International Food Research Journal 23(1): 211-219 (2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

In vitro antioxidant capacities and antidiabetic properties of phenolic extracts from selected citrus peels

[1]Lim, S.M. and [1,2*]Loh, S.P.

[1]*Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Research Centre of Excellence, Nutrition and Non-communicable Diseases, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

Article history

Received: 10 September 2014
Received in revised form: 16 June 2015
Accepted: 23 June 2015

Keywords

Free phenolic extract
Bound phenolic extract
α-amylase
α-glucosidase
Citrus peels

Abstract

This study aims to determine the antioxidant capacities (AC) and antidiabetic properties of phenolic extracts (free and bound) from white Tambun pomelo peels, kaffir lime peels, lime peels and calamansi peels. AC, total phenolic content (TPC) and antidiabetic properties of selected citrus peels extracts were determined spectrophotometrically using 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging, ferric-reducing antioxidant power (FRAP), Folin-Ciocalteu (FC) and α-amylase and α-glucosidase inhibition assay, respectively. This study found that the methanolic extract of kaffir lime showed the best AC with the lowest IC_{50} value of DPPH radical (7.51 ± 0.50 mg/ml) and highest FRAP value [369.48 ± 20.15 mM Fe (II) E/g DW]. TPC of free phenolic extracts of all citrus peels were significantly ($p< 0.05$) higher compared to the bound phenolic extracts with extract of calamansi showed the highest TPC. Free- and bound phenolic extract of calamansi also had the highest α-amylase inhibition activity (61.79 ± 4.13%; 45.30 ± 5.35%) respectively. The highest inhibitory effect in α-glucosidase inhibition assay of free- and bound phenolic extracts were white Tambun pomelo (41.06 ± 10.94%) and calamansi (43.99 ± 22.03%) respectively. Hence, the citrus peels could be furthered study for their potential in management and/or prevention of diabetes.

Introduction

Diabetes is a condition where the pancreas produce insufficient amount of insulin or when the body unable efficiently use the insulin or both (WHO, 2012). In 2010, WHO had estimated that about 285 million people worldwide were living with diabetes (Roglic and Unwin, 2010). According to ADA (2012), type 2 diabetes mellitus (T2DM) is the most frequent diagnosed cases of diabetes among adults. Roglic and Unwin (2010) had predicted that the prevalence of diabetes, one of the risk factor for premature dead, will reach an estimation of 438 million by 2030 and 80% of the cases will be coming from developing countries. Statistics also had revealed that the prevalence of diabetes is getting younger and younger (CDC, 2007, 2011).

The priority in the management of diabetes is to decrease the postprandial hyperglycaemia (Jo *et al*., 2011; Wongsa *et al.*, 2012). α-Amylase and α-glucosidase are the two enzymes that responsible in hydrolysis reactions of complex carbohydrate to simple sugar that are more readily absorbed by the villi of the small intestine (Kwon *et al*., 2006). Nevertheless, α-glucosidase is the earliest metabolic abnormality that occurs in T2DM and renowned as a therapeutic target for glycaemic index control (Yao *et al*., 2010). Synthetic oral antidiabetic drugs that designed for blood glucose control do come along with adverse effects such as abdominal distension, bloating, flatulence, abdominal pain and possibly diarrhoea (Suzuki *et al*., 2009; Holt and Kumar, 2010; Wu *et al*., 2012). People with diabetes need to take these drugs for possibly their entire live may cause them to suffer from the associated side effects (Alberti *et al*., 2004). Therefore, isolated of natural α-amylase and α-glucosidase inhibitors may provide an alternative as a hypoglycaemic agents with least side effects (Kwon *et al*., 2006).

Worldwide production of citrus is over 80 million tons per year and one-third of them were being processed (Marín *et al*., 2007). Meantime, citrus wastes can be produced up to an estimation of more than 15 million tons worldwide (Marín *et al*., 2007). Peels (flavedo and albedo), a residue after juice and essential oil extraction, represent about 50 to 65% of total weight of the fruits and remain underutilized (Abd El-aal and Halaweish, 2010). Citrus peels are

*Corresponding author.
Email: *sploh@upm.edu.my*
Tel:+603-8947-2432; Fax: +603-8942-6769

rich in numerous active compounds like phenolic acids and antioxidant properties (Manthey and Grohmann, 2001; Naczk and Shahidi, 2006). Research done by Yusof *et al*. (1990) showed that the flavonoids content was richer in citrus peels instead of seeds and it's are long used in folk medicine (Ma *et al*., 2008). Thus, it could potentially offer a great source of natural α-amylase and α-glucosidase inhibitors.

The cultivation of citrus plants originated at least 4000 years ago in Asian continent and the Malaysian archipelago (Dugo and Di Giacomo, 2002). *Citrus maxima* was probably the first ancestor which originated in Malaysian archipelago (Dugo and Di Giacomo, 2002). In Malaysia, citrus fruits have been traditionally used for various purposes especially in traditional medicine, cuisine and religious uses (Herbal Medicine Research Center, 2002). Several studies done on orange peels (Oboh and Ademosun, 2011), grape fruit peels (Oboh and Ademosun, 2011a) and shaddock peels (Oboh and Ademosun, 2011b) showed that the phenolic extracts had mild α-amylase inhibition activity but stronger inhibition activity against α-glucosidase. However, there is still lacking of scientific information and researches about the inhibition activity on α-amylase and α-glucosidase on inedible citrus peels such as white Tambun pomelo, kaffir lime, lime and calamansi that have long time use in Malaysian culture.

