al.*, 2015), and exhibit insulin-like effect (Borradaile *et al.*, 2003). In addition, traditionally the epicarp is burnt in a room to serve as mosquito repellent. This is solely attributed by the presence of bioactive compounds in materials investigated that exhibited antioxidant activity. Previous investigations on oranges showed the evidence of phenolic compounds include hydroxycinnamic acid and flavonoids, among which flavanones are the most prevalent (Klimczak *et al.*, 2007). Both lemon and lime are dominated by hesperidin and eriocitrin in their flavanone profiles (Peterson *et al.*, 2006). Similar compounds were detected in *Citrus hystrix* (Ghafar *et al.*, 2010). The existences of these compounds are responsible for antioxidant activities reported in literature.

Citrus hystrix is a citrus native to tropical Asia. Studies showed leaves (Abirami *et al.*, 2015), juice (Abirami *et al.*, 2014), and peel (Jamilah *et al.*, 2007; Putri *et al.*, 2013) exhibited antioxidant activity (Jamilah *et al.*, 2007), anticancer (Tunjung *et al.*, 2015), antidiabetes (Abirami *et al.*, 2014), tyrosinase inhibitory (Abirami *et al.*, 2014), cardioprotective (Putri *et al.*, 2013) and hepatoprotective (Putri *et al.*, 2013; Abirami *et al.*, 2015) effects. Several work (Gorinstein *et al.*, 2001; Li *et al.*, 2006; Park *et al.*, 2014) confirmed the substantially higher amount of phenolic compounds in the peel towards the flesh and thus, higher antioxidant activity was observed in the peel part. Higher amount of phenolics in peel has been claimed due to the protection action to inner material deterioration from insects and microorganisms (Jeong *et al.*, 2004).

Despite a lot of research work carried out on *Citrus hystrix* as stated previously, the investigation on native Indonesian fruits and their derivatives products is still limited. Several work reported the antioxidant activity of *Citrus hystrix*-based products collected from Sumatra (Ernawita *et al.*, 2017), Central Java (Putri *et al.*, 2013; Wulandari *et al.*, 2017) and East Java (Adrianto *et al.*, 2014; Warsito, *et al.*, 2016). Investigating the antioxidant activity grown at particular location is important since plant activity is greatly influenced by phytochemicals content that the latter is determined by environmental and edaphic factors such as rainfall, temperature, humidity, sunlight or ultraviolet irradiation, and soil nature during the growth of plants. These factors affect the production of secondary metabolites, i.e. phytochemicals, to certain extent (Li *et al.*, 1993; Gebruers *et al.*, 2010; Baniasadi *et al.*, 2014).

For example, less water in soil may reduce the photosynthetic rate and this condition promotes the production of reactive oxygen species. As a result, more phenolics compounds will be produced as a defense mechanism (Reddy *et al.*, 2004). Another factor is temperature. Similar to the case of water content in soil, at high temperature, plants contain more phenolics which in turn enhance the antioxidant activity. The presence of toxic metal of cadmium in soil has also been reported to increase the amount of antioxidant or phytochemicals in *Erica andevalensis* as its survival technique (Márquez-García *et al.*, 2012). In addition, the treatment of solar irradiation in the range of 280 to 320 nm on a series of *Arabidopsis* wild plant showed the amount of phenolic compounds were improved with higher ultraviolet intensity (Li *et al.*, 1993). Moreover, phenolics compounds have been observed as the least stable phytochemicals when the same plant variety was experimentally grown across environments (Shewry *et al.*, 2010; Baniasadi *et al.*, 2014; Tang *et al.*, 2017). It is clear that environment conditions determine the plant growth and its metabolism which in turn results specific phenolics compounds. Those environmental conditions has been claimed to provide major contribution on plant metabolome compared to genetic factor (Baniasadi *et al.*, 2014). The investigation of antioxidant activities of Indonesian *Citrus hystrix*-based products collected from several areas has been performed on specific application. For instance, Ernawita *et al.* (2017) reported their work on the antioxidant and antidiabetic activities of pulp and peel extract of *Citrus hystrix* collected in Sumatra (Ernawita *et al.*, 2017). Putri *et al.* (2013) collected *Citrus hystrix* fruits from Central Java and used the peel extract as cardioprotective and hepatoprotective agents (Putri *et al.*, 2013). Adrianto *et al.* (2014) used leaves (from Sidoarjo-East Java) extract against *Aedes aegypti* larvae (Adrianto *et al.*, 2014). Therefore, study on phytochemicals/phenolics compounds of Indonesian *Citrus hystrix*, especially from East Java, and assess the antioxidant activities is another challenge and the peel part becomes focus in this study. The fruit peel is generally discarded as waste and therefore, the utilization of peel as antioxidant is potential to be further developed (Rafiq *et al.*, in press).

