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Optimization of vitexin and isovitexin compounds extracted from dried Mas Cotek leaves using one-factor-at-a-time (OFAT) approach in aqueous extraction

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Abstract

Mas Cotek or *Ficus deltoidea* is regarded as one of the most precious herbal plants in Malaysia due to its content of beneficial active compounds and appreciation as a traditional remedy. This research investigated performance of aqueous or water extraction of dried Mas Cotek leaves for vitexin and isovitexin compounds using three regiments of temperatures (50, 70 and 100°C) in sample-to-water ratios of 1:10, 1:20 and 1:30g/mL during 8 hours of extraction. The optimum yield recorded for vitexin compound was 0.463 ± 0.045 (%w/w) from; 1:30 g/mL sample-to-water ratio, 50°C temperature at 4th hour of extraction. While for isovitexin compound, the optimum value recorded was 0.136 ± 0.015 (%w/w) from; 1:20 (g/mL) sample-to-water ratio, 50°C temperature at 5th hour of extraction. The morphological characterization of the extracted dried leaves particles performed using FE-SEM showed an intact and less damaged surface of extracted sample after 8 hours of extraction. The experimental values under best conditions were consistent with the predicted values, which suggested that aqueous extraction of Mas Cotek leaves is a good platform to serve as baseline study for more advanced extraction techniques.

Introduction

An immense interest in the use of herbal preparations and active ingredients isolated from medicinal plants is observed in recent years. At present, herbal products are obtaining popularity in the treatment and prevention of various diseases. Approximately 60% of world's population still relies on medicinal plants for their primary healthcare. For primary healthcare purpose, 80% of the population in some Asian and African countries depend on these natural medicines (WHO 2013). In many developed countries, 70% to 80% of the population have used some form of complementary or alternative medicine with herbal treatments (Oreagba *et al.*, 2011).

Compared to synthetic medicines, plant herbs are often considered to be harmless and served as a rich source of numerous novel biologically active compounds. Natural products from plants have provided the pharmaceutical industry with one of its most important sources of lead compounds and up to 40% of modern drugs are derived from natural sources, using either the natural substance or a synthesized version (Cragg and Newman, 2013).

Malaysia is recognized as one of the 12 mega diversity countries in the world, with estimated 1,300 plants used in traditional medicine. The conservation and sustainable utilization of this biodiversity resource are important to preserve the biological and medicinal products derived from this natural fountainhead. Realizing the importance of these medicinal plants, the government of Malaysia had established Herbal Medicine Research Centre in 2001 to provide scientific evidence for the efficacy and safety of herbal products.

Medicinal plants contain phytochemicals such as flavonoids and phenolic acids used to show remarkable biological activities. Mas Cotek, a plant of the family Moraceae is one of the most popular and well-known plant with a long history of use among the Malays. This plant is traditionally claimed to exhibit antidiabetic properties and was widely used for treatment of diabetes. Two exceptional compounds in flavone C-glycosides, vitexin (apigenin-8-C-glucoside) and isovitexin (apigenin-6-C-glucoside) that appeared as light yellow powder form were study thoroughly. As one of the important subclasses of the flavonoids family, the flavone C-glycosides were usually presents in food nutrients and functional foods. They are reported to exhibit anti-inflammatory (Borghi *et al*., 2013), anti-nociceptive (Bunawan *et al*., 2014) and recently had received much attention received because of their antioxidant (Misbah *et al.*, 2013) and anticancer properties (Lee *et al*.,

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2011; Soib *et al*., 2015). Other various biological and pharmacological activities have been attributed to these two components, such as antimicrobial antispasmodic, radio protective effects, antioxidant, and hypotensive or free radical scavenging (Fu *et al*., 2008; Koolen *et al.*, 2013; Wang *et al*., 2015; Calixto Júnior *et al.*, 2015; Fernandes *et al.*, 2017).

There are several methods of activity determination and bioactive compounds extraction from Mas Cotek (*Ficus deltoidea* var. *deltoidea*). Abdullah *et al*. (2009) and Mohd *et al.* (2014) used soxhlet extraction and methanol as solvent to release vitexin and isovitexin compounds from Mas Cotek leaves. A study conducted by Mohammad *et al.* (2013) used acid digestion method to determine the mineral content in the leaves of this particular herbal plant. Other methods used methanol and water extracts (Abdullah, 2009; Woon *et al.*, 2014) and also common aqueous extraction (Dzolin *et al*., 2010) to extract flavonoids, tannins and other phenolic compounds from the leaves and fruits of Mas Cotek plant.

Based on these established studies, it is confirmed that Mas Cotek leaves exhibited some medicinal properties other than anti-diabetic such as anti-inflammatory (Abdullah *et al*., 2009), anti-hypertensive (Abdullah, 2009), anti-adipogenic (Woon *et al.*, 2014) and anti-oxidant (Dzolin *et al.*, 2010) under certain amount of consumption and application, depending on the nature state of experimental subjects. Other previous studies (Abdullah *et al*., 2009; Farsi, 2011; Farsi *et al.*, 2014) were emphasizing on using water as extraction medium with related parameters but only focusing on the activity determination of Mas Cotek leaves such as antioxidant, anti-inflammatory and anti-diabetic. Contrarily, this study is highlighting the optimum extraction yield of two antioxidant compounds, vitexin and isovitexin, also using water (aqueous) as an extraction solvent, but with an investigation on the influence of three regimes parameters; temperature, sample-to-water ratio and extraction time on the optimum extraction of these two compounds.