Therefore, this study aims to determine the antioxidant capacities (AC) of the peels of white Tambun pomelo, kaffir lime, lime and calamansi and to determine the antidiabetic properties of its phenolic extracts (free and bound) against key enzymes linked to T2DM (α-amylase and α-glucosidase).

Materials and Methods

Samples collection and preparation

Tambun pomelo (white pulp) was purchased at Tambun, Ipoh, Perak, Malaysia while kaffir lime, lime and calamansi were purchased at Pasar Borong Selangor, Selangor, Malaysia with only firm texture, no obvious physical or microbial damage were chosen. The peels were separated manually with a knife and cut into small pieces before freeze-dried in freeze dryer (Virtis, New York, USA). The freeze dried peels were grounded, sieved and kept at -20°C before used for extraction.

Methanolic extraction

Citrus peels were extracted as described by Siahpoosh and Javedani (2012). Citrus peels powder (10 g) was extracted with 100 ml of methanol (1:10, w/v) for 24 hours in a shaker (SHO-2D Wise shaker, Daihan Sientific Co., Ltd., Korea) with 150 rpm at room temperature. The mixture was then centrifuged (Rotofix 32A, Hettich Zentrifugen, Germany) at 3500 rpm for 20 min and the rest was re-extracted under the same conditions. The combined filtrates were filtered through Whatman No.1 filter paper and evaporated with a rotary evaporator (Büchi Rotavapor R-200, Büchi Labortechnik AG, Switzerland) below 50°C. The obtained extracts were used for determination of AC.

Extraction of free soluble phenolic

Extraction of free and bound phenolic of citrus peels were carried as reported by Chu *et al*. (2002) with modification in term of volume where 1:10 w/v were used instead of 1:2 w/v. Citrus peels powder (10 g) was extracted with 100 ml of 80% acetone with a shaker at a constant speed of 150 rpm for 10 min. The extraction was repeated for another two times under the same conditions. The mixture was then filtered with filter paper (Whatman no. 1) on a Büchner funnel under vacuum. The filtrate was then evaporated using a rotary evaporator at 45°C. The extracts were stored at -20°C until analysis, while the residues kept for the extraction of bound phenolic.

Extraction of bound phenolic

The dried residues from free soluble extraction were hydrolysed with about 20 ml of 4 M NaOH solution and flushed with nitrogen gas at room temperature for 1 hour with shaking. Then, the pH of the mixture was adjusted to pH 2 with 6 M HCl. The mixture was extracted three times with ethyl acetate (1:4 v/v) by using a shaker at a constant speed of 150 rpm for 10 min. The ethyl acetate fractions were then filtered with filter paper (Whatman no. 1) on a Büchner funnel under vacuum before evaporated at 45°C by using rotary evaporator and stored at -20°C until analysis. All the yields of extractions were calculated as Eq. 1.

$$\%\ \text{yield of extraction} = \frac{DWe}{DWs} \times 100 \quad (1)$$

where DWe is the dry weight of sample extract after evaporation of solvent and DWs is the dry weight of the sample powder.

DPPH free radical-scavenging assay

DPPH free radical-scavenging assay was performed as described by Brand-Williams *et al.* (1995). An aliquot of 0.1 ml of various concentrations of extracts was added to 2.9 ml of 0.1 mM DPPH solution. The mixture was then shaken vigorously

using vortex (REAX top, Heidolph, Germany) and incubated at room temperature for 30 minutes under dark conditions. The absorbance was measured at 517 nm by using spectrophotometer (UV-1800, Shimadzu Co., Japan). The control contained all reagents without the sample, whereas methanol was used as a blank. The DPPH radical scavenging activity was calculated using the Eq. 2.

$$\text{DPPH free-radical scavenging activity (\%)} = \frac{(A_{517}^{Control} - A_{517}^{Extract})}{A_{517}^{Control}} x\,100 \quad (2)$$

where $A_{517}^{Control}$ is the absorbance of DPPH solution without extract and $A_{517}^{Extract}$ is the absorbance of the tested extract. The scavenging ability was expressed as IC_{50}, represented the effective concentration required to scavenge 50% of DPPH free radical. A lower IC_{50} value means higher DPPH radical-scavenging activity. Ascorbic acid (0.05-1.00 mg/ml) was used as reference control.

FRAP assay

FRAP assay was performed as described by Benzie and Strain (1996). The FRAP reagent contained 10 mM TPTZ solution in 40 mM HCl plus 20 mM $FeCl_3 \cdot 6H_2O$ and 0.3 M acetate buffer at pH 3.6 in a ratio of 1:1:10. The FRAP reagent was prepared fresh daily and warmed to 37°C in a water bath (One 14, Memmert GmbH+ Co., KG., Germany) prior to use. Freshly prepared FRAP reagent (1.5 ml) mixed with 50 µl of extract and 150 µl of distilled water. The reaction mixtures was later incubated for 4 min at 37° C. Absorbance at 593 nm was read with reference to reagent blank containing methanol, which was also incubated for 30 min at 37°C. Aqueous solutions of known ferrous concentrations in the range of 0.25-2.5 mM ($FeSO_4 \cdot 7H_2O$) was used for prepared the calibration curve. FRAP values was expressed on a dry weight basis as mM of Fe (II) equivalent per g of dried weight sample [mM Fe (II) E/g DW].