Owing to different antioxidant compounds from different samples, the antioxidant compounds extracted from the samples relies on solvent media employed in the system and thus, it will influence the measurement of antioxidant activity (Zhou and Yu, 2004; Turkmen *et al.*, 2006; Ardestani and Yazdanparast, 2007). Ethanol, acetone, methanol, and water are most commonly solvent employed to

extract phenolics from citrus fruits (Bocco *et al.*, 1998; Zhou and Yu, 2004; Chan *et al.*, 2009; Park *et al.*, 2014). Accordingly, phenolic compound **6** in the extract with their specific characteristics will respond to different radical sources in a different manner (Park *et al.*, 2014). Further treatment or purification employed on the extract by using different solvents such as in fractionation process is aimed to improve the performance of the samples towards their antioxidant capacities. In addition, the investigation on *Citrus hystrix* peel for *in vitro* antidiabetes activity has not been performed yet.

The present study was designed to evaluate the antioxidant and antidiabetic activities of several samples (hexane fraction, ethyl acetate fraction and water residue) derived from ethanolic crude extract of *Citrus hystrix* peel. In the antioxidant activity, DPPH free radical compound was employed to assess the ability of the fractions to neutralize the DPPH. In addition, the metal chelation ability of the samples was also determined. The antidiabetic activity relates to the performance of the samples to inhibit α -amylase and α -glucosidase, carbohydrate-digesting enzymes, that compounds extracted from *Citrus hystrix* peel may inhibit starch digestion to produce sugars and thus, lowering the level of postprandial blood glucose.

Materials and Methods

Sample preparation and fractionation

Citrus hystrix was purchased from local market in the period of January to April and the voucher sample of the citrus identification was kept for further references. *Citrus hystrix* was peeled and cut into the size of 0.5 x 0.5 cm prior to dry it for 48 hr at ambient temperature. The peel was then stored at 4°C until further processing. Phenolic compounds of kaffir lime peel were extracted by soaking 5g of dried peels with 200 mL of aqueous ethanol (41% v/v) in an amber bottle for 8hr at room temperature. After separating the solid part, the crude extract was concentrated by using a rotary vacuum evaporator (IKA, RV10) at 55°C and weighed to know yield obtained. After that, the crude extract was subjected to fractionation with hexane by adding hexane to the extract (1:1 v/v) and the mixture was shaken vigorously and let to stand until two phases were observed. The hexane fraction was concentrated at 40-45°C until dryness. Remained phenolics in residue were further fractionated with ethyl acetate, followed by evaporation to obtain the fraction of ethyl acetate. The percent yield of hexane fraction, ethyl acetate fraction and water residue was calculated by using equation (1). The

schematic fractionation step carried out in this work is shown Figure 1. The hexane fraction, ethyl acetate fraction and water residue were weighed and stored at 4°C for future use. Each fraction and water residue underwent phytochemical screening to detect the presence of potential phytochemical constituents such as alkaloids, flavonoid, saponins, tannins, carbohydrates, phenolics, and sugars (Harbone, 1973).

$$\text{Yield (\%)} = \frac{\text{weight of extract}}{\text{weight of dry peel}} \times 100\% \quad (1)$$

DPPH radical scavenging assay

DPPH (2,2-diphenyl-1-picryl-hydrazil) radical scavenging activity was measured according to (Irfan *et al.*, 2011) with minor modification. In brief, an aliquot of 1 mL of 0.2 mM DPPH ethanol solution and 2 mL of sample were mixed and incubated at room temperature in dark conditions. The absorbance was then measured 30 mins later using a spectrophotometer (Shimadzu, UVmini-1240) at 520 nm. For the control, the assay was performed in the same procedures but ethanol was used instead of sample. The radical scavenging activity was measured as a decrease in the absorbance and was calculated by using the following equation:

$$\text{Percentage inhibition} = [(1 - A_s/A_c)] \times 100 \quad (2)$$

where A_s and A_c are the absorbances of sample and the control, respectively.

