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Effects of extraction solvent system, time and temperature on total phenolic content of henna (*Lawsonia inermis*) stems

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Abstract

Henna plant (*Lawsonia inermis*) is an Indian medicinal plant used in traditional medicine for the treatment of various diseases, besides its popularity as a natural dye to colour hand and hair. Research in the recent past has accumulated enormous evidence revealing henna plant to be an excellent source of antioxidants such as total phenolics. In this study, the extraction of total phenolics from henna stems was evaluated using the Folin-Ciocalteu assay. A set of single factor experiments was carried out for identifying the optimum condition of each independent variable affecting total phenolic content (TPC) extraction efficiency of henna stems, namely the solvent type, solvent concentration (v/v, %), extraction time (min) and extraction temperature (°C). Generally, high extraction yield was obtained using aqueous acetone (about 40%) as solvent and the extraction yield could further be increased using a prolonged time of 270 min and a higher incubation temperature of 55°C. Under these optimized conditions, the experimental maximum yield of TPC of 5554.15 ± 73.04 mg GAE/100 g DW was obtained.

Introduction

Henna (*Lawsonia inermis*), an Indian medicinal plant, is a perennial shrub native to India, North Africa, Asia and Australia (Wyk and Wink, 2004). It has been recorded that different parts of henna plant are a rich source of various bioactive principles and has been used in traditional medicine (Dasgupta *et al*., 2003). Recent study on phytochemical content in henna has shown that it is rich in phenolic antioxidants such as lawsone, flavonoids, tannins and coumarins (Khare, 2007).

Phenolic compounds, cyclic derivatives of benzene with one or more hydroxyl groups associated with the aromatic ring, account for one of the largest and most widely distributed group of phytochemicals (Andjelkovic *et al*., 2006). They vary considerably in structure with over 8000 naturally-occurring compounds having been identified (Balasundram *et al*., 2005). They may exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-viral, cardioprotective and vasodilatory effects (Balasundram *et al*., 2005; Tabart *et al*., 2007). The beneficial effects derived from phenolic compounds in human life have been attributed to their antioxidant activity that mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and metal-chelators (Rice-Evans *et al*., 1996). Therefore, phenolic compounds have been used in many antioxidant capacity assays before testing the properties of a biological system.

Extraction is the first important step in the recovery and purification of active ingredients from plant materials. The aim of an extraction process should be to provide for the maximum yield of substances and of the highest quality which consist of high concentration of target compounds and antioxidant power of the extracts (Spigno *et al*., 2007). Many techniques have been developed to extract phenolics, such as conventional solvent extraction, microwave-assisted, ultrasound-assisted and supercritical fluid extraction, among which solvent extraction (solid-liquid and liquid-liquid extraction techniques) is the most commonly used, and has

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proven to be a reliable and efficient method (Chirinos *et al.*, 2007; Banik and Pandey, 2008). The efficacy of solvent extraction is affected by many factors such as the type of solvent, solvent concentration, time, temperature, pH, number of steps, liquid-to-solid ratio and particle size of the plant material (Cacace and Mazza, 2003).

There are two most commonly used optimization studies, the classical single factor experiments and the response-surface methodology (RSM). The former is a one-factor-at-a-time approach, in which only one factor is varying at a time while all others are kept constant. Herein we used the single-factor experiments, despite being having some drawbacks, such as time-consuming, expensive, possible interaction effects between variables cannot be evaluated and misleading conclusions may be drawn (Bas and Boyaci, 2007; Bezerra *et al.*, 2008). However, single factor experiments are able to provide fundamental information on the ranges for significant extraction parameters on the extraction of phenolic compounds from plant materials. Up to now, solvent extraction of phenolic antioxidants from henna (*Lawsonia inermis*) stems using single factor experiments has not been reported. In considering the growing interest in assessing antioxidant capacity of herbal medicine, this study was therefore aimed (1) to determine the best extraction conditions (type of solvent, solvent concentration, extraction time and extraction temperature) for henna stems, in order to maximize simultaneously the yield of total phenolic content (TPC) by single factor experiments, and (2) to quantify the extracted phenolic contents in henna stems. This study provides an opportunity to evaluate the potential of henna stem as a natural source of potent antioxidants with potential medicinal value. In addition, the optimal ranges (minimum and maximum values) obtained for all extraction parameters in the present study can also be served as key information for the scale-up extraction of antioxidant compounds from henna stems.

