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Ergogenic, anti-diabetic and antioxidant attributes of selected Malaysian herbs: characterisation of flavonoids and correlation of functional activities

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Abstract

In the present work, aqueous ethanolic (60% ethanol) extracts from selected Malaysian herbs including *Murraya koenigii* L. Spreng, *Lawsonia inermis* L., *Cosmos caudatus* Kunth, *Piper betle* L., and *P. sarmentosum* Roxb. were evaluated for their ergogenic, anti-diabetic and antioxidant potentials. Results showed that the analysed herbs had ergogenic property and were able to activate 5'AMP-activated protein kinase (AMPK) in a concentration dependant manner. The highest AMPK activation was exhibited by *M. koenigii* extract which showed no significant ($p > 0.05$) difference with green tea (positive control). For anti-diabetic potential, the highest α-glucosidase inhibition was exhibited by *M. koenigii* extract with IC50 of 43.35 ± 7.5 µg/mL, which was higher than acarbose (positive control). The determinations of free radical scavenging activity and total phenolics content (TPC) indicated that the analysed herbs had good antioxidant activity. However, *C. caudatus* extract showed superior antioxidant activity with IC_{50} against free radical and TPC of 21.12 ± 3.20 µg/mL and 221.61 ± 7.49 mg GAE/g, respectively. RP-HPLC analysis established the presence of flavonoids in the herbs wherein *L. inermis* contained the highest flavonoid (catechin, epicatechin, naringin and rutin) content (668.87 mg/kg of extract). Correlations between the analyses were conducted, and revealed incoherent trends. Overall, M. *koenigii* was noted to be the most potent herb for enhancement of AMPK activity and *α*-glucosidase inhibition but exhibited moderate antioxidant activity. These results revealed that the selected herbs could be potential sources of natural ergogenic and anti-diabetic/antioxidant agents due to their rich profile of phenolics. Further analysis *in vivo* should be carried out to further elucidate the mechanism of actions of these herbs as ergogenic aids and anti-diabetic/antioxidant agents.

Introduction

Metabolic process occurs in human's body to release energy to function in everyday life. Imbalance in the energy uptake and expenditure has led to metabolic syndrome epidemic, which increases the risk of other health disorders such as diabetes and cardiovascular heart disease (Sun, 2016). This epidemic has been increasing and resulted in an alarming situation in many countries. One of the suggested solutions against these problems is by using ergogenic aid (Halim *et al.,* 2018). Ergogenic aids, initially introduced in sport activities, are now used to boost the performance of low metabolic persons and subsequently increases energy production and this could help in inducing weight loss for body weight management (Palou and Bonet, 2007).

Besides that, ergogenic aids can also be used to fight metabolic syndromes, especially the syndromes that are related to obesity and type 2 diabetes (Abdul Majid *et al.,* 2020). Currently, ergogenic aids are mostly based on synthetic materials such as 5-Aminoimidazole-4-Carboxamide Ribonucleotide (AICAR), protein such as creatine, and some plant-derived compounds including caffeine and flavonoids (Gurley *et al.,* 2015; Halim *et al.,* 2017). Since there are limitations on prolonged consumption of synthetic ergogenic aids, studies on natural ergogenic agents are being carried out (Gurley *et al.,* 2015; Halim *et al.,* 2018).

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Plants are potential sources of ergogenic agents with efficacy to enhance performance and increase energy production, thus balancing energy expenditure with energy uptake. Plants that have been reported with ergogenic aids are ginseng (Lee *et al.,* 2016), green tea (Jówko, 2015), longjack (Chen *et al.,* 2012), coconut water (Halim *et al.,* 2018) and *Morinda citrifolia* (Abdul Majid *et al.,* 2020). The ergogenic properties of plant extracts might be due to the presence of bioactive compounds such as flavonoids. Besides, these plant extracts are also well-known for their antioxidant property and can neutralise free radicals (Halim *et al.,* 2018; Abdul Majid *et al.,* 2020).

During normal biochemical processing, such as exercise or exposure to hazardous substances, free radicals are generated from the body (Halim *et al.,* 2017). Usually body can naturally eliminate free radicals in the presence of antioxidant enzymes such as superoxide dismutase and catalases. However, formation of free radicals at an excessive level could lead to diseases such as diabetes, inflammation and aging process (Suroowan and Mahomoodally, 2018; Rengasamy *et al.,* 2019). Therefore, the consumption of sufficient antioxidant could ameliorate these metabolic disorders. In addition, high antioxidant activity also helps to stimulate AMP-activated protein kinase (AMPK) activity and central nervous system which prove the usage of herbs as ergogenic aids (Bucci, 2000). Besides that, the activation of AMPK might be useful for the treatment of metabolic syndrome, such as type 2 diabetes (Hardie, 2016). These functional properties can be derived from phytochemicals found in the herbs.