TPC assay for free and bound phenolic

FC reagent was used for analysis of TPC according to Singleton *et al.* (1999), with modifications. Briefly, 0.5 ml of each extract (prepared in DMSO) and 2.5 ml of 1/10 aqueous dilutions of FC reagent (v/v) were mixed well. After 5 minutes, 2.0 ml of 7.5% Na_2CO_3 was added and incubated at 45°C for 45 minutes. The absorbance was then measured at 765 nm by using a spectrophotometer. The blank consisted of all reagents excluded the extract. A standard calibration of curve was prepared, using a standard solution of gallic acid (0.01-0.20 mg/ml). TPC was expressed as mg Gallic acid equivalents per 1 g of dried weight sample (mg GAE/g DW).

α-Amylase inhibition assay

The α-amylase inhibition was determined by an assay described from Worthington Biochemical Corp. (1978). A starch solution (0.5% w/v) was obtained by stirring 0.125 g of potato starch in 25 ml of 0.02 M sodium phosphate buffer pH 6.9, with NaCl 6.7 mM at 65°C for 15 min. The enzyme solution was prepared by mixing 0.0253 g of α-amylases in 100 ml of cold distilled water. The colorimetric reagent was prepared by mixing a sodium potassium tartrate solution (12.0 g of sodium potassium tartrate tetrahydrate in 8.0 ml of 2 M NaOH) and 96 mM DNSA solution. All the extracts were dissolved in DMSO to give final concentration of 200 µg/ml. A total of 500 µl of the extracts and 500 µl of the enzyme solution were incubated at 25°C for 10 min. After pre-incubation, 500 µl of 0.5% starch solution was added and incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of DNSA colour reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min and cooled in cold water bath for 10 min. The absorbance was measured at 540 nm with a spectrophotometer. The absorbance reading was compared to the control, which contained 500 µl of buffer solution, instead of the extract. The percentage of enzyme inhibition was calculated as Eq. 3.

$$\text{\% Inhibition} = \frac{(A_{540}^{Control} - A_{540}^{Extract})}{A_{540}^{Control}} x\,100 \quad (3)$$

where $A_{540}^{Control}$ is the absorbance without extract and $A_{540}^{Extract}$ is the absorbance with extract.

α-glucosidase inhibition assay

The inhibition of α-glucosidase was determined by using the method described by da Silva Pinto *et al.* (2008), with modifications in term of volume of extracts. Briefly, yeast α-glucosidase (1 unit/ml) was dissolved in 0.01 M phosphate buffer (pH 6.9). The substrate solution, 5 mM p-nitrophenyl-α-D-glucopyranoside, was prepared in the same buffer. All the sample extracts were dissolved in DMSO to give final concentration of 200 µg/ml. The extracts (20 µl) and 50 µl of enzyme solution were incubated in 96-well plates at 25°C for 5 min. After pre-incubation, 50 µl of substrate solution was added to each well at timed intervals. The mixture was then incubated at 25°C for 5 min. Before and after incubation, absorbance readings were recorded at 405 nm by microplate reader and compared to a control that had 50 µl of buffer solution instead of the extract. The results were expressed as percentage of α-glucosidase inhibition and calculated as Eq. 4.

$$\% \text{ Inhibition} = \frac{(\Delta A_{405}^{Control} - \Delta A_{405}^{Extract})}{\Delta A_{405}^{Control}} x\ 100 \quad (4)$$

where $\Delta A_{405}^{Control}$ is the absorbance without extract and $\Delta A_{405}^{Extract}$ the absorbance with extract.

Statistical analysis

Data analysed by using IBM SPSS software version 20.0. All the data were expressed as means ± standard deviation (SD) of six replicates. Significant differences at $p< 0.05$ among means were determined using one-way analysis of variance (one-way ANOVA) and followed by LSD (least significant difference).

Results and Discussion

Yields of extraction

Extraction is essential for the recovery of the phenolic compounds from the samples in order to carry out the assays. Different solvents have different chemical properties and the type of solvent may influence the extract yield of the sample that give impact to the result of the experiments (Yang *et al*., 2011). In this study, there are three different types of extraction were used namely methanolic extraction for phenolic compounds, 80% acetone extraction for free phenolic extract and ethyl acetate extraction for bound phenolic extract. The yield of extracts ranged from 13.13 ± 0.39 to 35.70 ± 0.85% for all three different type of extraction (Table 1). This findings were found in close agreement with the findings of Chatha *et al.* (2011) that reported 17.92 to 30.80% extract yield from grape fruit, lemon and mussambi peels.