Some researchers used boiling water temperature (100°C) to extract phytochemicals such as total phenolics and antioxidant activities from Mas Cotek (Adam *et al.*, 2007; Oh *et al*., 2011; Hasham *et al*., 2013; Misbah *et al*., 2013). While Sulaiman *et al.* (2008) and Dzolin *et al*. (2010) had selected water temperature of 60°C to be used in their study. Wahid *et al.* (2010) varied the water temperature at 55, 75 and 100°C. The findings from this study showed that the total phenolics decreased but the antioxidant increased as the water temperature increased from 55 to 100°C. Additionally, some of these studies assigned their sample-to-water ratio at 1:16 (g/mL) (Oh *et al*., 2011; Hasham *et al.*, 2013). Other researchers including Abdulla *et al.* (2010), Dzolin *et al.* (2010), Hakiman and Maziah (2009), Misbah *et al.* (2013) used sample-to-water ratios of 1:5, 1:10, 1:20 and 1:50 (g:mL). Wahid *et al*. (2010) observed that the highest total phenolics and antioxidant activity are recorded at lowest sample-to water ratio. Among these studies, the longest extraction period carried out was 48 hours. The common extraction periods were allocated for 1 to 4 hours in most recent studies available (Adam *et al*., 2007; Abdulla *et al*., 2010; Adam *et al*., 2012; Misbah *et al.*, 2013).

Some of the methods use distilled water as an extraction solvent because it offers better choice over other types of solvents such as chloroform, hexane, petroleum ether and methanol (Aris *et al.*, 2009; Suryati *et al.*, 2011; Farsi *et al*., 2014) due to its merit of being non-toxic. It is also safer in handling and more importantly acceptable for human consumption (Chew *et al*., 2011). As a polar solvent, water has superior extraction efficacy which also offers a better option in obtaining antioxidant-rich extracts like Mas Cotek leaves (Wong *et al.*, 2006). Previous related studies were focusing on the activity determination of Mas Cotek leaves such as antioxidant activity (Farsi, 2011), anti-inflammatory (Abdullah *et al*., 2009) and antidiabetic (Farsi *et al*., 2014) and their effects on hyperglycaemia. Contrarily, this study is emphasizing on the extraction of two antioxidant compounds, vitexin and isovitexin using water as extraction solvent under three regimes parameters of temperature, sample-to-water ratio and extraction time. It also highlights the effects of extraction on mass transfer, vapour pressure and effects of natural properties of these particular phenolic compounds on water polarity, solubility and surface structure of dried extracted Mas Cotek leaves samples.

This study aimed to obtain the optimum yield of vitexin and isovitexin from Mas Cotek leaves using aqueous extraction by investigation of the influence of extraction temperature, time and sample-to-water ratio on the optimum extraction of these compounds.

Materials and Methods

Most experimental work and chromatographic analyses were performed in the Bioprocess Laboratory of Faculty of Chemical and Natural Resources Engineering (FKKSA-UMP, Kuantan, Pahang, Malaysia).

Chemicals

Distilled water was used for the extraction of

vitexin and isovitexin compounds from Mas Cotek leaves using conventional technique of extraction (aqueous extraction). Solvents of high performance liquid chromatography grade (methanol, formic acid (98-100%) and acetonitrile) which were used for HPLC chromatographic analysis were obtained from Merck (Darmstadt, Germany). Standards of vitexin and isovitexin (ChemFaces-CFN98601, CFN98620) were in ≥98% purity purchased from Wuhan ChemFaces Biochemical CO., Ltd. (Wuhan, Hubei, PRC).

Plant material

Mas Cotek or *Ficus deltoidea* (var. *deltoidea*, 2 kg) leaves used in this study were harvested from a local plantation in Muadzam Shah, Pahang, Malaysia. The Mas Cotek leaves were sent to a milling factory in Johor Bahru to undergo further processes of drying, cleaning and grinding into smaller particle size of ~850 µm (ASTM No.20, 20 Mesh) and then sieve through a wire mesh on a sieve shaker (Fritsch, Germany) to remove the midrib materials. The samples were kept in a freezer at -4°C until experiments were on.

Apparatus and equipment

Aqueous extraction of samples was done in a 500 mL beaker placed on a magnetic stirrer hotplate MS 300 with a TP-100P special-purpose sensor probe for temperature control from Bante Instruments Limited, Shanghai, PCR. A laboratory thermometer was used to monitor the effectiveness of the temperature control. The mixture of distilled water and samples were stirred thoroughly at uniform stirring of 250 rpm. Aluminium foil was used to cover the top of the beaker to minimize the evaporation and to maintain the mixture ratio throughout the extraction process. The crude extracts were centrifuged in a Tabletop Centrifuge Model 4000 from Kubota, Japan, and then filtered using a vacuum pump from Zhejiang Value Mechanical and Electrical Products Co., Ltd., Zhejiang, PCR. The filtered extracts were analysed by HPLC (Agilent, CA, USA).