Materials and methods

Plant material

Henna (*Lawsonia inermis*), a traditional medicinal plant species, was collected from lowland beside the main city campus of UCSI University, Cheras, Kuala Lumpur. The species has been identified and confirmed by Forest Research Institute Malaysia (FRIM), Kuala Lumpur, Malaysia.

Chemicals and reagents

All chemicals and solvents used were of analytical reagent (AR) grade. Folin-Ciocalteu's phenol reagent, sodium carbonate anhydrous (Na_2CO_3) (≥99.9% purity), absolute acetone (CH_3COCH_3) (≥99.5% purity) and methanol (CH_3OH) (≥99.8% purity) were purchased from Merck KGaA, Darmstadt, Germany. Gallic acid ($C_7H_6O_5$) (98% purity) was obtained from Acros Organics, Belgium, USA. Absolute ethanol (C_2H_5OH) (≥95% purity) was purchased from R&M Chemicals, Essex, UK. The distilled water (reverse osmosis (RO) water) used for the analysis was purified by Milli-Q Millipore water purification system (Millipore Corporation, Billerica, MA, USA).

Sample preparation

The fresh henna plants collected were first thoroughly washed upon arrival at the laboratory. The leaves removed in order to obtain the stems (approximately 400 g) which were then cut into a constant size of 0.5 × 2.0 cm. The cut-stems were then evenly oven-dried for 24 hours at 40°C. After drying, dried stems were ground into fine powder (0.5 mm) by a miller (Model MF 10 basic, IKA Werke, Germany) at 4000 rpm. The dried ground samples were subsequently vacuum-packaged into nylon-linear low-density polyethylene (LDPE) pouches by a vacuum-packaging machine (Model DZQ 400/500, Clarity, China) and stored in a sealed container (dark, dry and room temperature environment) for extractions.

Solvent extraction of phenolic compounds from henna stems

Solvent extraction was performed in a temperature controlled water bath shaker (Model WNB 7-45, Memmert, Germany) with a useful volume of 14 L (internal dimensions: 365 × 315 × 150 mm) at a constant shaking speed of 130 rpm. Firstly, 2 g of dried-ground samples were weighed accurately and placed into a conical flask made up to 20 ml volume with extracting solvent (solvent-to-solid ratio of 10:1). The flask was then covered with parafilm (Pechiney plastic packaging, USA) and wrapped with aluminium foil (Diamond, USA) in order to provide dark environment, and incubated for different lengths of times at the required temperature. After the extraction, the flask was removed from the water bath shaker and cooled to room temperature by cold running tap water. The henna stem extract was filtered through a sand core glass funnel with Whatman No. 1 filter paper (Whatman International Ltd., England, UK), and the clear solution of crude extract (filtrate) was collected in a light-protected amber bottle for the determination of analysis without further treatment. All the extractions were carried out in replicates.

Experimental design

Extraction conditions for TPC from henna stems, namely solvent type, solvent concentration, extraction time and extraction temperature, were determined by varying one factor at a time while keeping the others constant for delimitation of the experimental region. The fixed factors were the particle size of 0.50 mm and the solvent-to-solid ratio of 10:1.

Extraction solvent type evaluation

Samples were extracted with 60% (v/v) methanol, 60% (v/v) ethanol, 60% (v/v) acetone, distilled water and boiling distilled water respectively, following the procedures as described in Section 2.4. The independent variables were constant solvent composition of 60% (v/v), extraction time (180 min) and extraction temperature (25°C). The optimal extraction solvent was selected upon the highest value of TPC (mg GAE/100 g DW).

