In vitro Inhibitory Potential of Selected Malaysian Plants Against Key Enzymes Involved in Hyperglycemia and Hypertension

Loh SP & Hadira O

Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

ABSTRACT

Introduction: This study was conducted to determine the inhibitory potential of selected Malaysian plants against key enzymes related to type 2 diabetes and hypertension. **Methods:** The samples investigated were *pucuk ubi (Manihot esculenta), pucuk betik (Carica papaya), ulam raja (Cosmos caudatus), pegaga (Centella asiatica)* and *kacang botol (Psophocarpus tetragonolobus)*. The inhibitory potential of hexane and dichloromethane extracts against the enzymes were determined by using α-amylase, α-glucosidase and angiotensin I-converting enzyme (ACE) inhibition assay. **Results:** In α-amylase inhibition assay, the inhibitory potential was highest in *pucuk ubi* for both hexane (59.22%) and dichloromethane extract (54.15%). Hexane extract of *pucuk ubi* (95.01%) and dichloromethane extract of *kacang botol* (38.94%) showed the highest inhibitory potential against α-glucosidase,while in ACE inhibition assay, the inhibitory potential was highest in hexane extract of *pegaga* (48.45%) and dichloromethane extract of *pucuk betik* (59.77%). **Conclusion:** This study suggests a nutraceutical potential of some of these plants for hyperglycemia and hypertension prevention associated with type 2 diabetes.

Keywords: *Carica papaya, Centella asiatica, Cosmos caudatus, Manihot esculenta, Psophocarpus tetragonolobus, in vitro* inhibitory potential

INTRODUCTION

Diabetes is a chronic disease which occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. This leads to an increased concentration of glucose in the blood (hyperglycemia). Approximately 171 million individuals worldwide had diabetes in the year 2000 and it is estimated that this will increase to 366 million by 2030 (WHO, 2009). On the basis of current trends, epidemiologists predict that almost half of all diabetic individuals will be in the Asia/Oceania region (Zaini, 2000). In Malaysia, the prevalence of diabetes has increased in recent decades, from 0.65% in 1960 to 2.1% in 1982, 8.4% in 1996 and 11% in 2006 (Ministry of Health Malaysia, 2006).

One therapeutic approach for treating diabetes is to decrease the post-prandial hyperglycemia. In particular, α-amylase and α-glucosidase are considered as key enzymes that can control post-prandial

---

* Correspondence author: Loh Su Peng; Email: *sploh@medic.upm.edu.my*

hyperglycemia (Lee *et al.*, 2007). An effective strategy for type 2 diabetes management is the strong inhibition of intestinal α-glucosidases and mild inhibition of pancreatic α-amylase (Krentz & Bailey, 2005). Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and, consequently, blunting the post-prandial plasma glucose rise (Rhabasa-Lhoret & Chiasson, 2004).

One of the long-term complications of type 2 diabetes is high blood pressure, or hypertension and one of the most important intermediary factors for controlling hypertension is the action of the angiotensin-converting enzyme (ACE) (Hernadez-Ledesma, Martin-Alvarez & Pueyo, 2003). Angiotensin I-converting enzyme (ACE) is an important enzyme that is involved in maintaining vascular tension by two different reactions that it catalyses: conversion of the inactive angiotensin I into a powerful vaso-constrictor and promoter of sodium retention, angiotensin II, and inactivation of the vasodilator bradykinin, which is conducive to lowering blood pressure (Johnston & Franz, 1992). Inhibition of ACE is considered a useful therapeutic approach in the treatment of high blood pressure.