For the extraction using methanol, the highest yield among the samples was white Tambun pomelo with significant differences ($p< 0.05$) observed among kaffir lime, lime and calamansi. In free phenolic extraction, kaffir lime showed the highest extract yield (19.28 ± 0.60%) while in bound phenolic extraction, calamansi had the highest extraction yield (35.70 ± 0.85%). Methanol was used in the extraction for antioxidant assay as it was shown to give good yields especially in phenolic compounds (Ma *et al*., 2008; Khan *et al*., 2010) that are polar in nature (Yang *et al*., 2011). Besides, extraction of the phenolic compounds can be influenced by many factors such as their chemical nature (simple to highly polymerized substances), the extraction method used, particle size of the sample, storage time and conditions, geographical and climate conditions, as well as presence of interfering substances (Naczk and Shahidi, 2004). All these factors might have contributed some effects on the result obtained in this study. However, in this study, the sample particle size was ensured to be as similar as possible by using the same sieve to sieve the powder of the samples before carrying out the extraction in order to reduce bias.

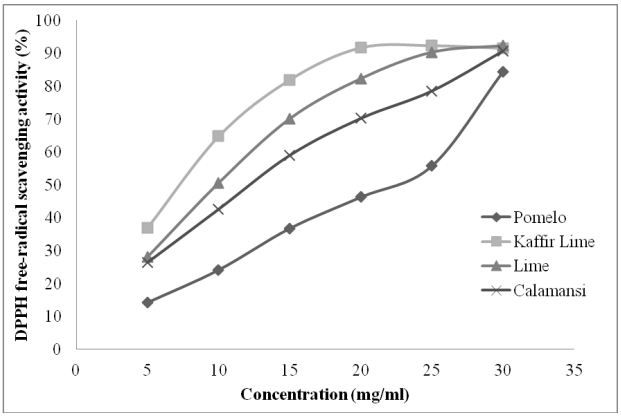

Figure 1. DPPH free-radical scavenging activity of the selected citrus peels from methanolic extraction

AC of methanolic extract

There are various types of methods can be used in measuring the AC of the sample. In this study, DPPH free radical scavenging activity and FRAP assay had been employed to evaluate the AC of methanolic extract of the four citrus peels.

In the DPPH free radical scavenging activity of the citrus peels, the scavenging activity increased with the increasing concentrations of test compounds. The scavenging activities for DPPH radical of the extracts ranged from 14.18 ± 1.85 to 92.43 ± 0.93% as showed in Figure 1 and their IC_{50} value is shown in Table 2 for comparison purpose. The methanolic extract of kaffir lime indicated the highest scavenging activity compared to other extracts with IC_{50} value of 7.51 ± 0.50 mg/ml. Ghasemi *et al.* (2009) reported that the IC_{50} value of DPPH radical scavenging activity of methanolic extract from 13 different types of citrus peels were found to fall between 0.6-2.9 mg/ml with the lowest IC_{50} values observed in *Citrus reticulata* var. Ponkan (0.6 mg/ml). Oppositely, Chatha *et al*. (2011) found that the IC_{50} value of DPPH radical scavenging activity from grape fruit peel *(Citrus paradise)*, lemon peel *(Citrus limon)* and mausambi peel *(Citrus sinensis)* extracts varied significantly in the range of 19.53-41.88 mg/ml with lowest value from mausambi citrus peels extract (19.53 mg/ml).

FRAP assay was another method used to evaluate the AC of the citrus peels in this study. The antioxidant compounds that present in the citrus peels acted as a reducing agent by donating H^+ to ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and thus interrupt the radical chain reaction. The higher the FRAP value is, the higher is the AC. The FRAP value

Table 1. Phenolic extract yield from three different extraction solvents of selected citrus peels

Sample	Scientific name	Local name	Phenolic extract yield (%)		
			Methanol	Free 80% Acetone	Bound Ethyl acetate
White Tambun pomelo	*Citrus maxima*	*Limau bali*	24.34 ± 0.48[a]	13.72 ± 0.45[a]	32.43 ± 0.39[a]
Kaffir lime	*Citrus hystrix*	*Limau purut*	19.95 ± 0.92[b]	19.28 ± 0.60[b]	21.43 ± 0.75[b]
Lime	*Citrus aurantifolia*	*Limau nipis*	13.13 ± 0.39[c]	14.64 ± 0.94[a]	15.90 ± 0.42[c]
Calamansi	*Citrus microcarpa*	*Limau kasturi*	19.05 ± 0.21[b]	18.05 ± 0.92[b]	35.70 ± 0.85[d]

[a-d]Values with different letters in the same column are significantly different at $p < 0.05$

Table 2. AC of methanolic extracts of different citrus peels from DPPH free radical-scavenging method and FRAP method

Sample	IC_{50} value (mg/ml)	FRAP value (mM Fe (II) E/g DW)
White Tambun Pomelo	22.30 ± 3.29[a]	141.25 ± 27.44[a]
Kaffir lime	7.51 ± 0.50[b]	369.48 ± 20.15[b]
Lime	10.29 ± 1.90[b,c]	234.98 ± 28.79[c]
Calamansi	12.91 ± 3.31[c]	290.65 ± 58.47[c]
Ascorbic acid	0.10 ± 0.03[d]	NA*

*NA- Not applicable
[a-d]Values with different letters in the same column are significantly different at $p < 0.05$

of the citrus peels is showed in Table 2 by reference to a standard curve ($y=0.7193x-0.06$, $r^2=0.99$). The methanolic extract of kaffir lime was significantly the highest FRAP value [369.48 ± 20.15 mM Fe (II) E/g DW] among other three citrus peels while methanolic extract of white Tambun pomelo had the lowest FRAP value [141.25 ± 27.44 mM Fe (II) E/g DW]. The results of this study showed lower FARP value compared to a study done on 21 types of citrus peels varieties with FRAP value ranged from 1420 ± 0.1 to 8130 ± 0.33 mM Fe (II) E/g fresh weight (Ramful *et al*., 2010).