Extraction solvent concentration evaluation

Aqueous acetone selected in Section 2.5(a) was used as the extraction solvent for the following experiments. Phenolics were extracted from henna stem powder using different acetone concentrations, ranging from 20% (v/v) to 100% (v/v) by the same procedure described in Section 2.4 while holding the other two independent variables, which were extraction time (180 min) and extraction temperature (25°C) at a constant level. The optimal solvent concentration was selected upon the highest value of TPC (mg GAE/100 g DW).

Extraction time evaluation

Based on the solvent type and solvent concentration selections in Sections 2.5(a) and (b), 40% (v/v) acetone was selected as the optimal extracting solvent to extract phenolics from henna stem powder. By repeating the same procedure as described in Section 2.4, dried henna stem powder was macerated with 40% (v/v) acetone and incubated for different time periods ranging from 30 to 450 min at constant temperature of 25°C. The optimal extraction time was selected upon the highest value of TPC (mg GAE/100 g DW).

Extraction temperature evaluation

Henna stem powder was macerated with the optimal solvent type and solvent concentration selected in Sections 2.5(a) and (b), which was 40% (v/v) acetone. Samples were incubated at different temperatures ranging from 25 to 55°C for 270 min, which was the optimal time determined in Section 2.5(c). The extraction procedure was repeated as described in Section 2.4. The optimal extraction temperature was selected upon the highest value of TPC (mg GAE/100 g DW).

Determination of total phenolic content (TPC)

Preparation of solutions

Folin-Ciocalteu (F-C) reagent (10-fold diluted) was freshly prepared by topping up 10 ml of F-C reagent to 100 ml with distilled water in a 100 ml volumetric flask. Sodium carbonate (Na_2SO_3) (7.5%, w/v) was prepared by accurately weighing 7.5 g of anhydrous Na_2SO_3 with an analytical balance (AB204-S, Mettler Toledo, Switzerland), dissolved with some distilled water in a 100 ml beaker, and topped up to total volume of 100 ml with distilled water in a 100 ml volumetric flask.

Procedure

The TPC of henna stem extracts was determined spectrophotometrically using Folin-Ciocalteu's reagent according to the method described by Lim *et al.* (2007) with slight modifications. Briefly, the crude extracts obtained from the extract preparation were first approximately diluted 40 times. Three hundred microlitres (300 µl) of henna stem crude extracts were introduced into aluminium foil-wrapped test tubes followed by 1500 µl of 10-fold diluted Folin-Ciocalteu's reagent and 1200 µl of 7.5% (w/v) sodium carbonate solution. The contents of the tubes were mixed thoroughly by using a vortex mixer (Model LMS, Japan) for 10 s, covered with parafilm and allowed to stand in the dark for 30 min at room temperature. After 30 min, the absorbance of the deep blue colouration developed was measured against a blank at 765 nm using Uvi Light Spectrophotometer (Model XTD 5, Secomam, France). Blank reagent was prepared by replacing 300 µl of sample with equal amount of distilled water. Measurements were carried out in triplicate and calculations were based on a calibration curve obtained with gallic acid, which was $y = 10.422x + 0.0042$ ($R^2 = 0.9977$). The TPC were expressed as mg gallic acid equivalents (GAE) per 100 g of dry weight (DW). All analyses were performed in triplicate.

Statistical analysis

All experimental results in this study were expressed as mean values ± standard errors (SE) of six measurements ($n = 6$). In these single factor experiments, the significant differences ($p < 0.05$) among treatment means were determined by one-way analysis of variance (ANOVA) with Tukey's test, using Minitab statistical software (Version 15.1.1.0, Minitab Inc., PA, USA).