Plants have been used as part of traditional medicine for thousands of years. Some of these plants are consumed as salads in Malaysia. They are believed to possess a variety of medicinal values and are used to treat various health conditions such as skin infections, digestive problems, mild pain, cuts and ulcers, fever, coughs and are beneficial to the anti-aging process (Khairana *et al.*, 2004). Recently some of these plant products have been shown to have some anti-diabetic properties (Babish *et al.*, 2010). Thus, the aim of this study is to investigate different traditionally used plants for their associated phenolic profiles and their inhibitory potential against key enzymes related to type 2 diabetes and

hypertension using *in vitro* models. The five plants were *pucuk ubi, pucuk betik, ulam raja, pegaga* and *kacang botol.*

METHODS

Sample preparation and extraction method

All fresh samples of *pucuk ubi (Manihot esculenta), pucuk betik (Carica papaya), ulam raja (Cosmos caudatus), pegaga (Centella asiatica)* and *kacang botol (Psophocarpus tetragonolobus)* were purchased from a local market in Serdang, Selangor (Malaysia). The edible portions of the fresh plants were cleaned and washed under running tap water. The edible parts were cut/sliced into small pieces and freeze-dried. The dried samples were then ground into powder using a grinder.

Samples (1 g) were blended with either hexane or dichloromethane (20 ml each) and filtered using Whatman No. 1 paper. The filtrates were then evaporated to dryness with a rotary evaporator, under reduced pressure at 40°C. The crude extracts were weighed and stored at -80°C for further experiments and analysis. The hexane and dichloromethane extracts were selected and tested for their inhibitory effect because they were deemed to contain negligible amounts of sugar, which, if present (as would be the case in water or alcoholic extracts), would complicate the detection of maltose in the assay (Hasenah, Houghton & Amala, 2006).

Total phenolics assay

Total phenolics were determined by an assay modified from Shetty *et al.* (1995). One milliliter of plant extract and all reagents were modified to 0.5 ml in this assay. An amount of 0.5 ml of plant extract was transferred into a test tube and mixed with 0.5 ml of 95% ethanol and 5 ml of distilled water. To each sample 0.5 ml of 50% (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 minutes, 0.5 ml of 5% Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 minutes. The

absorbance was read at 725 nm. The absorbance values were then converted to total phenolics and expressed in microgram equivalents of gallic acid per milliliter of the sample. Standard curves were established using various concentrations of gallic acid in 95% ethanol.

HPLC analysis of phenolic profiles

Phenolic profiles were determined by a method modified by Marcia Da *et al.*(2008). Two milliliters of plant extracts instead of 5 μL from the original method were filtered through a 0.2 μm filter. A 5 μL volume of the sample was injected using HPLC equipped with a DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 minutes and to 100% over the next 7 miutes. It was then decreased to 0% for the next 3 minutes and maintained for the next 7 minutes (total run time 25 minutes). The analytical column used was an Agilent Zorbax SB-C18, 250 × 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at ambient temperature. During each run, the absorbance was recorded at 306 and 333 nm, and the chromatogram was integrated. Pure standards of quercetin and rutin in 100% methanol were used to calibrate the standard curve and retention times. The results are expressed as micrograms per gram of plant fresh dry weight.

α-Amylase inhibition assay

The α-amylase inhibition was determined by an assay modified from Kwon, Apostolidis & Shetty (2008). Five hundred microliters of plant extract and all reagents were modified to 40 μl in this assay. A total of 40 μl of plant extract and 40 μl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (Porcine pancreatic α-amylase) (0.5 mg/ml) were incubated at 25 °C for 10 min. After pre-incubation, 40 μl

of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at 5 s intervals. The reaction mixtures were then incubated at 25 °C for 10 minutes. The reaction was stopped with 100 μl of dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 minutes and cooled to room temperature. The reaction mixture was then diluted after adding 900 μl distilled water and the absorbance was measured at 540 nm.

$$\% \ Inhibition = \left(\left[\frac{A_{540}^{Control} - A_{540}^{Extract}}{A_{540}^{Control}} \right] \right) \times 100$$

α-Glucosidase inhibition assay

The assay was performed based on a method from Marcia Da *et al.* (2008). α-glucosidase (1 unit/mL) was assayed by using 50 μL plant extracts and 100 μL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution and was incubated in 96-well plates at 25°C for 10 min. After pre-incubation, 50 μL of 5 mM*p*-nitrophenyl- α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 minutes. 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 in place of the extract. The results are expressed as percent of α-glucosidase inhibition.