The results were compared with ascorbic acid which has been selected as positive control. IC_{50} value of DPPH radical scavenging activity was calculated by using regression linear analysis.

Metal chelating activity

The Fe^{2+} -chelating activity was determined by measuring the formation of the Fe^{2+} -ferrozine complex according to (Liu *et al.*, 2015) with slight modification. 0.5 mL of sample was mixed with 0.2 mL of 1 mM iron (II) sulphate. The mixture was then incubated for 30 mins at room temperature and subsequently 0.4 mL of 2.5 mM ferrozine. 96% Ethanol was added into the mixture to make the total volume was 10 mL. After 30 mins, the absorbance was measured by a spectrophotometer (Shimadzu, UVmini-1240) at 562 nm. For control, the procedure was repeated by using ethanol instead of the sample. The percentage of inhibition of Fe^{2+} -ferrozine complex formation was calculated as

$$\text{Percentage inhibition} = [(A_c - A_s) / A_c] \times 100 \quad (3)$$

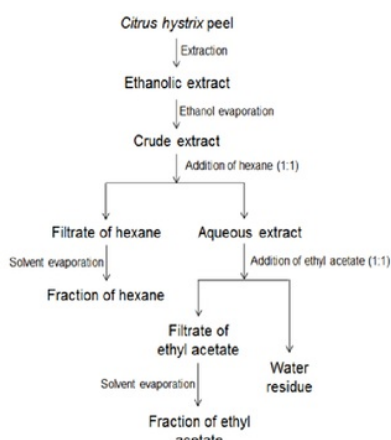


Figure 1. Schematic diagram for fractionation of *Citrus hystrix* peel extract

where A_c is the absorbance of the control and A_s is the absorbance of the sample.

α -amylase inhibitory activity assay

α -amylase inhibitory activity assay was performed by starch-iodine color assay (Pimstone, 1964; Xiao *et al.*, 2006; Hossain *et al.*, 2009) with slight modification. Briefly, the reaction mixture contained sample, 0.2 mL of α -amylase solution, and 0.4 mL of phosphate buffer (pH 6.9 containing sodium chloride) was prepared. Following the incubation of the mixture at 37°C for 10 mins, 0.2 mL of starch solution was added and the mixture was re-incubated for 1 hr. Hereafter, 0.2 mL of iodine solution and 10 mL of distilled water were subsequently added. Following the color development, the iodine-treated sample was transferred to a transparent cuvette and the intensity of blue color developed was then measured at 575 nm using a spectrophotometer (Shimadzu, UV mini-1240). The instrument is set to zero with iodine blank containing neither enzyme nor substrate. The control reaction representing 100% enzyme activity did not contain any sample was performed under the same conditions. To eliminate the absorbance produced by the sample, appropriate control without the enzyme was also included. The activity is presented as IC_{50} . The percentage of inhibition was calculated by using equation (2).

α -glucosidase inhibitory activity assay

α -glucosidase inhibitory activity assay was performed according to (Lordan *et al.*, 2013) with modification. 0.15 mL of *Citrus hystrix* fraction was added to a test tube containing 0.1 mL of 5 mM p-nitrophenyl- α -D-glucopyranoside solution (in phosphate buffer, pH 6.9) and the mixture was

Table 1. Yields of crude extract, hexane fraction, ethyl acetate fraction and water residue

| | Yield (%) |
|------------------------|-----------|
| Crude extract | 33.4 |
| Hexane fraction | 13.1 |
| Ethyl acetate fraction | 2.0 |
| Water residue | 15.0 |

incubated at 37°C for 5 mins. The reaction was initiated by adding 0.1 mL of enzyme solution (0.1 U/mL) followed by 30 mins incubation at 37°C. The reaction was stopped by adding 2.5 mL of 200 mM Na_2CO_3 . The absorbance of p-nitrophenol released from p-nitrophenyl- α -D-glucopyranoside was measured at 400 nm. The procedure was repeated with no enzyme for blank reading. Blank reading was subtracted from each sample measurement. Acarbose was selected as positive control. The α -glucosidase inhibitory activity of sample is presented as IC_{50} .