The HPLC used in this study consisted of a computer-controlled system with G1379A Degasser, G1311A QuatPump and G1321A fluorescence detector. A reverse-phase Phenomenex (Torrance, CA, USA) column (Synergi, C18, 4 µ, 250 x 4.60 mm i.d.) was used for the detection of both vitexin and isovitexin compounds. Data acquisition was performed by Agilent Chemstation B.04.03[16]. The column temperature was maintained at 25°C. Isocratic HPLC elution was used that consist of [A] 1.0% formic acid, water and [B] methanol. The mobile phases were filtered through a 0.2 µm nylon membrane and degassed using an ultrasonic bath (Crest Ultrasonics, Trenton, New Jersey) prior to use. The flow rate was set at 1.0 mL/min. Fluorescence detection was conducted at 335 nm wavelength.

Screening study procedures using temperature, sample-to-water ratio and extraction time parameters for vitexin and isovitexin analysis

A screening study on the aqueous extraction of vitexin and isovitexin compounds from Mas Cotek leaves was carried out to determine the optimum parameters (Temperature: 50 -100°C; Sample-to-water ratio: 1:10 – 1:50 g/mL; Extraction time: 240 minutes) that would be appropriate to be used in further experiments. These parameters were chosen based on the results from a study conducted prior to this preliminary study. In the preliminary study, three ranges of sample-to-water ratios were tested, which are 1:10, 1:27.5 and 1:45 (g/mL) in 2 hours and 30 minutes. Five samples with three reaction temperatures of 55, 77.5 and 100°C gave the concentration of vitexin and isovitexin compounds ranging from 29.19 to 155.06% and 1.85 to 5.78% respectively. The main purpose of this study is to confirm the availability of vitexin and isovitexin compounds in the sample of Mas Cotek leaves. The findings from this study had also become a benchmark for the selection of parameters in the preliminary study of aqueous extraction.

These findings also align with some previous studies conducted by some researchers and their findings (Mohd Lip *et al.*, 2009; Sirisha *et al*., 2010; Wahid *et al*., 2010). For the determination of extraction parameters, the results obtained from this preliminary study were analysed and the best parameters were selected for both vitexin and isovitexin compounds extraction. Temperatures of 50, 70 and 100°C were assigned at each sample-to-water of 1:10, 1:20 and 1:30 (g/mL) for 8 hours' extraction.

In order to find out the quantitative yield of vitexin and isovitexin compounds in the extracted leaves of Mas Cotek, HPLC analysis was carried out for all samples. For HPLC analysis, 100 ppm of vitexin and isovitexin stock solution was prepared and diluted in ultrapure water (Merck Millipore, USA). Calibrating curves of individual compounds of vitexin and isovitexin (ranging between 0.01 – 0.08 mg/ml) were prepared by diluting stock solution with the ultrapure water. A series of mixed dilution of vitexin and isovitexin standards were also prepared. The calibration curves obtained by plotting peak area of a series of analyses versus vitexin and isovitexin concentration (ranging between 0.01 – 0.10 mg/

ml) gave the linear regression equation with a determination coefficient (R^2) of 0.9889 and 0.9954 respectively.

The concentration of both compounds in Mas Cotek leaves extracts were calculated by using the calibration equation of reference compounds. Based on the obtained concentration, actual percentage weight (%w/w) of both vitexin and isovitexin were calculated by using the following formula (Equation 1).

$$(\%w/w)= [C][V][DF]/[W] \ X\ 100 \qquad (Eq.1)$$

where C is the concentration of compounds calculated from HPLC analysis (mg/L), V is the volume of solvent/water used during the extraction, DF is the dilution factor and W is the weight of sample used during the extraction.

Effect of sample-to-water ratio on vitexin and isovitexin extraction yield

Sample-to-water ratios of 1:10, 1:20, 1:30, 1:40 and 1:50 (g/mL) were initially utilized at 100 ± 0.06°C. Boiling temperature of 100°C was chosen in this method because it is commonly applied in majority decoction system for the extraction of active compounds involving heat. As the duration at which the optimum yield of active compound is unknown the extraction was completed within 4hr. Vitexin and isovitexin compounds were analysed using HPLC.

Effect of temperature on vitexin and isovitexin extraction yield

The effect of temperature for the screening study of vitexin and isovitexin extraction was done using 50, 60, 70, 80, 90 and 100°C. In this study, the extraction time remained constant at 4 hours. The optimum parameter of sample-to-water ratio (1:30 g/mL) from previous method was applied for all temperature ranges.