$$\% \ Inhibition = \left(\left[\frac{\Delta A_{405}^{Control} - \Delta A_{405}^{Extract}}{\Delta A_{405}^{Control}} \right] \right) \times 100$$

Angiotensin I-converting enzyme (ACE) inhibition assay

ACE inhibition was assayed based on a method modified by Kwon, Vattem & Shetty (2006). The substrates were hippuryl-histidyl-leucine (HHL) and angiotensin I-converting enzyme (ACE) from rabbit lung.
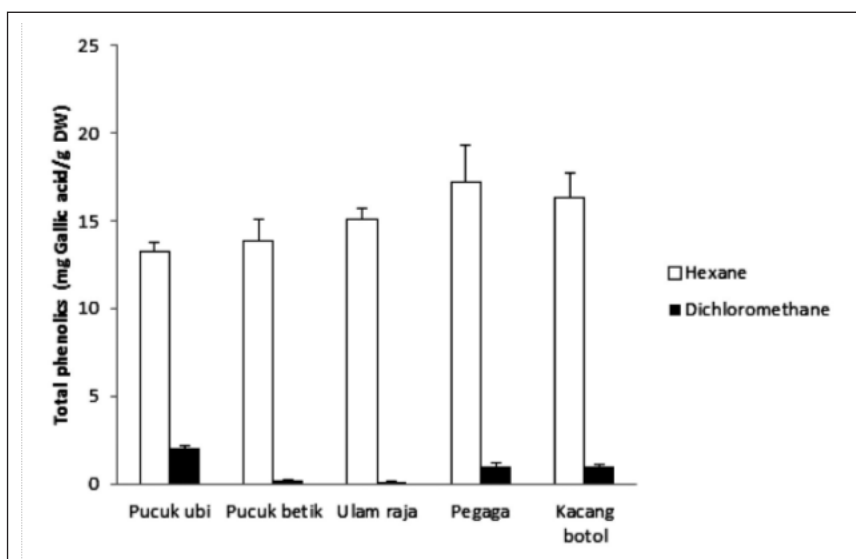
Figure 1. Total phenolic content of hexane and dichloromethane of six selected plants. Bars with different letters are significantly different ($p < 0.05$).

Fifty microliters of the extractwasincubated with 100 μl of 1.0 M NaCl-borate buffer (pH 8.3) containing 2.0 mU ACE solution at 37 °C for 10 minutes. After pre-incubation, 100 μl of a 5.0 mU substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 hour. The reaction was stopped with 150 μl of 0.5 N HCl. The hippuric acid formed was detected and quantified by the HPLC method. A volume of 5 μl of sample was injected into the HPLC equipped with DAD 1100 diode array detector. The solvents used for gradient were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 minutes and to 100% for the next 5 minutes, then decreased to 0% for the next 5 minutes (total run time, 18 minutes). The analytical column used was Nucleosil 100-5C18, 250 × 4.6 mm i.d., with a packing material of 5 μm particle size at a flow rate 1 ml/minute at ambient temperature. During each run the chromatogram was recorded at 228 nm and integrated for the detection of liberated hippuric acid. Pure hippuric acid was used

to calibrate the standard curve and retention time. The % inhibition was calculated by:

$$\% \ Inhibition = \left(\left[\frac{E^{Control} - E^{Sample}}{E^{Control} - E^{Blank}} \right] \right) \times 100$$

E; efficacy (area of peak, HPLC).

Data analysis

The data was analsed by the SPSS program, version 17.0 with values expressed as a mean ± SD of the triplicate measurements. Data were subjected to one-way ANOVA means compared using Duncan's multiple range test. The correlation between variables was analysed by Pearson correlation. Statistical significance was accepted at a level of P < 0.05.

