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Comparison of Alkaloid Yield Obtained using Conventional and Ultrasound-assisted Extraction from *Chromolaena odorata*

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Abstract. *Chromolaena odorata* or commonly known as 'Pokok Kapal Terbang' in Malaysia is a medicinal plant that has many important bioactive compounds for human health. In this study, berberine, one of the alkaloid compounds, was investigated. Berberine is famous for its broad range of bioactivities such as anti-inflammatory, antibacterial, antidiabetic, and antiulcer. The main purpose of this study is to evaluate and compare the effects of two extraction methods on berberine yield from the whole plant of *Chromolaena odorata*. The extraction process was carried out using the conventional method which is maceration and non-conventional extraction using ultrasound-assisted extraction (UAE). UAE is known to be an environmentally green extraction method and is expected to have a high extract yield, shorter time, and eco-friendly extraction technologies compared to a conventional method. Comparison between both methods was done at different extraction times (20−60 minutes). First, phytochemical screening was performed to observe the presence of berberine yield. Next, berberine concentration was measured using High-Performance Liquid Chromatography Diode Array Detector (HPLC/DAD) at gradient system, and the surface morphology was observed using FESEM. HPLC analysis showed that maximum berberine yield was obtained with the extraction yield of 2.7228% at 40 min for UAE, while only 0.2193% at 60 min for the conventional method. From the FESEM image, a small rupture was observed from the conventional sample, while a large perforation can be seen from the UAE sample. The larger perforation supported the idea that more cells were ruptured to release more yields. This finding showed the effectiveness of the UAE method in extracting berberine from *Chromolaena odorata*.

INTRODUCTION

Chromolaena odorata or *Eupatorium odoratum* (Asteraceae) is an herb shrub that can be found in tropical Asia, North America, West Africa, Texas to Mexico, and Australia. *Chromolaena odorata* is also known as "Awolowo", "Independence weed", Siamese weed, bitter bush, triffid weed, jack in the bush [1], Christmas Bush, Common Floss Flower [2], and Pokok Kapal Terbang or Pokok Malaysia [3]. The leaf extract of *Chromolaena odorata* has antioxidant, anti-inflammatory, analgesic, anti-microbial, cytoprotective, and many other medicinally significant properties. The ability of wound healing is attributed to the antioxidant property of the drug which helps in conserving the fibroblast and keratinocyte proliferation on the site [4,5]. These valuable pharmacological activities come from a

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phytochemical compounds such as flavonoids, alkaloids, essential oil, phenolic, tannins, and saponins. In this study, the main significant chemical compound that needed to be extracted was berberine.

Berberine is a quaternary benzylisoquinoline alkaloid that is essential in the synthesis of other alkaloid derivatives. It is famous for its broad range of bioactivities such as anti-inflammatory, anti-bacterial, anti-diabetes, anti-ulcer, sedation, protects from myocardial ischemia-reperfusion injury, expands blood vessels, inhibits platelet aggregation, and causes hepatoprotective and neuroprotective effects [6,7]. Several studies have reported that berberine has been used in folk medicine in Asia, Greece, China, the Middle Eastern, Ayurvedic, and South America. In Asia, Berberis sp. (the main source of berberine) has been widely used for more than 3000 years. For instance, Chinese and middle Easterners have used berberine extracts to prevent microbial infections such as fungi, protozoa, bacteria, viruses, and helminths. Berberine's exciting benefits possess essential insights.

The extraction of berberine yield is the first step for their utilization and further research. In this study, berberine was extracted by two different extraction methods, conventional extraction and ultrasound-assisted extraction (UAE). The conventional extraction method is widely used for plant material extraction for many years as it is easy to conduct, does not require high-end instruments, and is generally much cheaper than the non-conventional method [8,9]. However, conventional extraction is time-consuming and has a low yield and low quality of extract. This is due to prolonged extraction time that leads to excessive agitation and heat transfer.