Effect of time on vitexin and isovitexin extraction yield

In order to study the effect of time on the extraction of vitexin and isovitexin, the optimum sample-to-water ratio (1:30 g/mL) and temperature 50°C were selected from the previous two methods. The experiment was carried out for 4 hours and 10mL extracts were sampled every hour, starting from the first hour of extraction until the experiment was terminated. All the crude extracts from screening study were further analysed by HPLC for the determination of quantitative yield of vitexin and isovitexin compounds.

Aqueous extraction procedures

Representative samples of 27.27 ± 0.002, 14.29 ± 0.004 and 9.68 ± 0.003 g dry Mas Cotek leaves were extracted with distilled water in sample-to-water ratios of 1:10, 1:20 and 1:30 g/mL respectively. The volume of infusion was set at 300 mL for each mixture ratio in order to extinguish the volume differences. The extraction was carried out at 50.0 ± 0.05, 70.0 ± 0.09 and 100.0 ± 0.08°C for 8 hours with uniform stirring. A 10 mL of extract was sampled every hour. The sampled extracts were left to cool at room temperature and centrifuged at 5800 rpm for 10 minutes. The supernatant was separated from the sediment by filtration and then kept at 4°C prior to HPLC analysis. The ratios and temperatures were selected from the results obtained in screening study. The procedures applied in this study were similar to the procedures exerted in screening study, except for the use of parameter ranges (temperature, ratio and time).

Morphological study using FE-SEM

The morphological study on the surface of dried extracted sample of Mas Cotek leaves was carried out by using Field Emission-Scanning Electron Microscopy (JEOL, Tokyo, Japan). In FE-SEM analysis, the solid remains of Mas Cotek leaves were collected and dried in a 60°C oven for overnight after the extraction was terminated. Prior to the FE-SEM observation, a thin layer of sample was mounted using colloidal graphite upon the stub and dried thoroughly before loading into the load lock. Several magnifications were taken into consideration in observing the morphological surface of samples.

Statistical analysis

The observations were replicated thrice for each parameter. Experimental results were expressed as mean values and standard deviation was calculated. Statistical analysis was performed using analysis of variance (ANOVA). Differences were considered to statistically significant at $p<0.05$.

Results and Discussion

Screening study

Effect of sample-to-water ratio on vitexin and isovitexin extraction yield

The first three lower ratios of 1:10 to 1:30 (g/mL) as shown in Figure 1(a) exhibited higher yield of both compounds compared to the larger ratio of sample-to-water. Among these three ratios, the highest yields of vitexin and isovitexin were recorded from 1:30

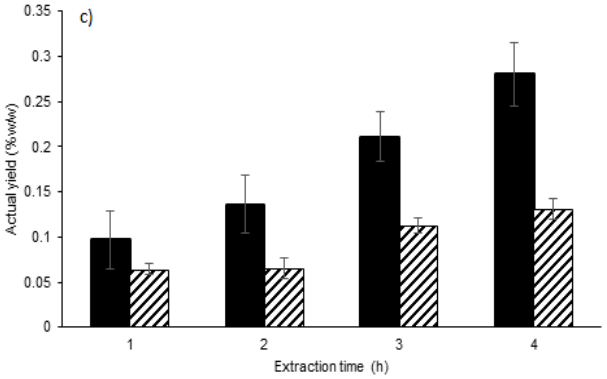

Figure 1. Effects of (a) sample-to-water ratio (4 h; 100 °C; n=6), (b) temperature (4 h; 1:30 g/mL; n=6) and (c) extraction time (50°C; 1:30 g/mL; n=6) on the average yield of vitexin and isovitexin from Mas Cotek leaves extracts.

(g/mL) ratio with values of 0.248% and 0.149% respectively. The increasing yield from 1:10 to 1:30 (g/mL) is attributed to the mass transfer principles of the process. The increase in the ratio of sample-to-water can result in higher concentration gradient during the diffusion so the analytes (solutes) will have higher tendencies to leave the matrix and move into the solvent (Rezaei *et al.*, 2013).

It can also be said that in 1:30 (g/mL) sample-to-water ratio, an appropriate amount of sample was added to an adequate amount of water to release the highest yield of active compounds at boiling temperature as higher concentration of phenolic compounds can be obtained from a higher concentration gradient with an increase in solvent amount. However, such increase in the yield of compounds was not observed as the ratio increase from 1:30 to 1:50 in this study.

This might be due to the limited content of the extractable phenolic compounds, which can be extracted with a 1:30 (g/mL) level, where additional increase in the ratio cannot locate any further polyphenolic compounds from Mas Cotek leaves. Excessive solvent volume could prevent sufficient stirring of the solvent in the extraction and may cause fewer yields of extracted compounds. Meanwhile in ratio 1:50 g/mL, the lowest yield of both vitexin and isovitexin compounds were observed due to the immoderate amount of solvent to the sample and caused much less detection of active compounds because of the insufficient stirring during the extraction (Savjani *et al.*, 2012).