Results and Discussion

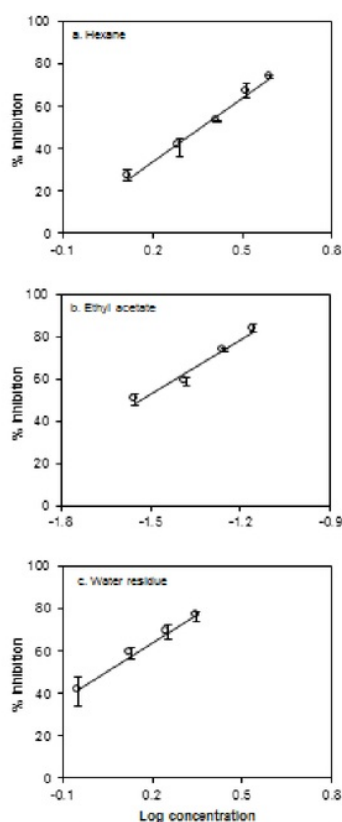
Extraction yield

Yields of crude extract, hexane fraction, ethyl acetate fraction and water residue obtained from *Citrus hystrix* peel is presented in Table 1. The results show different amounts of extractable soluble compounds. Crude extract obtained from this work is 33.4%. The yields of hexane and ethyl acetate fraction were 13.1 and 2.0%, respectively. The remained extract, named water residue, has higher yield compared to the other two fractions, i.e. 15.0%.

Compared with other studies, the yield reported in this study is relatively higher. For example, Safdar *et al.* (2017) used 50% ethanol to extract *Citrus reticulata* L. peel by using 50% ethanol and the same extraction method as employed in the present study. They found that yield obtained from the extraction was 15.64% (Safdar *et al.*, 2017). Furthermore, Bimakr *et al.* obtained the yield of 25.8% when they used ethanol 70% to extract using soxhlet extraction method. They also found that the yield was decreased up to 35% when they carried out the extraction using different method, i.e. supercritical carbon dioxide extraction. However, the later exhibited more flavonoid compounds being extracted (Bimakr *et al.*, 2011).

DPPH radical scavenging assay

DPPH (2,2-diphenyl-1-picryl-hydrazil) assay has been widely used to determine the free radical-scavenging potential of various plants. The performance of a sample on DPPH radical



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Figure 2. Dose-dependency of the DPPH free radical scavenging activity of fractions of hexane, ethyl acetate, and water residue.

scavenging activity is often correlated to its phenolics and flavonoids content (Ardestani and Yazdanparast, 2007; Hayat *et al.*, 2010; Patel *et al.*, 2011; Sarepoua *et al.*, 2015). Hydrogen donation performed by phenolics/flavonoids compounds will decolorize the deep purple color of DPPH solution. Consequently, high color reduction of DPPH is related to high scavenging activity performed by the sample. Antioxidant activity is depicted as % inhibition of the fraction to slow down the neutralization of DPPH radical compound and thus, higher amount of the fraction tested would lead to higher inhibition (Figure 2). Radical DPPH scavenging activity was affected by the three fractions (hexane, ethyl acetate, and water residue) in a dose-dependent manner as shown in Figure 2. As seen, the DPPH inhibition percentage is enhanced with the increased of the tested concentration, presented as log concentration, of each fraction as expected. Similar result was reported by other work (Ardestani and Yazdanparast, 2007; Park *et al.*, 2014). The straight lines were obtained from the three fractions. The DPPH inhibition decreased in

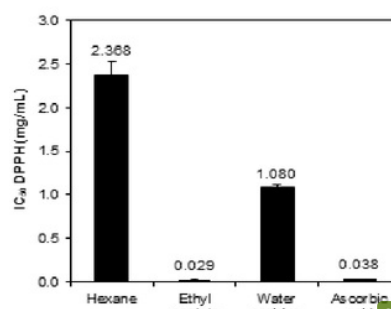


Figure 3. IC_{50} values to the inhibition of DPPH. Ascorbic acid was selected as positive control.

the order of fraction of ethyl acetate > water residue > hexane.