RESULTS

Total phenolics and HPLCphenolic profiles

Figure 1 shows the total phenolic contents of the plants. In hexane extraction, *pegaga*
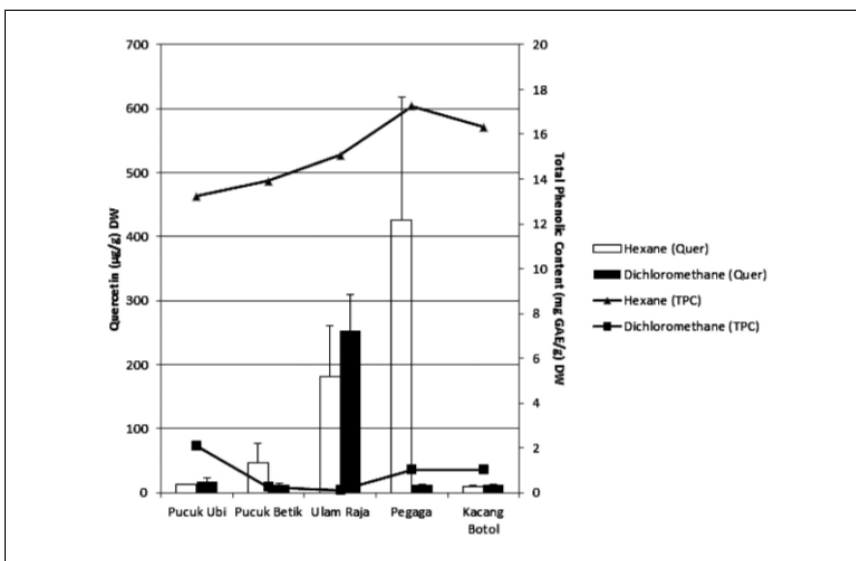
Figure 2. Quercetin and total phenolic content of the plants. Bars with different letters are significantly different ($p < 0.05$).

$(17.25 \pm 2.06$ mg GAE/g) showed the highest phenolic contents followed by *kacang botol* $(16.31 \pm 1.40$ mg GAE/g), *ulam raja* $(15.08 \pm 0.64$ mg GAE/g), *pucuk betik* $(13.92 \pm 1.22$ mg GAE/g) and *pucuk ubi* $(13.23 \pm 0.60$ mg GAE/g). In dichloromethane extraction, *pucuk ubi* $(2.09 \pm 0.09$ mg GAE/g) showed the highest phenolic content followed by *pegaga* $(1.04 \pm 0.21$ mg GAE/g), *kacang botol* $(1.03 \pm 0.10$ mg GAE/g), *pucuk betik* $(0.26 \pm 0.05$ mg GAE/g) and *ulam raja* $(0.13 \pm 0.08$ mg GAE/g). Overall, the total phenolic content in the hexane extract wassignificantly higher than that found in the dichloromethane extract.

According to HPLCphenolic profiles, quercetin and rutin were found in both the hexane and dichloromethane extraction of all the samples (Figures 2 and 3). Quercetin ranged from 12.6 to 426.2 μg quercetin/g dried weight in hexane extracts while in dichloromethane extracts, the range was from 5.73 to 253.3 μg quercetin/g dried weight. Significant differences ($p< 0.05$) were found in the quercetin value between hexane and dichloromethane extracts of *ulam raja* and *pegaga* samples. As for the rutin found

in the hexane extracts, their value ranged from 17.7 to 1715 μg rutin/g dried weight and in dichloromethane extracts, it ranged from 17 to 963 μg rutin/g dried weight. Most samples of hexane extraction showed higher rutin value compared to the same sample from dichloromethane extraction with the exception of *kacang botol*. Only *pucuk ubi* and *kacang botol* did not show a significant difference ($p> 0.05$) between hexane and dichloromethane extraction. The correlation between the total phenolic content with rutin was significant ($p<0.05$, r = 0.402). However, the total phenolic content was not correlated with quercetin ($p> 0.05$).