Results and Discussions

Effects of various solvent types on extraction of total phenolic compounds

Solvent extraction is the most common method used for isolation of phenolic antioxidants, and both extraction yield and extraction activity are strongly dependent on the solvent. Antioxidative potential of phenolic compounds is strongly affected by the polarity of solvent used in extraction. Hence, the selection of extraction solvents is critical for the complex plant samples. An extraction solvent system is generally selected according to the purpose of extraction, polarity of the interested components, polarity of undesirable components, overall cost, safety and environmental concern (Wang *et al*., 2008). Aqueous mixtures of acetone, ethanol and methanol have been widely used for extracting phenolic components from botanical materials, especially herbs and medicinal plants (Tabart *et al*., 2007; Wang *et al.*, 2008). Figure 1 shows the TPC results of henna stem extracts from five types of solvents as described in Section 2.5 were used. Aqueous acetone (60%, v/v) significantly ($p < 0.05$) showed the highest extraction capacity for phenolics from henna stems in comparison to the other solvents in this order: acetone (60%) > ethanol (60%) > methanol (60%) > distilled water > boiling water. Acetone, ethanol and methanol seem to have their distinct specificity in the extraction of polyphenolic substances. Many studies showed that acetone is the best solvent for proanthocyanidins and tannins extraction; ethanol effectively extracts flavonoids and their glycosides, catechols and tannins; whereas a better yield for phenolic acids and catechin is obtained with methanol (Chirinos *et al*., 2007; Mane *et al.*, 2007; Spigno *et al*., 2007; Tabart *et al.*, 2007). These facts are in accordance with polarity of the solvent used for the extraction and solubility of phenolic compounds in them. It is interesting to note that the polarity of acetone, ethanol and methanol is 0.355, 0.654 and 0.762, respectively. Aqueous acetone is a good solvent for polar antioxidants and more useful for extracting polyphenols from protein matrices, since they appear to degrade the polyphenol–protein complexes. Meanwhile, ethanol and methanol are more effective in extracting polyphenols linked to polar fibrous matrices (Chirinos *et al*., 2007; Al-Farsi and Lee, 2008). In fact, the use of aqueous acetone has several advantages to the use of aqueous ethanol, aqueous methanol and pure water, for example higher extraction efficiency, suggesting the use of aqueous acetone as extraction solvent for the following stages in this study. However, there is still a need to check if using different water percentages in acetone (%, v/v) is possible to increase the extraction efficiency of TPC from henna stems in the present experiment.

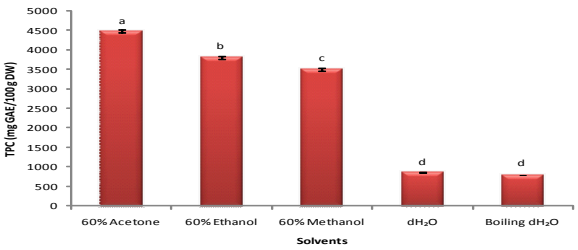

Figure 1. Influence of solvent type on extraction efficiency of total phenolic content (TPC) from henna stems. Values are means ± standard errors (SE) of six determinations (n = 6) from two extract replicate. Values marked by different letters indicate significantly different ($p < 0.05$).

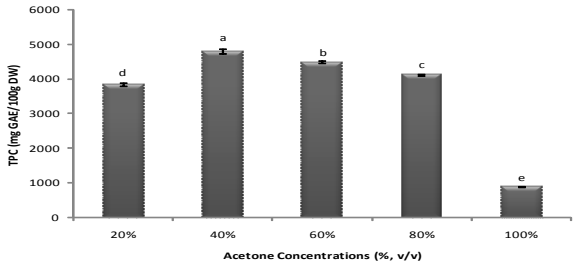

Figure 2. Influence of acetone concentration on extraction efficiency of total phenolic content (TPC) from henna stems. Values are means ± standard errors (SE) of six determinations (n = 6) from two extract replicate. Values marked by different letters indicate significantly different ($p < 0.05$).