As an alternative, UAE has been proven as a more effective extraction method. It is simple, requires relatively low cost that can be used in a small and large industry, reduces extraction time, reduces the use of a large amount of solvent, reduces energy, reduces equipment size, a quick start-up, increases production, and discards process steps [10]. UAE also helps in the diffusion of solvent across the cell wall, breaking the cell wall, enhancing the release of bioactive content, and increasing the extraction yield [11]. There have been no studies on *Chromolaena odorata* and owing to its medicinal importance, the present work is carried out. Thus, the study herein evaluates and compares the berberine yield of *Chromolaena odorata* using different extraction methods with selected parameters. In addition, the surface morphology obtained from both methods is compared using Field Emission Scanning Electron Microscopy (FESEM).

MATERIALS AND METHODS

Chemicals

All chemical reagents used in this experiment were of analytical grade: hydrochloric acid and Wagner's and Mayer's reagents. Distilled water was directly taken from the laboratory to be used as the solvent. Berberine standard was purchased from Sigma Aldrich Chemical Company. The solvent used for HPLC purposes such as acetonitrile and acetic acid were HPLC grade solvents.

Sample Preparation

The plant material preparation was conducted according to a combination of methods as used by previous research with some modifications [12,13]. A fresh *Chromolaena odorata* plant was collected around Universiti Malaysia Kelantan, Jeli Campus. The parts collected involved leaves, stems, and roots. The plant was washed thoroughly 2−3 times with running tap water and then with distilled water to remove extraneous materials. Next, the plant was dried for 2 weeks under the shade in a clean environment until it was completely dried. The dried plant leaves were milled using a blender and sieved to obtain a powder of 425μm in size. After that, the powder was placed inside the zip-lock bag and stored in a desiccator at room temperature until used to prevent the powder from gathering moisture.

Phytochemical Screening

The qualitative test for alkaloids was conducted according to the previous study [14]. About 2 mL of extract was dissolved in 6 mL of dilute hydrochloric acid. After that, the mixture was cooled and filtered. The filtrate was separated into two portions. For the first portion, 2 drops of Wagner's reagent were placed, and the formation of reddish-brown precipitate represented a positive result on alkaloid. For the second portion, 2 drops of Mayer's reagent were added, and the formation of creamy-white precipitate indicated the presence of alkaloid.

Conventional Extraction

This method has been described with slight modifications [15]. A sample-to-water ratio of 1:10 (g/mL) was used throughout the study. The mixtures were stirred thoroughly in a 250 mL beaker and heated on a hotplate at 50°C for 20, 40, and 60 minutes with uniform stirring. The extract was left to cool at room temperature and centrifuged at 5800 rpm for 15 minutes. The supernatant was separated from the sediment and kept at 4°C before analysis.

Ultrasound-assisted Extraction

Table 1 shows the constants and manipulative parameters used for the three types of extraction. Ultrasound-assisted extraction was used to extract berberine compounds from *Chromolaena odorata*. A 250 mL beaker containing the mixture of sample and distilled water was used in this experiment. The sample-to-water ratio was also set at 1:10 (g/mL). Then, the sample was placed in the sonicator probe. An overhead stirrer was used to agitate the solution in the designed reactor throughout the experiment. The tip of the 15 mm diameter stirrer was submerged at half the height of the sample solution to achieve good results in extraction. The optimum values of the parameters were used as constant parameters. The parameters fixed in this extraction were 40 minutes of sonication time and 75% of the duty cycle. The parameter that was varied during ultrasound extraction was sonication time (20, 40, and 60 min). All experiments were done in triplicate. After that, the solution was left to cool at room temperature before being centrifuged. A clear solution of extract was obtained using a centrifuge with the following conditions: 5800 rpm, temperature of 21°C, and time of 30 minutes. The supernatant was taken and stored at 4°C before characterization.