α-Amylase inhibition assay

α-Amylase inhibition was observed in all tested samples (Figure 4). *Pucuk ubi*showed the highest percentage inhibition in both the hexane and dichloromethane extracts. The hexane extracts of *pucuk ubi*, *ulam raja* and *kacang botol* showed higher inhibitory activity compared to the dichloromethane extract, in which *pucuk betik* and *pegaga* showed significant higher inhibitory activity
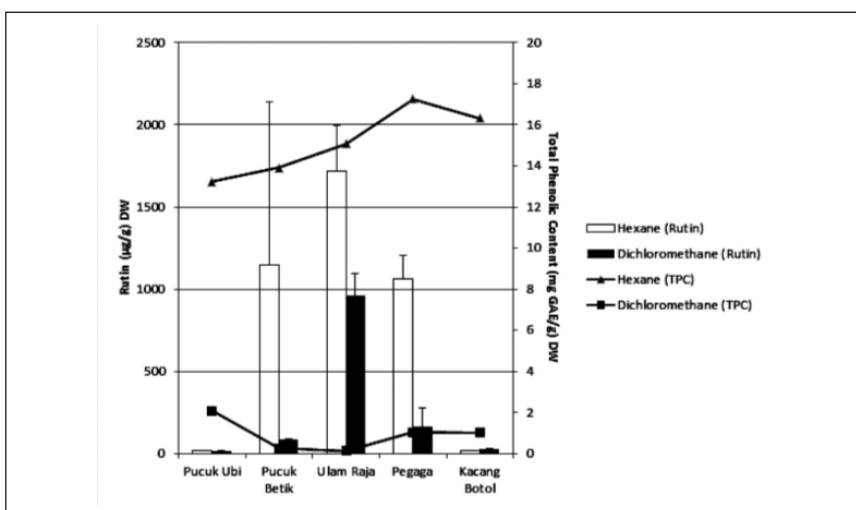
Figure 3. Rutin and total phenolic content of the plants. Bars with different letters are significantly different (*p*< 0.05).
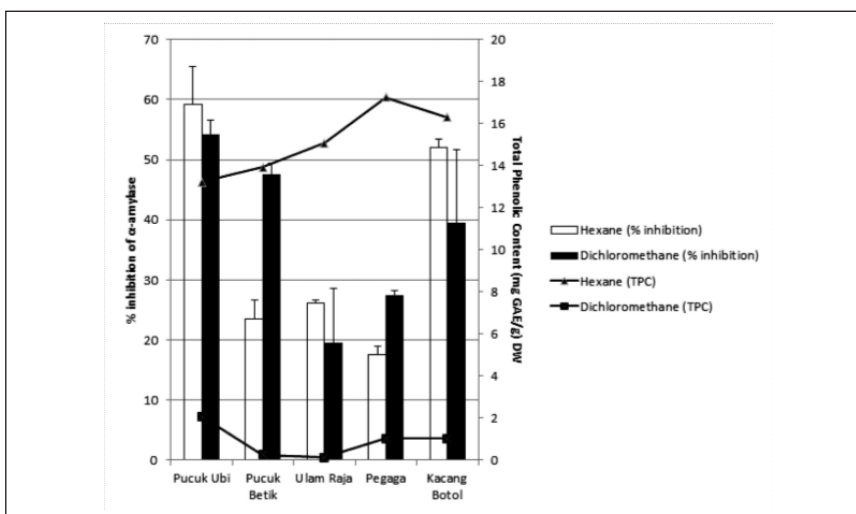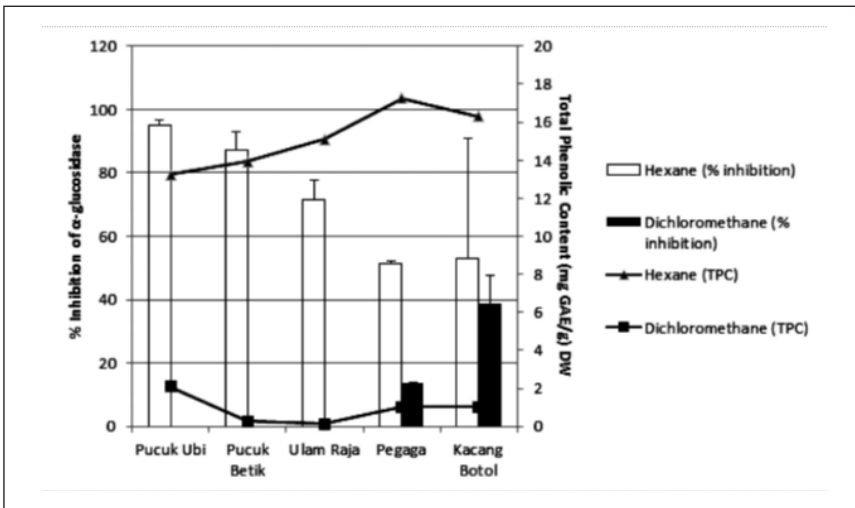