Both DPPH free radical and FRAP assay showed the similar trend in the AC of the citrus peels in this study. A significant negative correlation between IC_{50} of DPPH free radical and FRAP assay (r-value = -0.853, $p < 0.01$) was observed in this study. This relationship could be due to the same mechanism that DPPH free radical and FRAP assay reacted upon. The mechanism of both assay rely on the ability of the phenolic compounds to reduce the DPPH radical and ferric ion, respectively.

TPC of free and bound phenolic extracts

The TPC of free and bound phenolic extracts of the citrus peels is shown in Table 3. Generally, the free phenolic extracts are significantly ($p < 0.05$) higher compared to the bound phenolic extracts of the same samples. Free and bound phenolic extract of calamansi showed the highest TPC. The TPC of the selected citrus peels for free phenolic extracts were in the range 60.77 ± 0.52 to 98.90 ± 0.82 mg GAE/g of DW and bound phenolic extracts were in the range of 13.10 ± 0.59 to 18.88 ± 0.35 mg GAE/g of DW. These results are comparable to studies done on shaddock peels and grape fruit peels which varied between 6.5 ± 0.8 and 13.5 ± 0.2 mg GAE/g of DW respectively for free phenolic extracts and 3.4 ± 0.3 and 0.7 ± 0.1 mg GAE/g of DW respectively for bound phenolic extracts (Oboh and Ademosun, 2011a, 2011b). This indicated that the TPC of the citrus peels in this study were much higher probably due to the freeze-drying method had been employed than oven dry method.

The higher TPC value of free phenolic extracts compare to bound phenolic extracts indicated that the main form of phenolic compounds in citrus peels existed in free form rather than in bound form. The bound phenolic compounds were associated with cell wall of citrus peels also influence the ability of the solvent to extract all the bound phenolic compounds. A study done on five types of different vegetables (carrot, tomato, taro, beetroot and eggplant) also showed the TPC of free phenolic extracts were significantly higher than those of the bound phenolic extracts (Hung and Duy, 2012). This trend also agrees with the study showed that the TPC of free phenolic extract of clove buds was higher than bound phenolic extract (Adefegha and Oboh, 2012).

α-Amylase inhibitory activity

The ability of phenolic extracts (free and bound) from four citrus peels in inhibiting the α-amylase is presented in Table 3. The α-amylase inhibition activity in this study ranged from 38.17 ± 9.71 to 61.79 ± 4.13% for free phenolic extracts and 38.04 ± 2.01 to 45.30 ± 5.35% for bound phenolic extracts, respectively. Generally, the free soluble phenolic extracts of citrus peels had slightly higher inhibitory α-amylase activity than the bound phenolic extracts of the same sample. The free and bound phenolic

Table 3. Total phenolic content and inhibition activities (α-amylase and α-glucosidase) of free and bound phenolic extracts of selected citrus peels.

Sample		Total phenolic content (mg GAE/g DW)	α-Amylase inhibition activity (%)	α-Glucosidase inhibition activity (%)
White Tambun Pomelo	Free	89.80 ± 5.30^{a}	38.17 ± 9.71^{a}	$41.06 \pm 10.94^{a,b}$
	Bound	15.92 ± 0.82^{b}	38.04 ± 2.01^{a}	$30.26 \pm 11.82^{b,c,d}$
Kaffir lime	Free	60.77 ± 0.52^{a}	$47.16 \pm 11.32^{a,b}$	$25.47 \pm 6.86^{c,d,e}$
	Bound	13.45 ± 0.13^{c}	43.80 ± 8.94^{a}	$26.98 \pm 6.54^{c,d,e}$
Lime	Free	98.90 ± 0.82^{d}	$53.95 \pm 14.34^{b,c}$	15.63 ± 3.93^{e}
	Bound	$18.88 \pm 0.35^{b,c}$	41.37 ± 5.45^{a}	$36.97 \pm 8.60^{a,c}$
Calamansi	Free	87.82 ± 2.53^{e}	61.79 ± 4.13^{c}	$32.66 \pm 9.17^{a,d}$
	Bound	13.10 ± 0.59^{f}	$45.30 \pm 5.35^{a,b}$	43.99 ± 22.03^{a}

[a-f]Values with different letters in the same column are significantly different at $p< 0.05$

Table 4. Correlation between AC with both α-amylase and α-glucosidase inhibitory activities

	α-Amylase inhibitory activities				α-Glucosidase inhibitory activities			
	Free phenolic extract		Bound phenolic extract		Free phenolic extract		Bound phenolic extract	
	r	p	r	p	r	p	r	p
IC_{50} of DPPH	-0.289	0.171	-0.264	0.213	0.457*	0.025	-0.128	0.550
FRAP value	0.245	0.249	0.362	0.083	-0.180	0.401	0.180	0.401
Free phenolic TPC	0.070	.745	0.278	0.189	0.533*	0.007	0.033	0.880
Bound phenolic TPC	0.502*	0.013	0.368	0.076	0.052	0.810	0.220	0.302

*Correlation was significant at the 0.05 level (2-tailed)

extracts of the calamansi showed the highest inhibition activity which was 61.79 ± 4.13% and 45.30 ± 5.35%, respectively. On the other hand, the free and bound phenolic extracts of white Tambun pomelo showed the lowest inhibition activity which was 38.17 ± 9.71% and 38.04 ± 2.01%, respectively. The results of the α-amylase inhibition activity of citrus peels extracts follow a similar trend with study done by Adefegha and Oboh (2012) which reported on the inhibitory activities of free soluble phenolic extract (IC_{50} = 497.27 µg/ml) had significantly higher ($p< 0.05$) inhibition activity than the bound phenolic extract (IC_{50} = 553.77 µg/ml) of clove buds.