TABLE 1. Constant parameters used throughout the extraction process

Parameter	Value
Sample size	425µm
Sample-to-water ratio	1:10
Sample weight: UAE	5g
The volume of solvent: UAE	50 mL
Sonication frequency	20 kHz
Centrifugation temperature	21˚C
Centrifugation speed	5800 rpm
Centrifugation time: UAE	30 min

High-Performance Liquid Chromatography (HPLC)

HPLC analysis was performed using HPLC-DAD (diode array detector) by Shimadzu Corporation. The separation was achieved by a reverse phase of the C-18 column. For standard preparation, 20 mg of each standard (quercetin and berberine) was weighed into a 20 mL volumetric flask for preparation of 1 mg/1mL stock solution. Methanol was added to the mark. A series of standard dilutions were prepared from stock solutions, which are 0.01, 0.05, 0.10, 0.15, and 0.20 mg/mL, and diluted with methanol up to 20 mL. Each dilution was filtered using a 0.45 µm syringe filter before being injected into the vial for HPLC analysis. For the preparation of the sample, the sample extract was diluted to 10 mL with HPLC water. After dilution, the sample was filtered through a 0.45µm syringe filter before being injected into the HPLC system. The sample was analysed by MetaChem C18 (250mm × 4.6 mm, 5 µm) column. The mobile phase used was HPLC water with acetic acid (99:1 v/v) in solvent A and acetonitrile (HPLC grade) in solvent B. The flow rate was fixed at 1.2 mL/min and the injection volume was fixed at 20 µL. The running time of each sample was 5 minutes. All the parameters and conditions are tabulated in Table 5 for HPLC analysis of *Chromolaena odorata* plant extract. Lastly, the chromatograms of sample extracts were compared with the chromatograms of standards to gain the concentration of berberine yield.

Field Emission Scanning Electron Microscopy (FESEM)

Field emission scanning electron microscopy (FESEM) was used to examine the complexity of plant structure such as external morphology (texture). The microstructures of the treated and untreated plants were analysed using scanning electron microscopy (Fei, Quanta FEG 450). The treated plant micro-structures (conventional, enzyme,

ultrasound, and ultrasonic enzyme-mediated) were observed and compared with untreated powder plants. All samples were kept in dry conditions to avoid any charging during scanning. The sample was placed on the metal support and sputter-coated with gold (sputtering apparatus Leica EM) to ensure no interruption charges. A magnificent depth was examined at 5000x [16]. The cavitation effect from the surface of the plant after using ultrasonic was observed and discussed.

RESULTS AND DISCUSSION

Preliminary Screening Analysis

The qualitative test for alkaloids has been demonstrated elsewhere [17]. The formation of reddish-brown precipitate indicated positive results on alkaloids. The darker reddish-brown colour indicated that a higher alkaloid was present. Alkaloid has an alkaline characteristic due to the presence of lone pair in nitrogen compounds. Iodine (I_2) was dissolved in potassium iodide (I^-) to give triiodide ion (I_3^-). The reddish-brown colour was formed due to the formation of a triiodide ion. Fig. 1 displays the reddish-brown colour of one of the sample extracts, which indicates the presence of alkaloids [18]. All samples show reddish-brown colour as shown in Table 2. Hence, the presence of berberine is confirmed in *Chromolaena odorata.*

FIGURE 1. Phytochemical screening of alkaloid (reddish-brown colour formed)

Phytochemical screening is first performed to detect the presence of bioactive compounds after the extraction. In Table 2, the phytochemical screening shows the presence of an alkaloid compound. The darkest colour of reddish-brown is observed at 60 minutes of extraction time obtained from the UAE method while the lowest colour with only slight opacity is observed at all extraction times of the conventional method. Overall, the presence of berberine is confirmed in *Chromolaena odorata* using these two methods at 20 until 60 minutes.

TABLE 2. The phytochemical results for bioactive compounds

Extraction time (min)	Berberine from the conventional method	Berberine from UAE method
20	+	++
40	+	++
60	+	+++

Note:(-): Absence of turbidity/ flocculation/ precipitation
(+): Slight opacity
(++): If the reactive product and not turbidity flocculation
(+++): Presence of heavy precipitate/ flocculation