Figure 3 depicts the potency of fractions of the fractions to scavenge DPPH radical compound as IC_{50} . The DPPH IC_{50} is defined as the concentration of the sample necessary to decrease the initial DPPH concentration by 50%. The IC_{50} values of DPPH radical scavenging activity observed in ethyl acetate and water residue were found 0.029 ± 0.001 and 1.080 ± 0.038 mg/mL, respectively. In comparison, the fraction of hexane revealed the highest IC_{50} value, i.e. 2.368 ± 0.156 mg/mL against the DPPH radical. Compared with other work reported in literature, the ability of the fraction to scavenge free radical was found higher. For example, Anagnostopoulou *et al.* (2006) used sweet orange peel and found the highest ability to neutralize DPPH radical compound was residue with IC_{50} value was around 9 mg extract per mg DPPH; while in the present work, the highest performance has been shown by ethyl acetate fraction which equivalent to 2.6 mg extract per mg DPPH.

The lowest IC_{50} value of DPPH radical scavenging assay of the fraction of ethyl acetate compared to other fractions indicates the highest performance of the fraction to neutralize the DPPH radicals. Accordingly, ethyl acetate is regarded as the most effective solvent to extract antioxidant compounds from ethanolic crude extract of *Citrus hystrix* peel. Similar result was reported by other work (Anagnostopoulou *et al.*, 2006) that the fraction of ethyl acetate from sweet orange possessed the highest antioxidant capacity towards the same free radical compound. The formation of a complex of phenolic compounds with other components which are more extractable in ethyl acetate medium than other solvents has been claimed as the reason for this (Zhao and Hall, 2008; Zhu *et al.*, 2011). Different phytochemical content in each fraction that may influence the ability of the fraction to scavenge the DPPH radical compound was carried out in this

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Table 2. Preliminary phytochemical analysis of the three fractions of *Citrus hystrix* crude extract

| Fraction | Components | | | | | | |
|---------------|------------|----------|---------|--------|---------------|-----------|------------|
| | Alkaloids | Saponins | Tannins | Sugars | Carbohydrates | Phenolics | Flavonoids |
| Hexane | + | - | - | - | - | + | + |
| Ethyl acetate | + | + | + | + | + | + | + |
| Water | + | + | + | + | + | + | + |

(+) or (-) sign indicates the presence or absence of the components in the tested fraction

study. Table 2 shows the preliminary phytochemical screening of the three samples (hexane fraction, ethyl acetate fraction and water residue) derived from *Citrus hystrix* peel extract. As seen, the samples contained some potent phytochemical constituents with different extents. Alkaloids, phenolics, and flavonoids were detected in the fractions of hexane, ethyl acetate, and water residue. Other phytochemicals were distributed specifically. For example, saponins, tannins, sugars, and carbohydrates were not identified in the fraction of hexane. However, those compounds were detected in other fractions of ethyl acetate and water residue. The high capability of phenolics and flavonoids on antioxidant activity [47] been well demonstrated in earlier reports (Choi *et al.*, 2007; Patel *et al.*, 2011; Zhu *et al.*, 2011). In addition, investigation on saponins resulted its ability to reduce the risk of atherosclerosis (Rodrigues *et al.*, 2005). Tan [41] s (Beninger and Hosfield, 2003; Ravichandiran *et al.*, 2012), polysaccharides (Zhao *et al.*, 2012) and flavones (Sufian *et al.*, 2013) were also reported to contribute to the antioxidant activity. Moreover, synergetic effects of phenolics and other substances present in the extract may also contribute to the antioxidant activity observed in this study. Without quantification test performed, however, the higher antioxidant activity exhibited by ethyl acetate cannot be clearly detailed. The least variety of phytochemicals detected in the fraction of hexane among the three fractions (Table 2) could be the reason why this fraction exhibited the lowest inhibition (Figure 3). The results of phytochemical analysis in the fractions of ethyl acetate and water residue suggest that bioactive compounds in *Citrus hystrix* peel tend to be polar compounds. Performing the same DPPH radical scavenging method as the fractions to ascorbic acid, [7] was found the IC_{50} value of the ascorbic acid was 0.038 mg/mL. The comparable IC_{50} values of ascorbic acid and the fraction of ethyl acetate (Figure 3) suggest the fraction possessed tremendous antioxidant property because ascorbic acid has been well known for its activity to neutralize free radicals, promising further

application as functional foods for health purposes.