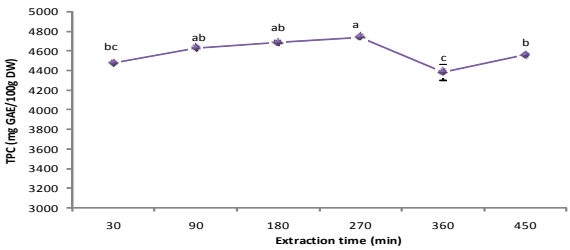

Figure 3. Influence of extraction time on extraction efficiency of total phenolic content (TPC) from henna stems. Values are means ± standard errors (SE) of six determinations (n = 6) from two extract replicate. Values marked by different letters indicate significantly different ($p < 0.05$).

Effects of solvent concentration on extraction of total phenolic compounds

The extraction of phenolic compounds from plant material is directly related to the compatibility of the phenolic compounds to the solvent and thus, when the compounds are well matched in polarity with the solvent they will be easily extracted. The effects of acetone concentration in the extraction solvent on the content of phenolics in henna stem extracts are shown in Figure 2. Based on Figure 2, the acetone concentrations had a significant effect ($p < 0.05$) on

the extraction efficiency of TPC from henna stems. As can be seen from Figure 2, the TPC as a function of acetone concentration follows a parabola shape. TPC of henna stem extracts reached a maximum at 40% acetone (v/v) (4796.78 ± 70.67 mg GAE/100 g DW) followed by a significant ($p < 0.05$) decrease with further increase in the concentration of the acetone in the extraction medium. Phenolic compounds in henna stem extracts might present a moderately polar profile. From Figure 2, it is evident that addition of a certain amount of water in acetone contributes to the creation of a moderately polar medium that ensures the extraction of polyphenols and thus improves the overall extracting efficiency. Acetone is a low-polar solvent while water is a strong polar solvent, and they can be blended with each other in any proportion. Hence, with the addition of water to acetone, the polarity of complex solvent will increase continuously. So the acquired ratio of more polar phenolic compounds in henna stem extracts increases with increasing water content according to "like dissolves like" principle (Chirinos *et al*., 2007; Zhang *et al.*, 2007). Another possible reason for the increased efficiency with the presence of some water might be due to the increase in swelling of plant material by water, which increases the contact surface area between the plant matrix and the solvent (Hemwimon *et al*., 2007). A moderately polar solvent of 40% acetone (v/v) was chosen for the determination of extraction time and extraction temperature.

Effects of extraction time course on extraction of total phenolic compounds

Extraction time was another important parameter influencing the extraction of phenolic compounds. Figure 3 showed that TPC of henna stem extracts increased gradually with increasing of the extraction time from 30 min (4478.22 ± 17.08 mg GAE/100 g DW) up to 270 min (4739.71 ± 10.08 mg GAE/100 g DW) and began to decline sharply until reaching a minimum of 4380.21 ± 78.51 mg GAE/100 g DW at 360 min. These phenomena could be well explained by the Fick's second law of diffusion, predicting that a final equilibrium between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) might be reached after a certain time, leading to deceleration in the extraction yield (Silva *et al*., 2007). Moreover, prolonged extraction time increases the chance of decomposition and oxidation of phenolics due to their long exposure to unfavourable environmental factors like temperature, light and oxygen (Naczk and Shahidi, 2004). On the other hand, the increased extraction time is uneconomical and time consuming from the industrialisation point of view, also potentially increasing the loss of solvent by vaporisation which directly affects the loss of solvent-to-solid ratio of extraction. Thus, extraction time of 270 min was selected as the optimum point for the subsequent step due to the practical and economical considerations, despite the higher yield of TPC.

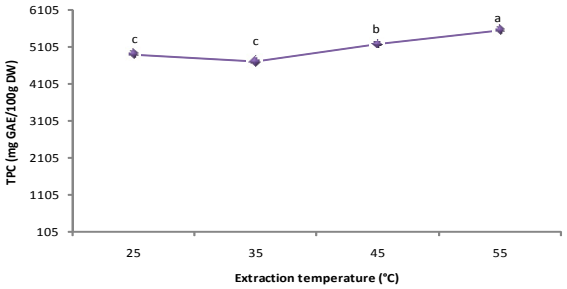

Figure 4. Influence of extraction temperature on extraction efficiency of total phenolic content (TPC) from henna stems. Values are means ± standard errors (SE) of six determinations (n = 6) from two extract replicate. Values marked by different letters indicate significantly different ($p < 0.05$).