Effects of Different Extraction Methods on the Yield of Berberine

Fig. 2 shows the results of the berberine yield using different extraction methods at different extraction times. For the conventional method, the yields increase when the extraction time increases from 20 to 60 minutes with the extraction yields from 0.1627 to 0.2193%. This observation indicates that the extraction time is an essential parameter for the extraction of berberine compounds. In Fig. 2, a drastic increase in berberine yield is obtained using the UAE method compared to conventional. Maximum berberine yield is obtained with the extraction yield of 2.7% at 40 minutes for the UAE method, and 0.2% at 60 minutes for the conventional method. The berberine yield of the UAE

method is more than ten times higher compared to the conventional method. This is because the conventional extraction is a slow process as it solely depends on the diffusion of solvent and agitation, while the UAE method uses an ultrasound probe which produces a cavitation effect [20]. Due to the cavitation process, the vibration disrupts molecular interactions while cell lysis causes the alkaloid compound such as berberine to be released from the cell. This proves that high berberine could be obtained when the cavitation effect from the UAE method is involved in the extraction process.

The previous study supports this result as the alkaloid extraction from soursop fruit using the UAE method is also 56.31 times higher than the extraction using the conventional method [19]. It has been reported that the alkaloid extraction from *Sophora alopecuroides* seeds produces a higher extraction yield than other methods of reflux heating by 4.6% [20]. Several previous studies also demonstrate that the UAE method could be the most efficient method for extracting alkaloids for different types of plants such as *Stephania cambodica* [21], potato peel [22], and *Rhizoma coptidis* [23].

For UAE, the prolonged extraction time at 60 minutes decreases the yield of berberine as a longer extraction time might induce the change of molecule structure and bioactivities of the berberine compound [24]. The longer the extraction time, the higher the ultrasonic amplitude or density of acoustic energy and constant acoustic irradiation which causes high cavitation, producing greater cellular lysis [19].

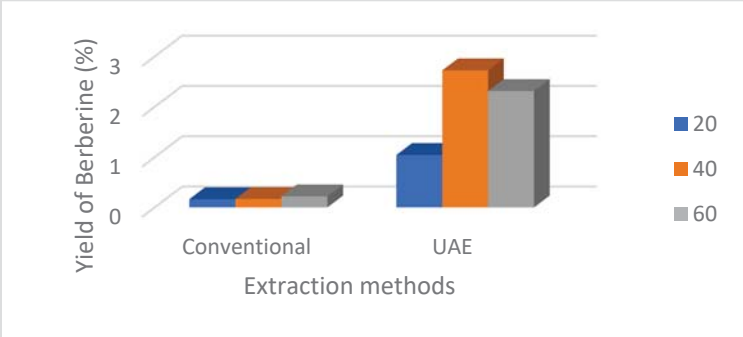

FIGURE 2. Extraction yield of berberine with different extraction times of different extraction methods

From a preliminary study, the yield of berberine from the conventional method is almost similar (2.6971) to the yield obtained from the UAE method (2.7228). However, the extraction time is hugely different in which the conventional method requires 12 hours (720 minutes), which is 18 times higher than the UAE method (40 minutes). Therefore, this finding supports the effectiveness of the UAE method in extracting berberine. This result supports that the UAE method has a great potential for extracting alkaloids from *Chromolaena odorata* at a shorter extraction time.

High-Performance Liquid Chromatography (HPLC)

A calibration curve is a standard curve used to determine the concentration of the substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration. In this study, the curve is constructed by injecting five different concentrations of standard solutions, and the peak area obtained is measured to form a graphical representation of the peak area (y-axis) versus concentrations (x-axis). Fig. 3 displays the peaks of the berberine standard, using high-performance liquid chromatography (HPLC) analysis. The correlation coefficients (R^2) show well-fitted lines as R^2 values for the calibration curves are greater than 0.95 [25]. Therefore, good linearity is demonstrated for berberine's standard curve.

Field Emission Scanning Electron Microscopy (FESEM)

The morphological analysis of the surface structure of the dried powdered sample of *C. odorata* is performed using field-emission scanning electron microscopy (FESEM). This analysis aims to study the influence of different extraction methods on physical changes and compare their physical morphology. Fig. 4(a) illustrates the FESEM image of conventional extraction, while Fig. 4(b) displays the FESEM image of the UAE. The images are captured at 5,000× magnification.