Figure 4. Relationship between α-amylase inhibition (%) of the plants and total phenolic content (mg GAE/g). Bars with different letters are significantly different (*p*< 0.05).

compared to the hexane extract. The increase in α-amylase inhibitory activity was not significantly correlated with the total phenolic for dichloromethane extracts (*p*>0.05). However, for hexane extracts, there was a significant inverse relationship between the total phenolic content with α-amylase (*p*< 0.05, r= -0.622).

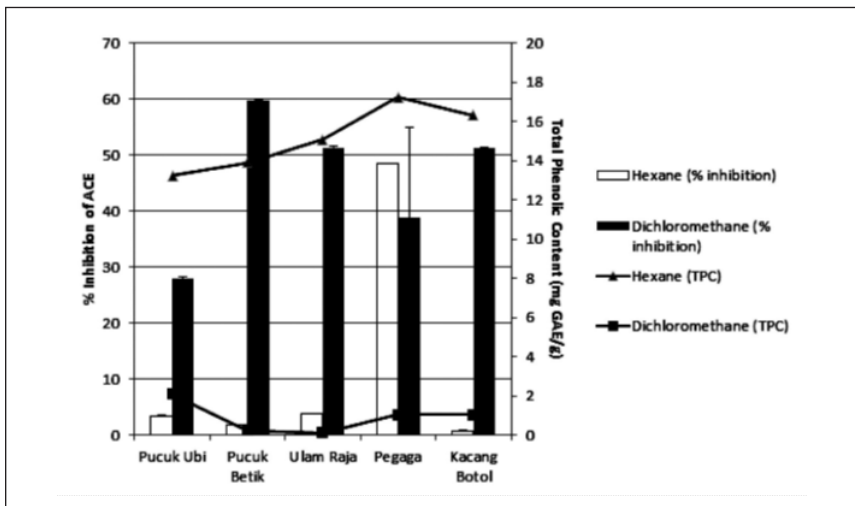**α- Glucosidase inhibition assay**

Not all tested samples showed α-glucosidase inhibitory activity (Figure 5). *Pucuk ubi* showed the highest inhibitory activity in the hexane extract while *kacangbotol* showed the highest inhibitory activity among dichloromethane extracts. Dichloromethane extracts of *pucuk ubi, pucuk*

Figure 5. Relationship between *á*-glucosidase inhibition (%) of the plants and total phenolic content (mg GAE/g). Bars with different letters are significantly different ($p < 0.05$).

Figure 6. Relationship between ACE-I inhibitory activity (%) of the plants and total phenolic content (mg GAE/g). Bars with different letters are significantly different ($p < 0.05$).

betik and *ulam raja* did not show any α-glucosidase inhibitory activity. On the whole the samples extracted using hexane showed higher inhibitory activity compared to the same sample from dichloromethane extraction. The total phenolic content was significantly correlated with α-glucosidase inhibitory activity with dichloromethane extracts showing r = 0.794 while the hexane extracts were inversely correlated (r=-0.829).

Angiotensin I-converting enzyme (ACE) inhibition assay

Overall, all extracts had some capacity in inhibiting angiotensin I-converting enzyme (Figure 6). In the hexane extracts, *pegaga*

showed the highest inhibitory activity while *pucu kbetik* was the highest in dichloromethane extracts. All samples for hexane extraction showed a lower percentage of inhibition compared to the same sample from dichloromethane extraction with the exception of *pegaga*. The ACE inhibitory activity of the extracts correlate with the total phenolic content ($p<0.05$) for both hexane (r=0.749) and dichloromethane extracts (r=-0.853).