Phytochemicals such as flavonoids and other polyphenols are responsible for antioxidant functionalities (Hardie, 2016). Antioxidant in plants functions by scavenging free radicals and subsequently prevent the formation of oxidative stress in the cells (Halim *et al.,* 2017). Plants with potent antioxidant property can also exert beneficial effects in diabetes because it preserves *β*-cell function and suppresses apoptosis in *β*-cells without changing the rate of cell proliferation (Mazumder *et al.,* 2012). Therefore, the use of herbs that have high antioxidant activity may activate AMPK and have anti-diabetic potential which can be potentially used as ergogenic aids. Those herbs can also be used as functional foods and become one of the solutions to fight this metabolic syndrome epidemic. The present work was conducted with the aim to analyse AMPK enhancement, antioxidant activity and *α*-glucosidase inhibition of selected Malaysian herbs including *Murraya koenigii* L. Spreng, (curry), *Lawsonia inermis* L. (henna), *Cosmos caudatus* Kunth (*ulam raja*), *Piper betle* L. (*sireh*), and *P. sarmentosum* Roxb. (*kaduk*). The flavonoids in these herbs were quantified using high performance liquid chromatography (HPLC).

Materials and methods

Plants material

Five selected herbs namely *Murraya koenigii* L. Spreng, (curry), *Lawsonia inermis* L. (henna), *Cosmos caudatus* Kunth (*ulam raja*), *Piper betle* L. (sireh), and *P. sarmentosum* Roxb. (*kaduk*) were collected from the University Agriculture Park, Universiti Putra Malaysia (UPM). The herbs were identified by the herbarium in Institute of Bioscience, UPM, and voucher numbers were given from SK2960/16 to SK2964/16. The herbs were cleaned with tap water and rinsed. The cleaned leaves were packed in bags and frozen overnight at -20°C. The frozen leaves were then dried in the freeze dryer (FreeZone, Labconco, Missouri USA) at -52°C for 3 d. The water content of the dried samples was then determined using moisture analyser and was ensured to be less than 10% for optimal quantity (Mustafa *et al.,* 2010). The dried leaves were then ground into fine powder. The fine powder was kept in airtight container at -20°C until extraction process.

Chemicals and reagents

Standard of ascorbic acid, gallic acid, catechin, epicatechin, rutin, quercetin, naringin, Folin-Ciocalteau reagent, 2,2-diphenyl-1-dipicrylhydrazyl radical (DPPH), sodium carbonate (Na_2CO_3), and glycine were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Methanol of high-performance liquid chromatography (HPLC) grade, ethanol, trifluoroacetic acid (TFA), and hydrochloric acid (HCl) were purchased from Fisher Scientific (Springfield, N.J., USA). Adenosine Monophosphate Kinase (AMPK) assay kit was purchased from CycLex Co., Ltd., (Nagano, Japan).

Solvent extraction of herbs

The ground dried leaves were weighed and placed in a 250 mL Erlenmeyer flask, and 100 mL of 60% ethanol was added into the flask at 1:10 sample:solution ratio (w/v). The extracts were then filtered with Whatman cellulose filter paper. The supernatants were then concentrated using a rotary evaporator at 40°C to remove the solvent. The concentrated extracts were then transferred into a Falcon tube and stored at -80°C overnight. The frozen extracts were placed in a freeze dryer (Labconco, Labconco Corporation, Kansas, USA) to

remove the water for at least 3 d or until dried. The dried extracts were then stored at -20°C until further analysis.

AMP-activated protein kinase (AMPK) analysis

The method used was conducted as described by Palanivel and Sweeney (2005) with slight modifications. The murine rat L6 skeletal muscle cells were incubated at a density of 8,000 cells/cm^2 (10 cm^2 dish) and were grown in DMEM containing 10% Foetal Bovine Serum and 1% penicillin-streptomycin (antibiotic) at 37°C in 5% carbon dioxide. When cells were grown at 100% confluence, these were transferred into differentiation medium (DMEM with 2% horse serum/bovine serum and 1% penicillin-streptomycin). The cells were then fed with fresh differentiation medium every day until the myotubes were fully formed.

The cells were treated with 20 µL of each herb sample for 24 h in a fresh DMEM containing 2% FBS and 1% penicillin-streptomycin. Prior to sample treatment, 1 mg of each extract was dissolved in 1 mL of DMSO. The solution was vortexed vigorously until dissolved completely. The samples were then stored at 4°C until used. The cells were washed three times with ice-cold phosphate buffer solution (PBS). The cells were then lysed by adding 0.1 mL of Cell Lysis Buffer for 60 - 90 min. The cell lysates were then transferred to microcentrifuge tubes and centrifuged at 15,000 rpm for 10 min at 4°C. The clear lysates were then transferred to a new 96 well microplate and ready for analysis. The assay method used was in accordance to the AMPK kit. After 24 h, the absorbance was read at 450 nm using microplate reader (BioTek Instruments, Inc., EL 800, Winooski, USA). The result of the relative AMPK activity was expressed in increase of energy fold as compared to the basal control and synthetic positive control of AICAR.