After conventional extraction, small ruptures and perforations could be seen (Fig. 4(a)). The cell wall is less permeable due to less rupture and perforation, thus, less cell content is released. This finding explains that conventional extraction only causes little damage to the vegetal cell wall [26], which explains the low yield obtained from the HPLC results of the conventional method.

Besides, using the UAE method, the plant sample receives heat treatment and vibration from ultrasound sonication, causing the internal surface of the plant to progressively be torn and broken, as seen in Fig. 4(b). The vibration from the ultrasound wave can damage the bond in lignin and cellulose, thus enhancing the mass transfer of the content. This vibration can also cause changes in chemical composition, releasing more bioactive contents. Similarly, the ultrasound effect is reported for *Strobilanthes crispus* by previous research [27]. The physical structure of the sample after UAE treatment is severely damaged, with large perforations and more ruptured cells (Fig. 4(b)) compared to the conventional extraction (Fig. 4(b)). A previous study claims that UAE could increase the swelling of vegetal tissues, hence enlarging pores in the plant cell wall, assisting diffusion, and increasing extraction yield [28]. Such evidence is corroborated by the HPLC results, which show a higher extraction yield of UAE than the conventional method. Overall, these results support the hypothesis that non-conventional extraction plays a vital role in breaking the plant cell wall and gives a significant effect compared to the conventional method.

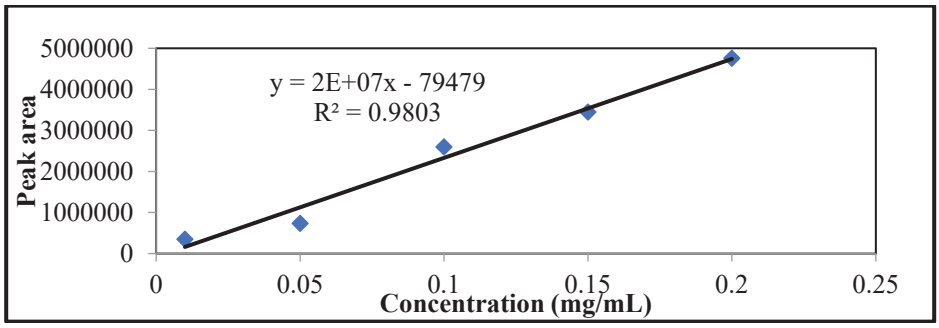

FIGURE 3. Calibration curve of Berberine

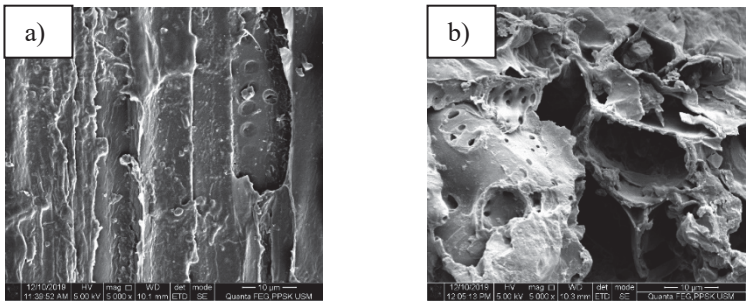

FIGURE 4. FESEM images of a) conventional extraction and b) ultrasound-assisted extraction

CONCLUSION

In this study, the comparison of the two extraction methods is discussed. From a preliminary study, the screening test shows the presence of berberine in these two methods. The UAE shows the darkest reddish-brown colour at 60 minutes, while conventional extraction shows the brightest colour of reddish-brown at 20 to 60 minutes. From the HPLC analysis, the maximum berberine yield is obtained at 40 minutes using UAE with an extraction yield of 2.6% compared to conventional which is 0.14% at 60 minutes. This is because the UAE cavitation effect enhances the extraction process. Meanwhile, the FESEM images of the two methods could also explain the reason behind the high extraction yield obtained from UAE. The FESEM image from the conventional extraction shows only small ruptures compared to UAE that shows large perforations and more fractures. The high extraction yield is directly proportional to high cell rupture and large perforation since more content can be released. Overall, the study shows that the UAE

method is better than the conventional method in extracting berberine from *Chromolaena odorata* at a shorter extraction time.

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