DISCUSSION

In animal models and a limited number of human studies carried out so far, polyphenols and foods or beverages rich in polyphenols have attenuated post-prandial glycemic responses and fasting hyperglycemia, and improved acute insulin secretion and insulin sensitivity. The mechanisms suggested include the inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic *β*-cells, modulation of glucose release from liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Hanhineva*et al.*, 2010).

α-amylases or α-1,4-glucan-4-glucano-hydrolases are a group of digestive enzymes widely present in microorganisms, plants and animals, which catalyse the hydrolysis of α-1.4 glycosidic bonds (Dojnov*et al.*, 2008). A relation between the activity against α-amylases inhibition and the chemical constituents sample was observed in this study. A higher presence of other compounds such as carbohydrates in samples could be associated with a real potential of the plant to produce substances that are capable of avoiding predatory action (Everton *et al.*, 2009).

The results from this *in vitro* study also provide the biochemical rationale that phenolic-linked ingredients of selected plants have the potential for intestinal α-glucosidase inhibition. This indicates the potential to reduce glucose absorption in the intestine. Additionally several plant samples (e.g., hexane extracts of *pucuk betik* and *ulam raja*) have high α-glucosidase inhibitory activity combined with low α-amylase inhibitory activity. Combinations of enzyme inhibitory activity found in the hexane extracts of *pucuk betik* and *ulam raja* have interesting functionality for potentially controlling glucose absorption with high α-glucosidase inhibition and not generating undigested starch linked side effects with low α-amylase inhibition. It has been suggested that such adverse effects might be caused by the excessive inhibition of pancreatic α-amylase resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Horii *et al.*, 1987). Therefore based on this study *pucuk betik* in hexane extraction with high α-glucosidase and lower α-amylase inhibitory activities could be considered potential candidates as a part of dietary and supplement designs to manage early stages of hyperglycemia linked to type 2 diabetes.

A further benefit from this study is the indication that the selected plants studied have the potential to inhibit ACE in vitro, which indicates potential anti-hypertension activity. The ACE inhibitory activity of the plants did not correlate positively with the total soluble phenolic content and may be potentially linked to other unknown factors. Kwon *et al.* (2006) tested several purified compounds in relation to the ACE inhibition and they observed that among these, quercetin had no inhibitory activity. The plants studied here showed a high quercetin level. Another possibility is that other compounds present in the plants are likely to be responsible for the inhibitory activity since there is evidence that ACE inhibition could be due to the presence of some water-soluble compounds other than the phenolics. There is also evidence that some soluble peptides that are naturally present in foods could be responsible for such inhibitory activity (Lee *et al.*, 2006; Gouda *et al.*, 2006).

CONCLUSION

Although this is an *in vitro* study, the results indicate the positive effect of some of the plant extracts on hyperglycemia risk factors and the biomarker of hypertension (ACE). The hexane extracts had a higher total phenolic content than the dichloromethane extracts. From these *in vitro* assays, *pucuk betik* and *ulam raja* were found to have a good inhibitory profile against carbohydrate modulating enzymes such as α-glucosidase, which are related to glucose absorption in the intestine. Further, *pucuk betik* and *ulam raja* have a moderate effect against ACE, especially dichloromethane extracts. These functiona-lities when combined in a diet are potentially useful to manage the glucose-induced hyperglycemia and hypertension-induced vascular complications and provide the biochemical rationale for further animal and clinical studies.

REFERENCES

Babish JG, Pacioretty LM, Bland JS, Minich DM, Hu J & Tripp ML (2010). Anti-diabetic screening of commercial botanical products in 3T3-L1 adipocytes and db/db mice. *J Med Food* 13:535-47.

Dojnov B, Bozic B, Nenadovic N, Ivanovic J &Vujcic Z (2008). Purification and properties of midgut α-amylase isolated from *Morimusfunereus* (Coleoptera: Cerambycidae) larvae. *Comp Biochem Physiol B: Biochem Mol Biol* 149: 153-160.