α-glucosidase inhibitory activity

The determination of anti-diabetic activity was conducted according to Mediani *et al.* (2015) with some modifications The enzyme in phosphate buffer of pH 6.5 was pipetted into 96-well microplate and mixed with sample at different concentrations (20 - 1000 µg/mL). The mixture was shaken thoroughly and was kept at room temperature for 10 min. Subsequently, *p*-nitrophenyl *α*-D-glucopyranoside (PNPG) was then added to the wells followed by incubation at 37°C for 30 min. The reaction was stopped by adding glycine (pH 10). Acarbose served as positive control. The absorbance of the plate was then read at 405 nm using the Biotek EL 800-microplate reader (BioTek Instruments, Inc., EL 800, Winooski, USA). Percentage inhibition was calculated using Equation 1. Different concentrations of acarbose were prepared (6.25 - 100 µg/mL). All samples were analysed in triplicate. The α-glucosidase inhibitory activity was expressed as IC_{50}. The blank sample was only needed when the sample was coloured.

$$\% \text{ inhibition} = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample)}{Absorbance\ of\ control} \times 100 \quad \text{(Eq. 1)}$$

2,2-Diphenyl-1-dipicrylhydrazyl (DPPH) scavenging activity

The determination of 2,2-diphenyl-1-dipicrylhydrazyl (DPPH) free radical scavenging activity was performed following the method of Sarian *et al.* (2017) with some modifications. Briefly, 50 µL of different concentrations (20 - 1000 µg/mL) of extracts were pipetted into 96-well microplate. Then, 100 µL of DPPH (5.9 mg/100 mL) solution was added into each well. The mixture was mixed thoroughly and was left at room temperature in the dark for 30 min. The absorbance was then measured at 515 nm using a microplate reader (BioTek Instruments, Inc., EL 800, Winooski, USA). Ascorbic acid served as positive control. The analyses were done in triplicate. The free radical scavenging activity was calculated using Equation 2:

$$\% \text{ inhibition} = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample)}{Absorbance\ of\ control} \times 100 \quad \text{(Eq. 2)}$$

Total phenolic content (TPC)

The total phenolic content (TPC) of the herbs was determined following the method described by Pak Dek *et al.* (2011). A diluted extract (0.5 mL) was mixed with 0.5 mL of Folin-Ciocalteau reagent in a test tube. The mixture was vortexed for 10 s before 10 mL of 7% Na_2CO_3 was added. The mixture was left at room temperature (25 ± 2°C) for 60 min for reaction to occur. The absorbance was then read at 715 nm using UV-Visible Spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid was treated with the same procedure as sample extract. Different concentrations of gallic acid for constructing a standard curve (20 - 100 µg/mL) were prepared. All analyses were done in triplicate. The result was expressed as milligram of gallic acid equivalent (GAE) per gram of extract (mg GAE/g).

Flavonoid detection using high-performance liquid chromatography (HPLC)

The method described by Pak Dek *et al.* (2011) was followed for flavonoid detection using HPLC, with slight modifications. The mobile phase consisted of methanol and TFA at pH 2.5. First, 1.5 mL of HPLC-grade methanol was added into a round bottom flask containing 5 g extract. It was then vortexed vigorously until all the samples were dissolved completely. After that, 1 mL of deionised water and 625 µL of 6 M HCl were added to the flask and were shaken thoroughly. It was then thermostated for 2 h at 82°C. Next, 0.5 mL of the hydrolysed sample was then filtered using 0.45 µm nylon membrane filters into a screw-capped sample vial.

The HPLC analysis was conducted using Agilent HPLC system equipped with Agilent 1200 series wavelength absorbance detector, Agilent 1200 series pump, degasser and auto sampler (Agilent Technologies, USA). Separation of samples was done using a reversed phase HPLC with Agilent C_{18} column (4.6 × 150 mm, 5 µm) as the stationary phase. As for the mobile phase, deionised water with TFA (pH 2.5) as solvent A and absolute methanol of HPLC grade as solvent B were used. The gradient that was used in this separation was as followed: 100 to 50% solvent A (0 to 20 min), 50 to 40% solvent A (20 to 30 min) and 40 to 100% solvent A (30 to 40 min). The flow rate for both mobile phases was maintained at 1.0 mL/min, and wavelength for the detector was set at 280 nm using a UV-Vis detector attached to the 1200 series model HPLC machine (Agilent technologies, USA). The retention time and area for the peaks of selected standards were used for comparison purposes in the identification and quantification of flavonoids found in the samples.

Statistical analysis

Statistical analyses were conducted using Minitab 16 (Version 16, Minitab Inc, State College, PA, USA). Analyses of variance (ANOVA) was applied to examine the significant differences between the means with $p < 0.05$ was considered as significantly different. The correlation between metabolite components and IC_{50} values for DPPH radical scavenging activity were converted to $1/IC_{50}$ in order to acquire the same trend as the functional properties' activity.