In this study, there was no significant correlation found between the AC (IC_{50} of DPPH radical and FRAP assay) with the α-amylase inhibition activity of the citrus peels (Table 4). However, McCue *et al*. (2004) suggested that the antioxidant activity of phenolic from clonal oregano extracts may affect the five sets of disulphide bridges located on the outer surface of α-amylase. Thus, the reduction of these cysteine residues may cause inhibition by modifying in the structure of the enzyme (McCue *et al*., 2004). Thus, the α-amylase inhibition activity of the citrus peels perhaps not due to AC but also involve other mechanisms that worth to be disclosed.

In general, the inhibition activity of α-amylase of the citrus peels extracts at concentration of 200 µg/ml ranged from 38.0-61.8%. This indicated that the inhibition of this enzyme was only moderate. Kumar *et al.* (2011) proposed that the unusual and excessive bacterial fermentation of undigested starches in the colon part may be due to strong inhibition of pancreatic α-amylase. Therefore, mild α-amylase inhibition activity is useful and more preferred than strong inhibition activity.

α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity of the extracts is summarized in Table 3. All the phenolic extracts (free and bound) showed inhibitory effects towards α-glucosidase. The free phenolic extract of white Tambun pomelo had the highest inhibitory activity (41.06 ± 10.94%) while lime extract had the lowest inhibition (15.63 ± 3.93%). In contrast, the bound phenolic extract of calamansi indicated the highest inhibition (43.99 ± 22.03%) while kaffir lime extract had the lowest inhibitory activity (26.98 ± 6.54%). The current findings were found to have lower inhibition activities compared with the findings done on shaddock peel that reported 74.45 ± 1.3 to 89.05 ± 4.2% for free phenolic extract and 45.99 ± 2.3 to 95.99 ± 2.1% for bound phenolic extract (Oboh and Ademosun, 2011b). One of the reasons probably due to some of the bound phenolic compounds has been extracted out in free phenolic extraction assay. Thus, more specified method in extracting the bound phenolic compounds should be studied.

The bound phenolic extracts of all the citrus peels had slightly higher inhibitory effect in α-glucosidase than the free phenolic extracts in the same samples except the pomelo extract. However, significant

differences ($p < 0.05$) between free phenolic extract and bound phenolic extract was only observed in lime extract. The investigation done by Adefegha and Oboh (2012) on clove buds reported that the α-glucosidase inhibition activity of bound phenolic extract (IC_{50} = 127.31 µg/ml) had higher than free soluble phenolic extract (IC_{50} =145.07 µg/ml). The α-glucosidase inhibition activity of bound phenolic extract is expected to be higher than free phenolic extract. This is because the bound phenolic is mostly existed in β-glycosides form that prefer aqueous phase than the free phenolic which is in the form of aglycones, a non-sugar group (Ademiluyi and Oboh, 2013). Thus, direct enzyme-inhibitor interaction is predicted to be higher in bound phenolic extracts in α-glucosidase assay.

Besides, the mechanisms of inhibition for α-glucosidase may different from α-amylase with regard to AC. McCue *et al.* (2005) reported that there are none disulphide bridges especially not on the surface of the molecule (possible site for interaction with antioxidants) on the structure of Baker's yeast α-glucosidase. This indicated that the inhibition of α-glucosidase activity of phenolic extracts might not involve the interaction of the AC with the disulphide bridges. Therefore, the inhibition caused by the phenolic extracts could be attributed through other mechanism. Conversely, there was a significant ($p < 0.05$) but moderate correlation between IC_{50} of DPPH radical with the free phenolic extract of α-glucosidase inhibition activity (Table 4) of the citrus peels. This deserves further study on the potential mechanisms of the α-glucosidase inhibition by phenolic extracts from citrus peels.

In addition, these results also suggested that phenolics were not the only component responsible for the α-glucosidase inhibitory activities. For instance, the bound phenolic extract of the citrus peels had much lower TPC compared to the free phenolic extract of same sample. However, the inhibitory activity of α-glucosidase of bound phenolic extract of the citrus peels except pomelo peel had slightly higher percentage of inhibition. This higher inhibitory activities could be credited to the presence of some non-phenolic phytochemicals that probably acted as enzyme inhibitors, exhibiting an additive or synergistic effect with the phenolics present in the sample (Oboh *et al*., 2010).