Effects of extraction temperature on extraction of total phenolic compounds

The selection of an appropriate extraction temperature was the final step in a series of single factor experiments. The phenolics extraction yield as a function of the extraction temperature is shown in Figure 4. Results indicated that a significant ($p < 0.05$) increase in the extraction of total phenolics from 4894.65 ± 74.81 mg GAE/100 g DW to 5554.15 ± 73.04 mg GAE/100 g DW when increasing the temperature from 25 to 55°C. An exception is incubation at 35°C which resulted in an insignificant ($p > 0.05$) decrease in the total phenolics extraction, down to 4702.11 ± 43.75 mg GAE/100 g DW. This is due to the increased solubility and diffusion coefficients of phenolics; decreased solvent viscosity; as well as the enhanced mass transfer and penetration of solvent into the plant matrix (Al-Farsi and Lee, 2008; Hemwimon *et al.*, 2007; Silva *et al.*, 2007; Wang *et al.*, 2008), thus accelerating the whole extraction. On the other hand, according to Shi *et al.* (2003), heating might soften the plant tissue and weaken the phenol-protein and phenol polysaccharide interactions in the plant materials. Consequently, more phenolics would transfer to the solvent portion. Despite the positive effects of higher temperatures on the phenolics extraction, this cannot be increased indefinitely. Elevating the temperatures up to a certain level might be followed by their possible concurrent decomposition of antioxidants which were already mobilized at lower temperatures (Liyana-Pathirana and Shahidi, 2005). Other than that, denaturation of

membranes and a possible degradation of polyphenolic compounds caused by hydrolysis, internal redox reactions and polymerizations which are detrimental to the extraction yield may happen and influence quantification of bioactive compounds (Abad-Garcia *et al*., 2007). Moreover, it was also reported by Cacace and Mazza (2003) that certain phenolic compounds such as flavonoid families (mainly anthocyanin and flavan-3-ol derivatives) are heat sensible, hence an upper limit must be respected to avoid degradation of the thermo-sensitive phenolic compounds. Most importantly, since 40% acetone (v/v) is used for extraction in this study, the temperature must not go very high as acetone has a boiling point of 56.2°C. Therefore, a very high extraction temperature may evaporate acetone from the aqueous acetone solution and subsequently changes the acetone-to-water ratio. Manipulation steps and extraction costs are expected to increase with increasing of the extraction temperature. Considering the above facts, moderate extraction temperature at 55°C was selected as the optimal extraction temperature for this optimization design, which extracted the total yield of 5554.15 ± 73.04 mg GAE/100 g DW of TPC.

Reproducibility

The reproducibility of the measurements were assessed for henna stem extractions in six determinations (n = 6) from two extract replicate at four different parameter combinations. It was observed that the overall percentage relative standard deviation (% RSD) is very small and falls between the narrow ranges of 0.52 – 4.39%, confirming the high reproducibility of this extraction method.

Conclusions

In a nutshell, the experimental optimum conditions that allow fast, quantitative and maximum extractions of TPC from henna stems were obtained through the effective classical solvent extraction method as well as single factor experiments. Acetone was found to be the best solvent for the extraction. Subsequently, the optimum conditions for maximum TPC was found to be acetone concentration 40% (v/v), extraction time 270 min and operating temperature 55°C. These optimum conditions can be useful for further optimization of the extraction of phenolic antioxidants from henna (*Lawsonia inermis*) stems using response surface methodology (RSM) in conjuction with central composite rotatable design (CCRD). However, solvent extraction give reasonable recovery but it poses some disadvantages like the solvent need to be evaporated, adding extra cost and possible loss of quality. Therefore, other methods should be considered to extract phenolic contents from plant materials.

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