The results obtained in Figure 1(a) were consistent with trends reported by Wahid *et al.*, (2010) but in terms of antioxidant activity. Sample-to-water ratio of 1:10, 1:20 and 1:30 (g/mL) were selected to be applied in the next extraction methods. The error bars

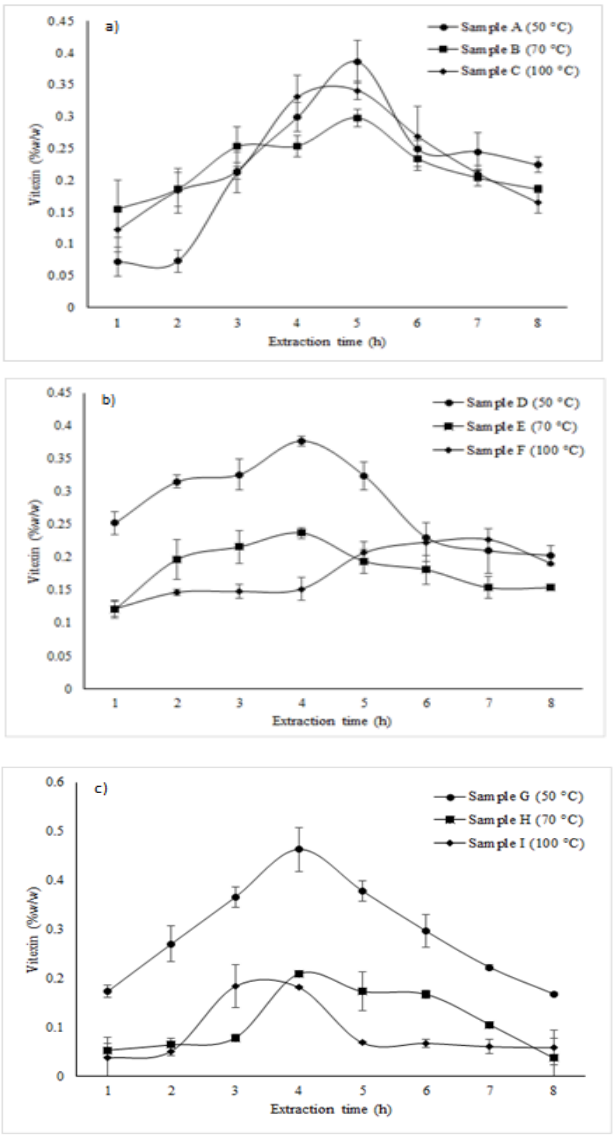

Figure 2. Yield of vitexin compound in aqueous extraction method from (a) 1:10, (b) 1:20 and (c) 1:30 (g/mL) at temperatures of 50, 70 and 100 °C in 8 hours of extraction.

in all graphs demonstrated a good reproducibility of the extraction. The screening study of AE was continued with a series of extraction temperatures at 50, 60, 70, 80, 90 and 100°C using the selected sample-to-water ratio.

Effect of temperature on vitexin and isovitexin extraction yield

Figure 2 shows the average yield recorded at each temperature for 4 hours of extraction. The yield of vitexin at the first three lower temperatures of 50, 60 and 70°C were consistent at 0.183% - 0.185%. Spigno *et al*. (2007) claimed that mild heating was found to have the ability to soften the plant tissues, weaken the cell wall integrity and favoured the release of bound active compounds. It seems that at starting temperature of 50°C, the extracted vitexin compound had achieved the high capacity even though the

temperature was increase to 60 and 70°C. It also explained the highest yield of isovitexin obtained at 50°C. However, at 80°C the yield decreased by 26% and shows a gradual increase at 90 and 100°C.

The decline values of vitexin and isovitexin at 80°C showed that at this point, more other phenolic compounds that present in Mas Cotek leaves extract had started to escape the cellulosic plant cell wall and some of them might had hindered or disguised the detection of these two compounds. The highest yield of vitexin compound (0.231%) was reported at 100°C. Juntachote *et al.* (2006) reported that elevated extraction temperature would increase the mass transfer of phenolic compounds by reducing the solvent viscosity and surface tension and hence, promoting the extraction of phenolic compounds. Increasing temperature favored extraction by enhancing solubility of active compounds and increasing the diffusion coefficient, which increased the extraction rate and explained the increased of mass transfer. The temperature dependence of solid solubility is determined not only by the ideal solubility, but also by changes in the activity coefficient with the temperature (Gracin *et al*., 2002).

In terms of vapor pressure, the higher the temperature of extracted solution, the kinetic energy of the molecules increased. As more molecules transitioning into vapors increase, the vapor pressure increases (Turnbull, 1956). However, in this case, the yield of both compounds increased as the temperature increased. It showed that these compounds are non-volatile, therefore; the vapor pressure of their molecules are low or negligible (Bhattacharyya *et al*., 2006).

As both compounds showed stable values at boiling temperature, it can be claimed that the bioavailability of these bioactive compounds relative to high temperature had almost no detrimental effects (González-Ferrero and Sáiz-Abajo, 2015). While a fluctuate trend was observed in the yield of isovitexin at 50 to 100°C with the highest yield recorded at 50°C with the value of 0.142%. Due to the differences in the optimum yield of vitexin and isovitexin, temperatures of 50, 70 and 100°C were selected to be applied in the next extraction methods.