Results and discussion

Five different herbs namely curry, henna, *sireh*, *ulam raja*, and *kaduk* were screened of their ergogenic property through the activation of 5'adenosine monophosphate-activated protein kinase (AMPK), antioxidant activity and *α*-glucosidase activity *in vitro*.

The ergogenic property of the selected herb extracts was assessed based on their ability to stimulate the AMPK activity in L6 skeletal muscle cell. The result of AMPK activation was expressed in relative fold increase of cellular energy. The relative fold increase of AMPK for studied samples were compared with green tea, a natural positive control and AICAR, a synthetic positive control as shown in Figure 1. The results showed that the relative fold increase of cellular energy in cell culture treated with the herbs extracts varied with the highest activity was shown by curry (5.38 ± 0.28), followed by *ulam raja* (4.84 ± 0.58), henna (4.01 ± 0.3), *kaduk* (3.31 ± 0.33) and *sireh* (2.58 ± 0.18).

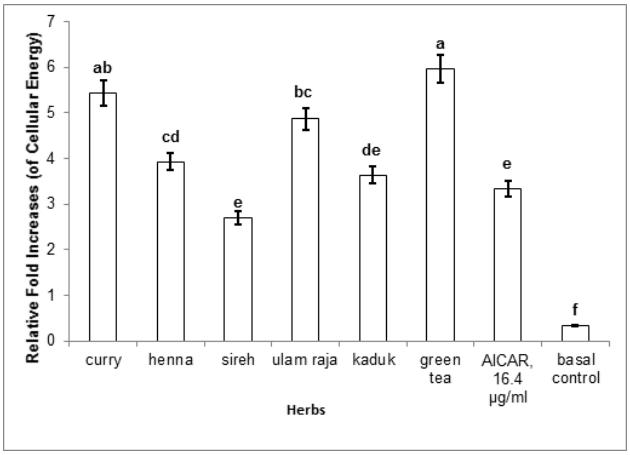

Figure 1. Relative AMPK activity of L6 skeletal muscle cells when treated with selected herbs extracts for 24 h. Values are means ± standard deviations (SD) of three replications ($n = 3$). Means that do not share a letter are significantly different ($p < 0.05$) as measured by Tukey's test. AMPK = Adenosine Monophosphate-activated Kinase; AICAR = 5-Aminoimidazole-4-Carboxamide Ribonucleotide

AICAR is an adenosine monophosphate (AMP) analogue where adenosine transporters are taken into the cells and phosphorylated by intracellular adenosine kinase into AICAR monophosphate (ZMP), an AICAR monophosphate (Hardie, 2016). It is interesting to note that the activity of the curry extract was not significantly ($p > 0.05$) different from green tea (5.88 ± 0.32). This shows that curry extract had a potent ergogenic aid property since the increment of AMPK activity was similar to a natural positive control (green tea). *Ulam raja* also exhibited high ability to activate AMPK activity as it was not significantly ($p > 0.05$) different from curry extract. Nevertheless, it can be observed that all the tested herbs were good AMPK activators because all

samples showed a higher relative fold increase of cellular energy as compared to the basal control (0.36 ± 0.02). Curry, henna, and *ulam raja* extracts activated AMPK activity significantly ($p < 0.05$) higher than synthetic positive control of AICAR (3.40 ± 0.60).

AMPK activation by plant extracts has been reported in various studies. These include the application of green tea extract (Banerjee *et al.,* 2012) and naringin from grapefruit (Pu *et al.,* 2012). The herb extracts used in the present work have never been analysed for their ability to activate AMPK activity. However, various studies have utilised other plant extracts and their specific compounds for the stimulation of AMPK. All the analysed samples showed the ability to activate AMPK based on their polyphenol contents. Unfortunately, the mechanism of the AMPK activation by these plants remains unknown (Hardie, 2016).

Based on available literature, most of the natural plant products that activated AMPK contained secondary metabolites which comprised of phytochemicals such as flavonoids, phenolic acids and alkaloids (Hardie, 2016). Previous study showed that curry contained not only flavonoid, but also tannin, alkaloid, steroid, and triterpenoid which may be the contributor to the potent AMPK activation (Kusuma *et al.,* 2011). From the AMPK activity result obtained in the present work, it can be deduced that curry extract can be the most potential ergogenic aid among the five analysed herbs. However, *ulam raja*, henna, *kaduk*, and *sireh* were also good AMPK activators due to comparable ergogenic activity.