Everton MS, Arnubio V, Maria FG, Thales LR, Erika F, Jose E &Laila SE (2009). Inhibitory action of Cerrado plants against mammalian and insect α-amylases. *Pestic Biochem Physiol* 95: 141-146.

Gouda KGM, GowdaLR, Rao AGA & Prakash V (2006). Angiotensin-I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (Glycine max). *J Agric Food Chem* 54: 4568-4573.

Hanhineva K, Törrönen R, Bondia-Pons I, Pekkinen J, Kolehmainen M, Mykkänen H &Poutanen K (2010). Impact of dietary polyphenols on carbohydrate metabolism. *Int J MolSci* 11:1365-402.

Hasenah A, Houghton PJ &Amala S (2006). α-Amylases inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllan-thusamarus*. *J Ethnopharmacol* 107: 449-455.

Hernadez-Ledesma B, Martin-Alvarez PJ&Pueyo E (2003). Assessment of spectrophotometric method for deter-mination of angiotensin converting enzyme activity: influence of inhibition type. *J Agr Food Chem* 51: 4175-4179.

Horii S, Fukasse K, Matrua T, Kameda K, Asano N & Masui Y (1987). Synthesis and α-D-glucosidase inhibitory activity of N-substituted valiolamine derivatives as potent oral antidiabetic agents. *J Med Chem* 29: 1038–46.

Johnston J I & Franz V (1992). Renin-angiotensin system: a dual tissue and hormonal system for cardiovascular control. *J Hypertens* 10: 13-26.

Khairana H, MohdShafie Z, Ibrahim J, JamiaAzdina J & Juriati J (2004). *Tinjauan etnobotani ke atas tumbuhan yang digunakan sebagai ulam di Malaysia. Jurnal Sains Kesihatan Malaysia* 2 (1): 19-36.

Krentz AJ & Bailey CJ (2005). Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs* 65: 385-411.

Kwon YI, Apostolidis E & Shetty K (2008). *In vitro* studies of eggplant (*Solanum melongena*) phenolics as inhibitors of key enzymes relevant for type 2 diabetes and hypertension. *Bioresour Technol* 99: 2981-2988.

Kwon YI, Vattem DA & Shetty K (2006). Evaluation of clonal herbs of Lamiaceae species for management of diabetes and hypertension. *Asia Pac J Clin Nutr* 15: 107-118.

Lee J, BaeIY, Lee HG & Yang C (2006). Tyr-Pro-Lys, an angiotensin-I-converting enzyme inhibitory peptide derived from broccoli (*Brassica oleracea italica*). *Food Chem* 99: 143–148.

Lee YA, Cho EJ, Tanaka T & Yokozawa T (2007). Inhibitory activities of proanthocyanidinis from persimmon against oxidative stress and digestive enzyme related to diabetes. *J Nutr Sci Vitaminol* 53: 287-292.

Marcia Da SP, Kwon YI, Apostolidis E, Franco ML, Maria IG & 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. *J Agr Food Chem* 56: 4386-4392.

Ministry of Health Malaysia (2006). Non-communicable Disease Risk factors in Malaysia Disease Control Division (NCD). Malaysian NCD Surveillance.

Rhabasa-Lhoret R & Chiasson JL (2004). Alpha-glucosidase Inhibitors (3rd ed.). In: *International Textbook of Diabetes mellitus,* Vol. 1. Defronzo RA, Ferrannini E, Keen H & Zimmet P (eds). John Wiley, UK.

Shetty K, Curtis OF, Levin RE, Wikowsky R & Ang W (1995). Prevention of verification associated with *in vitro* shoot culture of oregano (*Origanum vulgare*) by Pseudomonas spp. *J Plant Physiol* 147: 447-451.

World Health Organization (WHO) (2009). Country and Regional Data. World Health Organization.

Zaini A (2000). Where is Malaysia in the midst of the Asian epidemic of diabetes mellitus? *Diabetes Res Clin Pract* 50: 23-28.