Effect of time on vitexin and isovitexin extraction yield

Based on the findings from previous sections, a graph of average active compounds' yield against extraction time was plotted as shown in Figure 1(c). The graph shows that both compounds exhibited increasing pattern from the first hour to the 4th hours of extraction. This increasing pattern of both compounds was contributed by their same molecular structure and mass. Huge differences in the values however were observed in the yield of vitexin compared to isovitexin. From this result, it can be observed that as the extraction time increase, the yield of active compounds increased due to the damage of plant cell wall caused by the heat. When this happened, more active compounds or intercellular constituents were released to the surrounding medium. Both vitexin and isovitexin compounds show the highest value of 0.280% and 0.131%, respectively at fourth hour of extraction. Due to this increasing pattern, there seemed to be a need for the extraction time to be prolonged because it cannot be ensured that which extraction time would give the optimum yield of compounds if the period is limited to 4 hours and also to proof that 4th hour is the best extraction time. For the next extraction, the reaction period was prolonged to 8 hours to make sure that all intercellular compounds of interest can escape its cellulosic wall in Mas Cotek extracts.

Aqueous extraction

Aqueous extraction was carried out using the parameters selected in screening study. Factors that might affect the aqueous extraction were studied including the temperature, sample-to-water ratio and extraction time interval in 8 hours. Total nine samples with replicates were analysed and labelled as A, B, C for sample-to-water ratio of 1:10 [refer to Figure 3(a) and 3(a)], D, E, F for 1:20 [refer to Figure 3(b) and 3(b)] and G, H, I for 1:30 (g/mL) [refer to Figure 3(c) and 3(c)]. Each sample was tested at temperatures 50, 70 and 100°C. The highest value of vitexin (0.463%) was recorded from sample G [Figure 2(c)] at 4th hour of extraction with sample-to-water ratio 1:30 at 50°C. This finding is in line with the result obtained from screening study.

Meanwhile, for isovitexin compound, the highest yield (0.136%) was obtained from sample D [Figure 3(b)] at 5th hour of extraction with sample-to-water ratio of 1:20 at 50°C. Both results indicated that at 50°C in 8 hours of extraction, more active compounds were released to the surrounding medium compared to other two tested temperatures at 70 and 100°C. As explained earlier, higher temperature not necessarily accompanies with high extraction of active compounds as the mild heating of 50°C would also favor the extraction process by promoting both solubility of solute and diffusion coefficient. However, at higher temperature, the increasing yield of compounds was mainly favored by the increasing in mass transfer of compounds. Both lower and higher temperature might enhance the productivity

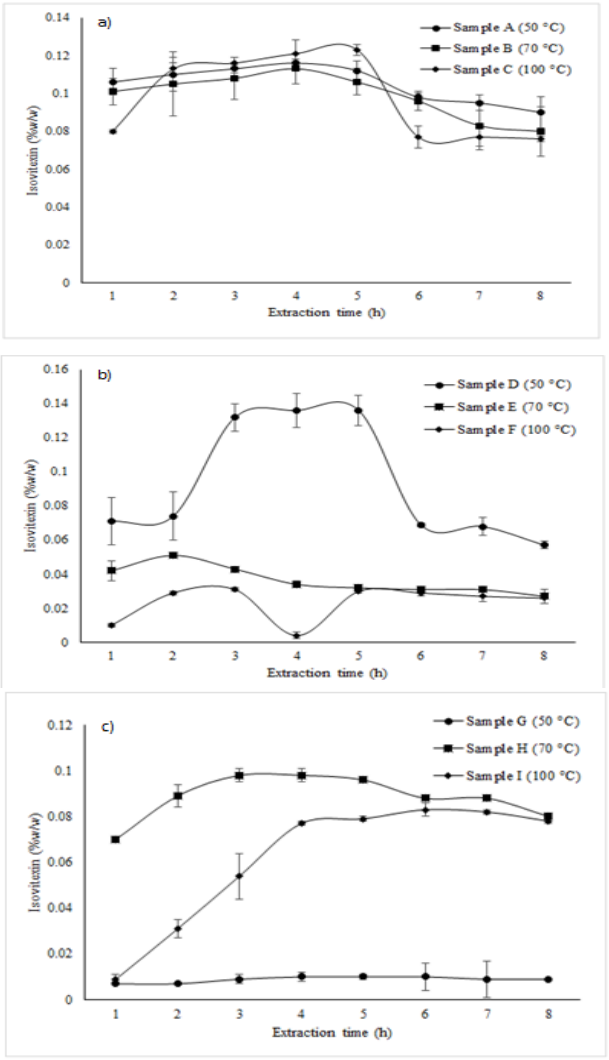

Figure 3. Yield of isovitexin compound in aqueous extraction method from (a) 1:10, (b) 1:20 and (c) 1:30 (g/mL) at temperatures of 50, 70 and 100 °C in 8 hours of extraction.

of extraction, but it also depends on other conditions of the extraction system including the ratio and time.