The *α*-glucosidase inhibitory activity was analysed in order to observe the potential of the extracts as anti-diabetic agents. This property can be used to counteract metabolic changes associated with type 2 diabetes (Vongsak *et al.,* 2018). The result showed that there was a variable inhibitory effect of plant extract against α-glucosidase activity as shown in Figure 2. It was also revealed that the highest *α*-glucosidase inhibitory activity was demonstrated by curry extract with IC_{50} of 43.35 ± 7.5 µg/mL, followed by henna and *sireh* with IC_{50} of 76.00 ± 5.42 µg/mL and 84.33 ± 6.12 µg/mL, respectively. The lowest activity was shown by *ulam raja* with IC_{50} of 128.76 ± 7.04 µg/mL. *Kaduk* extract showed no α-glucosidase inhibition at 1000 µg/mL. It is interesting to note that the inhibitory effect of the curry extract was significantly ($p < 0.05$) higher than the control, acarbose. Meanwhile, the inhibitory effect of henna and **sireh** was similar with acarbose. In contrast, the inhibitory effect of *ulam raja* was significantly lower than acarbose.

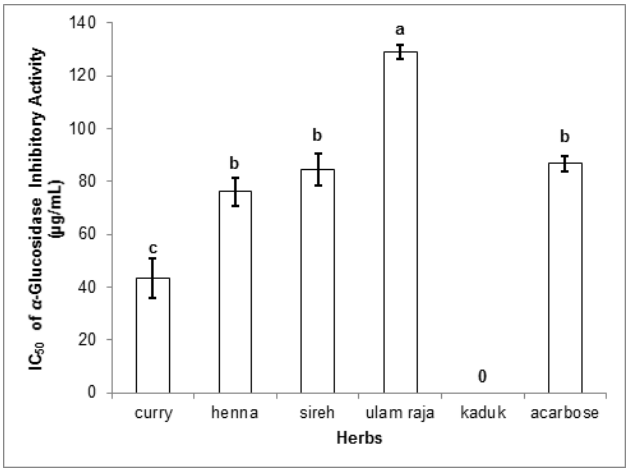

Figure 2. The *α*-glucosidase inhibitory activity of the selected herbs' extracts. Values are means ± standard deviations (SD) of three replications ($n = 3$). Means that do not share a letter are significantly different ($p < 0.05$) as measured by Tukey's test. IC_{50} = the concentration of extract that is required to inhibit 50% of *α*-glucosidase activity.

Based on the result, curry showed a significantly ($p < 0.05$) higher inhibitory activity than positive control of acarbose (IC_{50} of 86.66 ± 3.08 µg/mL). Acarbose is an *α*-glucosidase inhibitor and a primary type of drug which is utilised to control hyperglycaemia (Vongsak *et al.,* 2018). The result obtained in the present work showed that curry was a potent *α*-glucosidase inhibitor. The higher inhibitory activity exhibited by curry might be due to the presence of some phytochemicals that probably acted as enzyme inhibitors (Ghafar *et al.,* 2018). The IC_{50} of *α*-glucosidase inhibition for *ulam raja* was higher than what was reported earlier. The difference might be due to different sample collection and processing method. Javadi *et al.* (2015) reported higher α-glucosidase inhibitory activity of ulam raja with IC_{50} between 12.6 µg/mL and 40.9 µg/mL for different storage times (between 0 h to 12 h). On the other hand, Gul *et al.* (2012) reported a lower activity for curry (IC_{50} at 174.74 µg/mL). From the *α*-glucosidase inhibition result, curry extract can be said to have a potent anti-diabetic activity where the activity was higher than the positive control, acarbose. On the other hand, both *sireh* and henna also possessed excellent anti-diabetic activity where their *α*-glucosidase inhibitions were similar with acarbose. Therefore, curry, henna, and *sireh* can be considered as potential sources for natural anti-diabetic agent and can be used to develop functional food in order to overcome metabolic syndrome endemic.

Antioxidant activity of these herbs was screened through DPPH radical scavenging activity. Result of DPPH activity is presented in IC_{50} values as shown in Figure 3(A). The result showed that the

highest free radical scavenging activity was exhibited by *ulam raja* (IC_{50} of 21.12 ± 3.20 µg/mL) and *sireh* (IC_{50} of 39.42 ± 1.81 µg/mL), while the lowest was by *kaduk* (IC_{50} of 480.44 ± 2.08 µg/mL). On the other hand, henna and curry showed moderate radical scavenging activities with IC_{50} of 60.48 ± 3.88 µg/mL and 277.87 ± 3.71 µg/mL, respectively. It should be highlighted that *ulam raja* exhibited no significant ($p > 0.05$) difference with ascorbic acid for free radical scavenging. This showed that *ulam raja* was a potent radical scavenger.