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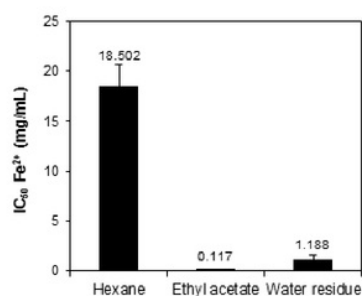
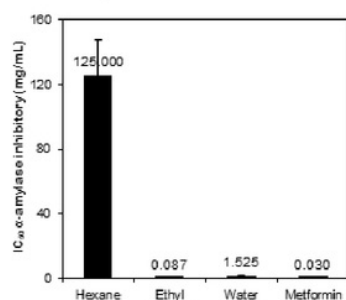
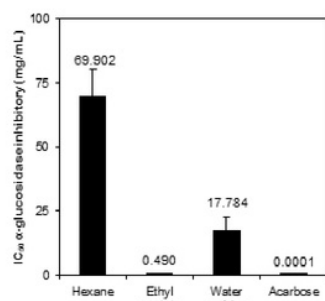
Metal chelating activity

Chelation of redox active metals such as iron, copper, cobalt and other metals prevents the production of reactive oxygen species in that inhibit the oxidative damage radicals in biological systems. The assay used to determine the chelating activity of Fe^{2+} was based on the interaction of the Fe^{2+} with ferrozine to form a magenta colored complex. The presence of other chelating agents such as phenolics compounds will disrupt the complex format [34] and thus, reduce the color intensity. Measurement the rate of color reduction therefore allows the estimation of the sample chelating activity. The ability of the fractions of *Citrus hystrix* peel to chelate Fe^{2+} , presented as IC_{50} , are shown in Figure 4. As seen, fraction of ethyl acetate exhibited the lowest IC_{50} value of 0.117 ± 0.014 , whereas fraction of hexane and water residue have IC_{50} values of 18.502 ± 2.154 and 1.188 ± 0.474 mg/mL, respectively. The results indicate that fraction of ethyl acetate chelated more iron than the other two.

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α -amylase inhibitory activity

Diabetes is characterized by high concentration of blood sugar and thus, slowing the digestion and breakdown of starch to simple sugars will reduce blood glucose level as well as the improvement of life quality of people with diabetes. The potential inhibitions of samples (hexane [19] fraction, ethyl acetate fraction and water residue) on the α -amylase inhibitory activity are shown in Figure 5. Similar to the antioxidant activity (Figure [37], the antidiabetes activity was represented as IC_{50} , i.e. the concentration of the fractions required to inhibit the starch conversion by 50%. The results [2] displayed in Figure 5 show the IC_{50} values of the hexane fraction, ethyl acetate fraction and water residue were observed 125.00, 0.09, and 1.53 [13] mL, respectively. Thus, based on the IC_{50} values, α -amylase inhibiting activities of the fractions were in the following order: ethyl acetate > water residue > hexane. Metformin hydrochloride

Figure 4. IC₅₀ values to the inhibition of Fe²⁺.Figure 5. IC₅₀ values of starch digestion inhibition by α -amylase compared to metformin hydrochloride as the reference drug.Figure 6. IC₅₀ values of starch digestion inhibition by α -glucosidase compared to acarbose as the reference drug.

is commercially available to treat type II diabetes and the IC₅₀ value of metformin hydrochloride was found 0.03 mg/mL. Lim and Loh have investigated the ability of *Citrus hystrix* peel extract derived from methanolic extract against α -amylase and found that the extract can retard the enzyme activity by 44-47% (Lim and Loh, 2016). The present work, however, employed ethanol 41% to extract the antioxidant and/or antidiabetic compounds followed by fractionation step and the results are presented as IC₅₀. Thus, these both works cannot be compared directly.