Figure 2 and 3 also show that the value of vitexin and isovitexin are mostly decreased after 4th and 5th hour of extraction until the end hour of the experiments. Previous studies have reported that prolonged extraction lead to a decrease in the phenolic content of crude extract because the prolong extraction results prolong exposure to environmental factors such as light and oxygen which causes oxidation of phenolic compounds (Chirinos *et al*., 2007; Chan *et al.*, 2009). Naturally, phenolic compounds are reactive species toward oxidation, notably the complex mixture of phenolics found in plant may undergo autoxidation during the ageing process. This explained the low yield of phenolic compounds caused by oxidation as the extraction takes place. In terms of economical point of view and also taking into consideration of the yield of active compounds, 4th and 5th hour was chosen as the optimal extraction time for vitexin and isovitexin, respectively as excessive extraction time was no longer useful to extract more active compounds from Mas Cotek leaves.

Statistical analysis using ANOVA for the yield of vitexin and isovitexin compounds shows that the factors of sample-to-water ratio and extraction time ($p<0.05$) were important parameters in aqueous extraction of both compounds. The effects of temperature (50, 70 and 100°C) on aqueous extraction were not significant maybe due to the insignificant difference in the yield of active compounds recorded.

Two stages of extraction could be observed, an initial increase in the yield of vitexin and isovitexin in the beginning of the process followed by slow extraction (after 5th hour) characterized by a low enhancement of vitexin and isovitexin content with the progress of extraction. This explained the small differences in the yield of isovitexin (Sample D) from 3rd to 5th hour extraction at 50°C [Figure 3(b)]. Type of solvent used in an extraction system gives a strong effect on the extraction activity (Chavan and Amarowicz, 2013; Nur Syukriah *et al.*, 2014).

As the selection of extraction solvents is critical for the complex plant materials, some considerations should be taken into accounts including the purpose of extraction, polarity of the interested components, polarity of undesirable components, overall cost, safety and also environmental concern (Wang *et al.,* 2008). Tan *et al*. (2013) claimed that active compounds can be easily extracted when they are well matched in polarity with the solvent. Otherwise, Dent *et al*. (2015) reported that as the difference in polarities of the extracting solvents might influence the solubility of the chemical constituents in a sample and its extraction yield; different polarities of the solvent and active constituents contribute the best yield.

In the present study, water is used as the extraction solvent. Other than its merit of being non-toxic and safe for human consumption, the strong polarity of water and low volatility had become the main reasons for the selection even though the influence in the qualitative composition of the extracted compounds are lower than those using other solvent such as methanol (Farsi *et al*., 2014), hexane and butanol (Farsi, 2011). A study conducted by Chen *et al.* (2016) reported that there are so many water soluble substances like sugar, protein and starch in plant sample. These water soluble substances can form a barrier that resists the extraction solvent to get into the plant material when other types of solvents were used (i.e. ethanol-based solvent). Thus, no

Table 1. Summary for the reference values and optimum experimental values of vitexin and isovitexin compounds from aqueous extraction

Extraction methods	Optimum value (%w/w)[a]	
	Vitexin	Isovitexin
Reference aqueous extraction		
Farsi *et al.* (2011)	0.427	0.191
Farsi *et al.* (2014)	0.243	0.098
Abdullah *et al.* (2009)	0.548	N/A
Aqueous extraction (AE)	0.386*	0.116*

Note: a Mean of six determinations (n = 6) from three replications.
*Significant at 0.05 level (when compared to reference value)

chemical marker will be extracted. When water is used as extraction solvent, it dissolves and removes these water soluble substances, therefore increases the availability of chemical marker to be extracted. Moreover, as vitexin and isovitexin are C-glucosides of apigenin, more phenolic hydroxyl groups exist in their molecules; therefore, they possess a moderate polarity and good solubility in water. Thus, water is the best choice to be used as solvent for the extraction of vitexin and isovitexin compounds from Mas Cotek leaves in this study.

Table 1 summarizes the experimental results of vitexin and isovitexin compounds from aqueous extraction method studied compared to the reference values (Abdullah *et al.,* 2009; Farsi 2011; Farsi *et al.*, 2014). There are some relative differences in the optimum yield obtained for vitexin and isovitexin compounds in this extraction method. As the same extraction solution is used to analyse both compounds, the disparity may mainly affect by numerous extrinsic and intrinsic control conditions and parameters (O'Donnell *et al.*, 2010). The differences in the yield of vitexin and isovitexin from Mas Cotek compared to the previous studies may be related to genuine interspecies differences or geographical and environmental effects. Although the same species of plant leaves were used in all four studies, the content of active compounds may vary from one study to another as the plant materials were collected from various sources (Omar *et al.,* 2011).