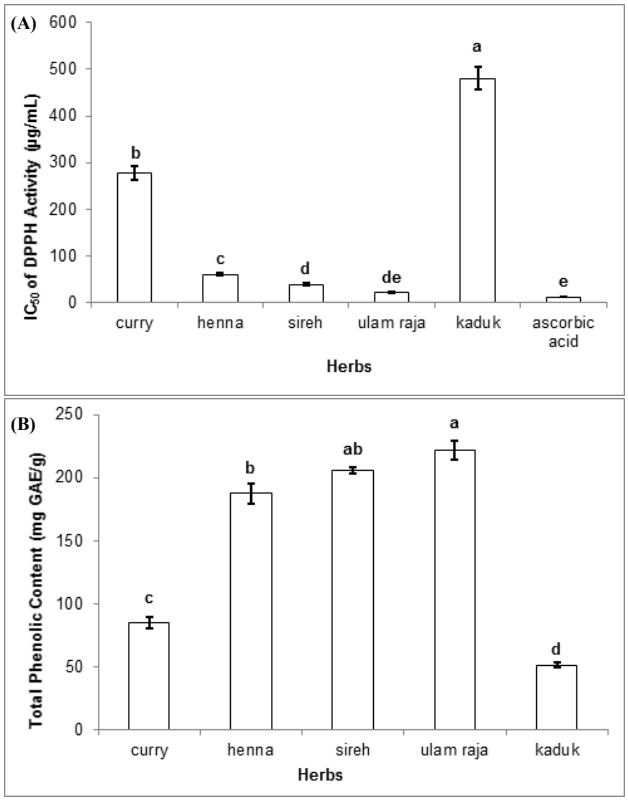

Figure 3. (A) Free radical scavenging activity, and (B) total phenolic content of the selected herbs' aqueous extracts. Values are means ± standard deviations (SD) of three replications ($n = 3$). Means that do not share a letter are significantly different ($p < 0.05$) as measured by Tukey's test. IC_{50} = the concentration of extract that is required to inhibit 50% of the free radicals. Total phenolic result was expressed as milligram of gallic acid equivalent (GAE) per gram of extract (mg GAE/g).

DPPH is stable nitrogen-centred free radicals and can be used to test free radical scavenging activity of the samples. The violet colour of DPPH solution is reduced to light yellow diphenylpicrylhydrazine radical when the stable DPPH radicals accept an electron from electron donor. Antioxidants and radical scavengers are substances that can perform this reaction (Pak Dek *et al.,* 2011). The high free radical scavenging activity of the extracts could be due to the presence of bioactive compounds (Andar wulan *et al.,* 2010). DPPH can be used to represent the oxidative stress in human metabolism and mechanism on how antioxidant could scavenge the free radical. Oxidative stress which results from the production of excessive free radicals in body is linked with onset of many metabolic processes such as type 2 diabetes, cancers and cardiovascular diseases (Ghafar *et al.,* 2018). The oxidative stress can also result from the impairment of glucose transport in adipose tissue and muscle, and from the disturbance of insulin secretion from pancreatic β cells which in turn can result in type 2 diabetes (Pu *et al.,* 2012). Protection from free radicals or oxidising agents can be achieved with adequate consumption of dietary antioxidants (Vongsak *et al.,* 2018).

Flavonoids are one group of the phenolic compounds that are known to possess high antioxidant activity due to their ability to donate electrons or hydrogen atoms and low redox potential (Nieman *et al.,* 2009). The present radical scavenging results concluded that *ulam raja* and *sireh* were the most potent radical scavengers as compared to henna, *kaduk* and curry. Shui *et al.* (2005) found that major antioxidant compounds in *ulam raja* were attributed to several proanthocyanidins that existed as dimers through hexamers, quercetin glycosides, chlorogenic, neo-chlorogenic and crypto-chlorogenic acids. Javadi *et al.* (2015) also found that metabolites existed in *ulam raja* included catechin, cyclohexen-1-carboxylic acid, vitamin E, stigmasterol, benzoic acid, lycopene, sucrose, *α-d*-galactopyranose, and turanose. Both activities of α-glucosidase and radical scavenging were in the range of control or better than control; henna and *sireh* were the good candidates to be used as natural anti-diabetic agents.

The herb extracts were then assessed for their TPC using Folin-Ciocalteau method. The TPC was found to vary between extracts. Based on Figure 3(B), *ulam raja* (221.61 ± 7.49 mg GAE/g) and *sireh* (205.46 ± 2.54 mg GAE/g) yielded the highest TPC, followed by henna and curry extracts with 87.48 ± 8.0 mg GAE/g and 84.61 ± 4.47 mg GAE/g, respectively. The lowest TPC was found in *kaduk* with only 51.27 ± 1.91 mg GAE/g.

Previous study done by Tharasena and Lawan (2014) showed similar results where the TPC value was quite low in *kaduk*, at only 0.87 ± 0.008 mg GAE/g. Andarwulan *et al.* (2010) also reported a lower TPC of *ulam raja* extracts at only 1.52 ± 0.11 mg GAE/g. Differences in the TPC of the herbs was probably due to the differences in weight comparison, extraction methods, climate, cultural practices, maturity levels during harvesting and storage conditions (Podsędek, 2007). In the present work, the

samples were collected from Universiti Agriculture Park, UPM and immediately dried using freeze dryer prior to extraction process. The application of freeze dryer could preserve phenolic compound in the extract, as reported by (Abascal *et al.,* 2005). Therefore, the TPC in the analysed samples including in *kaduk* and *ulam raja* was higher than the previous reports. It can thus be concluded that *ulam raja* and *sireh* showed the highest total phenolics among all the herbs studied.