α -glucosidase inhibitory activity assay

The ability of the three samples derived from

the *Citrus hystrix* ethanolic crude extract (hexane fraction, ethyl acetate fraction and water residue) on the α -glucosidase inhibitory activity are shown in Figure 6. The results show the IC₅₀ values of the three samples were found approximately 69, 0.5 and 18 mg/mL, respectively. On the other hand, acarbose, one of commercial drugs for diabetic patient, exhibited very small IC₅₀ value of 0.0001 mg/mL. Based on the results, the fraction of ethyl acetate has been considered as the best sample against α -glucosidase. Other work on *Citrus hystrix* peel extract was reported in literature by (Lim and Loh, 2016). They compared the ability of free and bound phenolic extracts derived from methanolic extract of *Citrus hystrix* peel to retard the α -glucosidase activity and found the extract inhibited the enzyme activity by 27% (Lim and Loh, 2016).

Similar to the antioxidant activity, the most active fraction to inhibit the conversion of starch to sugars was demonstrated by the fraction of ethyl acetate. Saponins and alkaloids, detected in our study (Table 2), has been claimed to prevent diabetes by blocking the breakdown of starch to reduce postprandial glucose level (Chen *et al.*, 2008; Hamden *et al.*, 2010; Sharma *et al.*, 2010; Babu *et al.*, 2013). In addition, previous work (Tadera *et al.*, 2006) has also claimed the phenolic compounds as the effective inhibitor of α -amylase. Any of the secondary plant metabolites (Table 2), individually or synergistically with others could be responsible for the inhibition of antidiabetic activity of the fractions observed in this study (Figures 5 and 6).

The finding suggests the inhibition activity against α -amylase could be part of the possible mechanisms of *Citrus hystrix* peel in therapeutic or dietary management of diabetes by retardation of the breakdown of starch or polysaccharides to produce oligosaccharides and disaccharides which are then hydrolyzed by α -glycosidase to monosaccharide to be absorbed through small intestines into the hepatic portal vein and increase postprandial glucose level (El-Kaissi and Sherbeeni, 2011). Comparing the antidiabetic activity with the reference drug metformin hydrochloride, it was found that all fractions exhibited lower activity than the reference one. The antidiabetic activity of the fraction of ethyl acetate was observed lower by a factor of one third compared to metformin hydrochloride. The α -amylase inhibitory potential of *Citrus hystrix* peel, such as metformin hydrochloride, would decrease the absorption of sugars and inhibit the increase of blood glucose. Thus, fractions derived from the ethanolic extract of *Citrus hystrix* peel, particularly ethyl acetate and water residue, potent to be further

developed for diabetes mellitus therapy application.

The inhibitory potential of the fractions against the target enzyme further support the traditional use of plant in medicine and thus, further structural elucidation and characterization are essential to be carried out to identify bioactive compounds responsible for activities observed in this study. In addition, in vivo antioxidant activity on animal models is also required to be investigated to evaluate the efficacy of phytochemicals fractionated on the impaired glucose tolerance, insulin resistance, and other biological parameters related to people with diabetes.

Conclusion

Different classes of *Citrus hystrix* peel extract are capable of providing different antioxidant and in vitro antidiabetic activity with regard to medium type to certain extent. The fraction of ethyl acetate exhibited the highest antioxidant and antidiabetes activities. The mechanism behind this may be due to the presence of more phytochemicals in the fraction of ethyl acetate. The comparable antioxidant activity of the fraction of ethyl acetate to the standard of ascorbic acid suggests the fractions may have beneficial implication for human health to alleviate oxidative stress. The findings demonstrated that ethyl acetate was the most effective solvent to extract phytochemicals from ethanolic crude extract of *Citrus hystrix* peel. Further studies are required to identify bioactive constituents to have insight on the compounds responsible for activities observed in this study, molecular mechanisms involved in antioxidant activity as well as in vivo studies to determine their efficacy prior to clinical trials.

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