Conclusions

In conclusion, methanolic extract of kaffir lime exhibited the highest AC among the four citrus peels extract. Both the free and bound phenolic extract of calamansi had the highest inhibition activity against pancreatic α-amylase while free phenolic extract of pomelo and bound phenolic extract of calamansi indicated the highest inhibition activity against α-glucosidase. This revealed that citrus peels extracts especially calamansi extracts maybe another great nutraceutical products that could be important in management of diabetes.

Acknowledgement

The authors acknowledge the assistance of laboratory staff from the Laboratory of Nutrition, Department of Nutrition and Health Sciences, Universiti Putra Malaysia throughout the research project.

References

Abd El-aal, H. A. and Halaweish, F. T. 2010. Food preservative activity of phenolic compounds in orange peel extracts (*Citrus sinensis* L.). Lucrări stiinţifice Seria Zootehnie 53(15): 233-240.

ADA. 2012. Diabetes Basics-Type 2. Downloaded from *http://www.diabetes.org/diabetes-basics/type-2/* on 30/10/2012.

Adefegha, S. A. and Oboh, G. 2012. In vitro inhibition activity of polyphenol-rich extracts from *Syzygium aromaticum* (L.) Merr. & Perry (Clove) buds against carbohydrate hydrolyzing enzymes linked to type 2 diabetes and Fe^{2+}-induced lipid peroxidation in rat pancreas. Asian Pacific Journal of Tropical Biomedicine 2(10): 774-781.

Ademiluyi, A. O. and Oboh, G. 2013. Soybean phenolic-rich extracts inhibit key-enzymes linked to type 2 diabetes (α-amylase and α-glucosidase) and hypertension (angiotensin I converting enzyme) in vitro. Experimental and Toxicologic Pathology 65(3): 305-309.

Alberti, G., Zimmet, P., Shaw, J., Bloomgarden, Z., Kaufman, F. and Silink, M. 2004. Type 2 Diabetes in the Young: The Evolving Epidemic. The International Diabetes Federation Consensus Workshop. Diabetes Care 27(7): 1798-1811.

Benzie, I. F. F. and Strain, J. J. 1996. The ferric reducing agent of plasma (FRAP) as a measure of "antioxidant power" : The FRAP assay. Analytical Biochemistry 239(1): 70-76.

Brand-Williams, W., Cuvelier, M. E. and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. Lebensmittel-Wissenschaft & Technologie, 28(1): 25-30.

CDC. 2007. National diabetes fact sheet: general information and national estimates on diabetes in the United States, 2007. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention.

CDC. 2011. National diabetes fact sheet: national estimates

and general information on diabetes and prediabetes in the United States, 2011. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention.

Chatha, S. A. S., Hussain, A. I., Asi, M. R., Majeed, M. and Iqbal, H. M. N. 2011. Evaluation of antioxidant potential of citrus peel extracts. Journal of the Chemical Society of Pakistan 33(6): 863-868.

Chu, Y. F., Sun, J., Wu, X. and Liu, R. H. 2002. Antioxidant and antiproliferative activity of common vegetables. Journal of Agricultural and Food Chemistry 50: 6910-6916.

da Silva Pinto, M., Kwon, Y. I., Apostolidis, E., Lajolo, F. M., Genovese, M. I. and Shetty, K. 2008. Functionality of bioactive compounds in Brazilian strawberry (*Fragaria x ananassa* Duch.) cultivars: evaluation of hyperglycemia and hypertension potential using in vitro models. Journal of Agricultural and Food Chemistry 56(12): 4386-4392.

Dugo, G. and Di Giacomo, A. 2002. Citrus: The Genus Citrus. Landon: Taylor & Francis.

Ghasemi, K., Ghasemi, Y. and Ebrahimzadeh, M. A. 2009. Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. Pakistan Journal of Pharmaceutical Sciences 22(3): 277-281.

Herbal Medicine Research Center. 2002. Compendium of Medicinal Plants Used in Malaysia. Institute for Medical Research Kuala Lumpur: Aktif Bestari Sdn. Bhd.

Holt, T., & Kumar, S. 2010. ABC of Diabetes (6 ed.). United Kingdom, UK: Wiley-Blackwell.

Hung, P. V. and Duy, T. L. (2012). Effects of drying methods on bioactive compounds of vegetables and correlation between bioactive compounds and their antioxidants. International Food Research Journal 19(1): 327-332.

Jo, S. H., Ha, K. S., Moon, K. S., Lee, O. H., Jang, H. D. and Kwon, Y. I. 2011. *In vitro* and in *vivo* anti-hyperglycemic effects of omija *(Schizandra chinensis)* Fruit. International Journal of Molecular Sciences 12(2): 1359-1370.

Khan, M. K., Abert-Vian, M., Fabiano-Tixier, A.-S., Dangles, O. and Chemat, F. 2010. Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis* L.) peel. Food Chemistry 119(2): 851-858.

Kumar, S., Narwal, S., Kumar, V. and Prakash, O. 2011. α-Glucosidase inhibitors from plants: A natural approach to treat diabetes. Pharmacogn Review 5(9): 19-29.

Kwon, Y.-I., Vattem, D. A. and Shetty, K. 2006. Evaluation of clonal herbs of *Lamiaceae* species for management of diabetes and hypertension. Asia Pacific Journal of Clinical Nutrition 15(1): 107-118.