In the present work, only targeted flavonoids (*i.e.*, catechin, epicatechin, rutin naringin, and quercetin) were identified and quantified from the herb extracts. The selection of flavonoids is based on the most found and studied flavonoids in herbs and vegetables, as indicated by previous studies. Table 1 shows the flavonoids identified in the present work. It is apparent that catechin was present at highest in henna (113.63 ± 28.37 mg/kg), followed by *kaduk* (59.10 ± 13.59 mg/kg) and *sireh* (35.04 ± 13.21 mg/kg). Interestingly, epicatechin was only detected in henna but not in other herbs. In additions, Mustafa *et al.* (2010) reported that curry leaf contained other types of flavonoids such as myricetin, kaempferol, apigenin and luteolin. These flavonoids might have influenced the AMPK activation and anti-diabetic activity of curry leaf.

Sulaiman and Ooi (2014) claimed that antioxidant activity and α-glucosidase inhibition in gooseberry (*Phyllantus acidus*) were attributed to flavanol compounds such as catechin because of structural configuration of double bonds which conjugated with hydroxyl groups and 4-oxo functions. Epicatechin is a monomeric flavanol which is linked to two aromatic carbon rings and a carbon bridge. It has been reported that the treatment of (-) epicatechin elevated protein expression of mitochondria and boost up time-to-fatigue and treadmill performance in mice. In fact, the combination of (-)epicatechin with treadmill exercise elevated mitochondrial biogenesis in muscle of mouse hence ameliorating tolerance towards exercise-induced fatigue. In another study, Taub *et al.* (2016) reported that daily intake of (-)epicatechin-rich cocoa increased AMPK activation in skeletal muscles after three months hence improving exercise performance.

Rutin is another flavonoid detected in the selected herbs as shown in Table 1. *Ulam raja* contained the highest rutin (310.33 ± 25.31 mg/kg) followed by henna (176.48 ± 85.00 mg/kg) and *sireh* (149.01 ± 33.03 mg/kg). However, rutin was not detected in curry and *kaduk*. It has been reported in previous study that rutin successfully lowered plasma glucose in streptozotocin-induced diabetic rats (Niture *et al.,* 2014). Seo *et al.* (2015) claimed that rutin could enhance mitochondrial biogenesis in muscle with activation of AMPK in induced-obese rats. In addition, rutin has been reported to have antioxidant and anti-hypertensive potential by controlling oxidative stress, lipid and glucose metabolism in adipose tissue and liver, respectively.

As shown in Table 1, the amount of naringin was found to be highest in henna (231.62 ± 24.02 mg/kg) followed by curry (85.19 ± 79.57 mg/kg) and *kaduk* (82.26 ± 20.92 mg/kg). The lowest naringin was found in *sireh* (5.50 ± 0.23 mg/kg). Meanwhile, naringin was not detected in ulam raja. Pu *et al.* (2012) reported that naringin possessed anti-oxidative stress which ameliorated lipogenesis and insulin resistance. In that study, naringin was found to activate AMPK significantly in liver which reduced the output of glucose into mice blood hence improving insulin resistance.

On the other hand, quercetin was only found in *ulam raja* (27.99 ± 8.82 mg/kg) and *sireh* (2.18 ± 3.02 mg/kg) as shown in Table 1. Nieman *et al.* (2009) reported that quercetin is a strong antioxidant agent and combination of quercetin with other polyphenols such as green tea extract resulted in the reduction of oxidative stress in athletes. In addition,

Table 1. Selected flavonoid contents of herbs' extracts as detected by HPLC.

Herb	Flavonoids (mg/kg Extract)					
	Catechin	Epicatechin	Rutin	Naringin	Quercetin	Total flavonoids
Curry	17.32 ± 3.71^{d}	ND	ND	85.19 ± 79.57^{b}	ND	102.51
Henna	113.63 ± 28.37^{a}	147.14 ± 39.86^{a}	176.48 ± 85.00^{b}	231.62 ± 24.02^{a}	ND	668.87
Sireh	35.04 ± 13.21^{c}	ND	149.01 ± 33.03^{b}	5.50 ± 0.23^{c}	2.18 ± 3.02^{b}	191.73
Ulam raja	8.38 ± 1.90^{e}	ND	310.33 ± 25.31^{a}	ND	27.99 ± 8.82^{a}	346.70
Kaduk	59.10 ± 13.59^{b}	ND	ND	82.26 ± 20.92^{b}	ND	141.36

Values are means ± standard deviations (SD) of three replications (n = 3). Means that do not share a letter are significantly different ($p < 0.05$) as measured by Tukey's test. ND = Not Detected.

quercetin has been utilised as a positive control for *in vitro* study of anti-diabetic activity using α-glucosidase inhibition (Sarian *et al.,* 2017). Quercetin also has anti-cancer property shown by its anti-proliferative and anti-inflammatory effects as highlighted by Kim *et al.* (2019).