It is important to understand that the selection of only two chemical markers, vitexin and isovitexin in this study is mainly to indicate the effects of parameters applied such as temperature, time and sample-to-water ratio. As stated by the European Medicine Agency (2008), any constituents or groups of a herbal medicinal products which are of interest for quality control purposes regardless any therapeutic activity they possessed are called chemical markers. Therefore, the use of these two antioxidant components are entirely justified and appropriate to show their higher values obtained from optimum parameters and lower values from less effective parameter conditions. It is ideal to classified chemical markers as unique components that contribute to the therapeutic effects of a herbal medicine (Li *et al.*, 2008). Under the category of therapeutic components, vitexin and isovitexin are not only exist in the extracted sample of Mas Cotek leaves, but also majorly contributed to the medicinal effects of this herb (Bunawan *et al.*, 2014). In this study, the proposed extraction conditions are related to the release of vitexin and isovitexin compounds, hence it will affect the strength of medicinal activity of Mas Cotek leaves extracts. Other compounds may exist as chemical markers, but with the same therapeutic effects as vitexin and isovitexin or listed under different categories such as characteristic or synergistic components (Li *et al*., 2008). In addition, as isomers compounds, the detection and separation of vitexin and isovitexin from Mas Cotek leaves extracts can be done using the same analytical method, which contribute to a time and energy consuming procedure.

Also, even though water was used as extraction medium, the parameters applied in each study such as parameters, sample-to-water ratio and duration of extraction may not be similar to one study to another. These differences may contribute to the low and high extraction of vitexin and isovitexin compounds. In fact, in the study conducted by Abdullah *et al*. (2009), the yield of isovitexin was not applicable because the dominant or main component in their sample of F. deltoidea var. deltoidea was vitexin. Therefore, it was observed that vitexin yield from their study was the highest compared to other studies. When compared to the present study, both vitexin and isovitexin compounds existed in the sample but the values were in-between and still comparable to other findings listed. Thus, in order to overcome the low yield of compounds, more advanced and efficient extraction methods may be needed (Mohd Abdul Alim *et al*., 2016).

Surface characterization using FE-SEM

The morphological study on the surface structure of dried Mas Cotek leaves sample was carried out using Field Emission Scanning Electron Microscopy (FE-SEM) after the extraction process from each method. The 300 and 1000x magnifications were chosen in this observation. Figure 4 shows the micrographs of Mas Cotek leaves from aqueous extracted sample to illustrate the cell damage caused

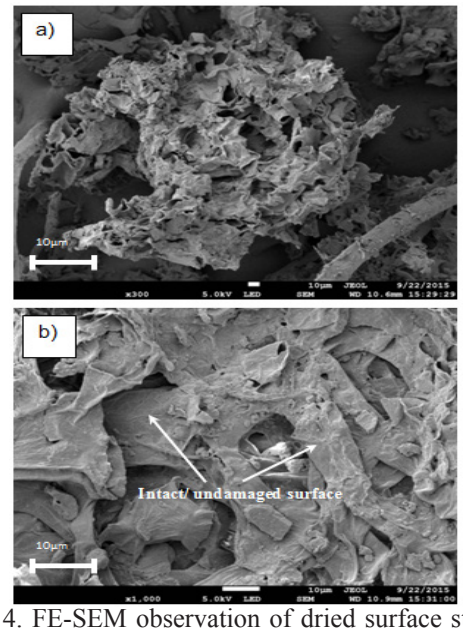

Figure 4. FE-SEM observation of dried surface structure of Mas Cotek leaves from aqueous extracted sample at (a) x300 and (b) x1000 magnifications (1:20 g/mL; 100 °C; 5th hour of extraction).

by the aqueous extraction method. The surfaces of dried Mas Cotek leaves exhibited significant variations in shape and size when viewed under FE-SEM. At x300 magnification, the bulk size with less damage of leaves particles could still be observed from the sample surface [Figure 4 (a)]. An intact, smooth surface could also be observed as shown in Figure 4 (b) under x1000 magnification with a few number of pores on the surface.

Based on these morphological observations, it generally shows that aqueous extraction caused a very little damage to the surface structure of Mas Cotek leaves sample. This may affect the amount of active compounds released from the plant cell, as less destruction occurred on the cell wall and low mass transfer rate between the sample constituents and solvent. Therefore, it explained the low yield of vitexin and isovitexin compounds from aqueous extraction method, as compared to other reference methods.

Conclusions

Extraction parameters of temperatures, sample-to-water ratio and time were successfully studied in aqueous extraction of Mas Cotek leaves. Temperature of 50°C gave the highest yield of both vitexin and isovitexin compounds. While the best sample-to-water ratios for extraction of vitexin and isovitexin were 1:30 and 1:20 g/mL, respectively. Higher yield of both compounds were recorded at 4th to 5th hour of extraction, which explained the increasing mass transfer of compounds at that particular time. Apparently, different temperatures combined with different sample-to-water ratio yields inconsistent amount of active compounds, thus need further research. The effect of aqueous extraction was also observed on the surface structure of dried Mas Cotek leaves under FE-SEM observation. A less damaged and intact surface was observed, indicating that less destruction of plant cell wall caused by aqueous extraction at boiling temperature. The low amount of vitexin and isovitexin released from Mas Cotek leaves may overcome by modern extraction techniques, hence there is a need to do further studies.

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