Ma, Y. Q., Ye, X. Q., Fang, Z. X., Chen, J. C., Xu, G. H. and Liu, D. H. 2008. Phenolic compounds and antioxidant activity of extracts from ultrasonic treatment of Satsuma mandarin (*Citrus unshiu* Marc.) peels. Journal of Agricultural and Food Chemistry 56(14): 5682-5690.

Manthey, J. A. and Grohmann, K. 2001. Phenols in citrus peel byproducts concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. Journal of Agricultural and Food Chemistry 49(7): 3268-3273.

Marín, F. R., Soler-Rivas, C., Benavente-García, O., Castillo, J. and Pérez-Alvarez, J. A. 2007. By-products from different citrus processes as a source of customized functional fibres. Food Chemistry 100(2): 736-741.

McCue, P., Kwon, Y.-I. and Shetty, K. 2005. Anti-diabetic and anti-hypertensive potential of sprouted and solid-state bioprocessed soybean. Asia Pacific Journal of Clinical Nutrition 14(2): 145-152.

McCue, P., Vattem, D. and Shetty, K. 2004. Inhibitory effect of clonal oregano extracts against porcine pancreatic amylase in vitro. Asia Pacific Journal of Clinical Nutrition 13(4): 401-408.

Naczk, M. and Shahidi, F. 2004. Extraction and analysis of phenolics in food. Journal of Chromatography A, 1054(1-2): 95-111.

Naczk, M. and Shahidi, F. 2006. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. Journal of Pharmaceutical and Biomedical Analysis 41(5): 1523-1542.

Oboh, G. and Ademosun, A. 2011. Phenolic-rich extracts from orange peels *(Citrus sinensis)* inhibit key enzymes linked to non-insulin dependent diabetes mellitus (NIDDM) and hypertension. La Rivista Italiana Delle Sostanze Grasse 88: 16-23.

Oboh, G. and Ademosun, A. O. 2011a. Phenolic extracts from grapefruit peels *(Citrus paradisi)* inhibit key enzymes linked with type 2 diabetes and hypertension Journal of Food Chemistry 35(6): 1703-1709.

Oboh, G. and Ademosun, A. O. 2011b. Shaddock peels *(Citrus maxima)* phenolic extracts inhibit α-amylase, α-glucosidase and angiotensin I-converting enzyme activities: A nutraceutical approach to diabetes management. Diabetes & Metabolic Syndrome: Clinical Research & Reviews 5(3): 148-152.

Oboh, G., Akinyemi, A. J., Ademiluyi, A. O. and Adefegha, S. A. 2010. Inhibitory effects of aqueous extract of two varieties of ginger on some key enzymes linked to type-2 diabetes in vitro. Journal of Food and Nutrition Research 49(1): 14-20.

Ramful, D., Bahorun, T., Bourdon, E., Tarnus, E. and Aruoma, O. I. 2010. Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: Potential prophylactic ingredients for functional foods application. Toxicology 278(1): 75-87.

Roglic, G. and Unwin, N. (2010). Mortality attributable to diabetes: estimates for the year 2010. Diabetes Research and Clinical Practice 87(1): 15-19.

Siahpoosh, S. and Javedani, F. 2012. Antioxidative capacity of Iranian Citrus deliciosa peels. Free Radicals and Antioxidants 2(2): 62-67.

Singleton, V. L., Orthofer, R. and Lamuela-Raventos, R. M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu

reagents. Method Enzymology 299: 152-178.

Suzuki, Y., Sano, M., Hayashida, K., Ohsawa, I., Ohta, S. and Fukuda, K. 2009. Are the effects of alpha-glucosidase inhibitors on cardiovascular events related to elevated levels of hydrogen gas in the gastrointestinal tract? FEBS Lett 583(13): 2157-2159.

WHO. 2012. Diabetes. Downloaded from *www.who.int/topics/diabetes_mellitus/en/* on 18/9/2012.

Wongsa, P., Chaiwarit, J. and Zamaludien, A. 2012. *In vitro* screening of phenolic compounds, potential inhibition against a-amylase and a-glucosidase of culinary herbs in Thailand. Food Chemistry 131(3): 964-971.

Worthington Biochemical Corp. (1978). Worthington enzyme and related biochemicals. Freehold, NJ: Worthington Biochemical Corp.

Wu, C. M., Shen, J. Y., He, P. P., Chen, Y., Li, L., Zhang, L., Li, Y., Fu, Y. J., Dai, R. Ji., Meng, W. W. and Deng, Y. L. 2012. The α-glucosidase inhibiting isoflavones isolated from Belamcanda chinensis leaf extract. Record of Natural Products 6(2): 110-120.

Yang, J., Kim, J. S., Jeong, H. J., Kang, H. H., Cho, J. C., Yeom, H. M. and Kim, M. J. 2011. Determination of antioxidant and α-glucosidase inhibitory activities and luteolin contents of *Chrysanthemum morifolium* Ramat extracts. African Journal of Biotechnology 10(82): 19197-19202.

Yao, Y., Sang, W., Zhou, M. and Ren, G. 2010. Antioxidant and α-glucosidase inhibitory activity of colored grains in China. Journal of Agricultural and Food Chemistry 58(2): 770-774.

Yusof, S., Mohd Ghazali, H. and Gan, S. K. 1990. Naringin content in local citrus fruits. Food Chemistry 37(2): 113-121.