In plants, flavonoid compounds exist as secondary metabolites which function as free radicals or singlet oxygen scavengers (Ghafar *et al.,* 2018). The intake of dietary antioxidants from fruits and vegetables has been linked with disease prevention, therefore, the active ingredients from medicinal plants become great interests in developing novel treatment for diabetes (Vongsak *et al.,* 2018). Among all the herbs analysed in the present work, henna showed the highest amount of flavonoids namely, catechin, epicatechin and naringin (Table 1). However, the flavonoid results obtained showed a different trend with TPC and DPPH activity of the herbs where *ulam raja* showed the highest activities for both analyses. This contradictory result might be due to the different targeted compounds between the TPC and DPPH. It has been suggested that TPC assay detects not only polyphenolic compounds but also other biological substances which are reactive such as amino acids and carbohydrates (Tulipani *et al.,* 2008). Other than that, the difference could also be due to the limitation observed in the present work, in which only five targeted flavonoids were detected and identified using HPLC.

Correlations between the analyses were conducted using Pearson correlation and the result is shown in Table 2. The TPC and DPPH free radical scavenging activity had a significant, positive and very strong correlation ($r^2 = 0.9409$, $p < 0.05$). Similarly, TPC also had positive correlation with *α*-glucosidase inhibition. However, TPC had no correlation ($p > 0.05$) with AMPK activation. This result was expected because usually plants rich in phenolic compounds will also have high antioxidant activity, as reported by Pak Dek *et al.* (2011). Phenolic compounds like flavonoids are known to possess potent antioxidant activity and the high polyphenol content could reduce the DPPH free radicals (Kim *et al.,* 2019).

In addition, TPC also had a significant, positive and moderate correlation ($r^2 = 0.6638$, $p < 0.05$) with *α*-glucosidase inhibitory activity. This is probably due to the presence of catechin that is known to be an active compound for the inhibition of *α*-glucosidase activity (Xiong *et al.,* 2016). Mediani *et al.* (2015) also asserted that phenolic acids such as chlorogenic acid and ellagic acid and other flavonoids in *ulam raja* which were not detected in the present work were highly linked to strong *α*-glucosidase inhibitory activity.

Table 2. Correlations between TPC of herbs and the activities.

	r^2	*p*-value
TPC and AMPK activity	0.0188	0.626
TPC and DPPH	0.9409	0.000
TPC and α-glucosidase inhibition	0.6638	0.004

Correlation is significantly different ($p < 0.05$) as measured by Pearson's correlation analysis. Strong correlation is when $R^2 = 1$.

On the contrary, TPC and AMPK activity showed no significant correlation ($p > 0.05$). This is probably due to the fact that phenolic compounds are not the only component that is responsible for the enhancement of AMPK activity (Hardie, 2016). As mentioned earlier, TPC assay also detects other biological substances which are reactive such as carbohydrates, ascorbic acid and amino acids other than polyphenols (Tulipani *et al.,* 2008). It has been claimed that certain phytochemicals other than polyphenols in plants can activate AMPK activity such as ginsenoside from *Panax ginseng* (Williams, 2006) and caffeine in guarana (Bucci, 2000). Since these phytochemicals were not measured in the present work, this could have lowered the correlation for AMPK activation with TPC and flavonoids.

Conclusion

The ergogenic property, and antioxidant and anti-diabetic activities of curry, henna, *sireh, ulam raja* and *kaduk* have been analysed in the present work. All the five herbs exhibited ergogenic property and exerted appreciable biological functionalities including as potent antioxidant and anti-diabetic agents, except for *kaduk*. Curry exhibited the ability to stimulate the AMPK activity in L6 skeletal muscle cells where it showed the highest ability to activate AMPK activity among the analysed herbs. Similarly for *α*-glucosidase inhibitory activity, all the herbs showed inhibitory activities, except for kaduk. The α-glucosidase inhibitory activity in the herbs might be attributed to the presence of phenolic compounds as shown by the correlation result. Similar to AMPK result, curry extract showed the highest inhibitory activity for *α*-glucosidase. Contrarily, *ulam raja* showed the highest total phenolic and a very potent free radical scavenging activity among the analysed herbs. It is noteworthy that henna consisted the highest catechin, epicatechin, and rutin among all the analysed herbs. Thus, due to their high antioxidant activity, ability to stimulate AMPK activity and ability to inhibit *α*-glucosidase activity *in vitro*, these

herbs can potentially be used as an ergogenic aid to fight metabolic syndrome such as diabetes. The *in vivo* study for the potency of these herbs as ergogenic aids and anti-diabetic agents should be carried out in the future.

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