Extraction of Asiaticoside from *Centella asiatica* using Ultrasonic-Assisted Enzyme Extraction

Nor Sarah Amira Ismail, Subash Nambiar SreePathia, Siti Aisyah Athirah Hazmi, Zubaidah Aimi Abdul Hamid and Mardawani Mohamad*

Affiliation, Faculty of Bioengineering and Technology, University Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia

*Corresponding author (email: mardawani.m@umk.edu.my)

Triterpene glycosides are secondary metabolites in *Centella asiatica* that can heal skin wounds with their anti-inflammatory properties. Ultrasonic-Assisted Extraction (UAE) is an extraction method focusing on green technology applications producing high-frequency cavitation bubbles. The addition of cellulase enzyme increases the leaching ability of the solvent used in this extraction, ethanol, by breaking down the plant cell walls. This study focused on extracting triterpene glycosides from *C. asiatica* in the form of asiaticoside using ultrasonic-assisted enzyme extraction (UAEE). The unified system of enzymatic ultrasonication enhances the effectiveness of the extraction process of bioactive compounds. The parameters studied were cellulase enzyme volume (0.1 mL, 0.2 mL and 0.3 mL) and solvent concentration (50 %, 70 % and 90 % (v/v)). Phytochemical screening using Salkowski's test showed the formation of a red brick precipitate, indicating the presence of glycosides in all the samples. From the high-performance liquid chromatography (HPLC) analysis, the most optimum parameters were 50 % ethanol combined with 0.2 mL cellulase for an extraction yield of 0.796 mg/mL. Fourier Transform infrared spectroscopy (FTIR) analysis showed the presence of C-O bonds in the extract, consistent with asiaticoside. Scanning Electron Microscopy (SEM) results showed a massive difference in the physical surface structure of the sample before and after extraction. In conclusion, UAEE showed good potential for extracting triterpene glycosides, while being a green and eco-friendly extraction method.

Keywords: Triterpene glycoside; asiaticoside; Ultrasonic; cellulase

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Herbs have been a part of human life since ancient times, as humankind sought treatment in nature and found that some plants possess the ability to treat and alleviate certain diseases and injuries [1]. *Centella asiatica* has been widely used in traditional medicine for many skin disorders and diseases such as inflammation and wounds. It is also used as a medicinal ingredient for diseases such as syphilis, epilepsy, dehydration, and diarrhoea [2]. *C. asiatica* has several benefits such as anticancer, antibacterial, antifungal, anti-inflammatory, neuroprotective, antioxidant and antidiabetic properties [3]. The major bio-constituents in this plant are triterpenoid saponins, which consist of triterpene glycosides, asiaticoside and madecassoside, and aglycones which contain asiatic acid and madecassic acid [4]. Several extraction studies have been conducted to obtain the triterpene saponin contents from plant leaves, including subcritical water extraction (SWE), microwave-assisted extraction (MAE), and solvent-based extraction [5-7]. The disintegration of the triterpene saponins and phenolic compounds occurred quickly during microwave-assisted extraction due to thermal degradation and oxidation reactions during processing.

However, the application of solvents goes against the initiatives of “green” extraction, as methanol, which is the most used solvent for this process, is toxic. Extra costs must be allocated for removing toxic pollutants, which adds to the total processing amount. Green extraction is the discovery and design of extraction processes that decrease energy usage, and use less harmful solvents and renewable natural products [8] that guarantee high-quality extracts and products. These solutions should improve and optimize the existing process using non-dedicated equipment. MAE and UAE are the closest processes that fulfil green extraction requirements, but the yield of triterpenoid saponin compounds by UAE was reported to be less than by MAE [9]. With MAE, as mentioned earlier, there were problems with heat degradation and oxidation of bioactive compounds [10].

As an alternative to the existing methods, ultrasonic-assisted enzymatic extraction (UAEE) was chosen as the focus of this study to achieve the maximum yield possible while promoting the principles of green extraction. UAE has been applied in the isolation of organic compounds due to its beneficial properties, low solvent consumption and shorter

extraction times compared to other methods. It also avoids the decomposition and oxidation of natural products because it can be carried out at room temperature [11]. Numerous studies have reported the capability of ultrasound as an extraction method, such as in the extraction of isoflavones from the root of *Radix Puerariae*, which showed that the ultrasound technique had a higher yield compared to pressurized solvent extraction [12]. Research on cannabinoids extracted from *Cannabis Sativa L* showed that 15 minutes extraction time with 130 W power and 80 % methanol were the optimal conditions [13].

Enzymes have been applied as catalysts for plant bioactive component extractions due to their safe and green properties. Some of the most common enzymes used are cellulase, hemicellulase and pectinases. The enzymes are usually obtained from microorganisms such as fungi, bacteria, and protozoa. The enzyme extraction mechanism is specific because of the high degree of enzyme selectivity. The principle of enzyme extraction relies heavily on the hydrolysation of the cell wall, which disrupts cell integrity, thus releasing its contents easily [14]. This study explicitly addresses variations in enzyme volume and solvent concentration as the parameters, and aims to identify the ability of the enzyme assisted ultrasonic method to extract asiaticoside from *C. asiatica*. For the outcome of this study, it was expected that using UAEE combined with the most optimum concentrations of solvent and enzyme would efficiently extract the asiacticoside. This process also aimed to produce less toxic waste, and thus be more eco-friendly. Enzyme pre-treatment may also contribute to an environmentally friendly method.

EXPERIMENTAL

Chemicals and Materials

Fresh *C. asiatica* plants were purchased and collected in Kelantan. The whole *C. asiatica* plant was used except for its roots. The samples were washed, air-dried, and ground until they passed through a 500 µm sieve. The drying process was run for 24 hours until a moisture content of 10 % was achieved.

The chemicals used in this study were mostly purchased from Sigma Aldrich, such as HPLC grade methanol with purity ≥ 99 %, distilled water, chloroform with purity ≥ 90 %, absolute ethanol with purity ≥ 99.8 % and concentrated sulphuric acid with purity ≥ 97 %. HPLC grade methanol and acetonitrile with purity ≥ 99 % were purchased from Merck (Darmstadt, Germany) and standard asiaticoside was purchased from Cayman Chemicals (Michigan, USA). The Celluclast in aqueous solution was used as cellulase enzyme with an activity of 75 FPU/mL.

Extraction Process

The extraction process involved two different parameters: cellulase enzyme volume and solvent concentration. An ultrasonic instrument (JY92-IIDN 20-25KHz, LCD, 900W (Scientz Biotech, China) was utilized in this study, working at 60° amplitude and with an input power of 60 W. The samples underwent similar procedures at the beginning, in which 5 g of ground sample was mixed with 100 mL distilled water and then immersed in a water bath shaker at 50 °C for 30 minutes. After 30 minutes of pre-heating, the samples were taken out, and a certain amount of enzyme was added before being immersed in the shaking water bath at 50 °C for 60 minutes. Then, it was left to cool at room temperature, after which 50 mL ethanol was added and subjected to ultrasonic treatment for 40 minutes. The sample to solvent ratio was set at 1:10 [15]. The parameters investigated in this study are presented in Tables 1 and 2, and all experiments were performed in duplicate.

After extraction, the samples were centrifuged at 5300 rpm for 15 minutes for solid-liquid separation. Then, the supernatant was filtered through filter paper and stored in the chiller at 4 ◦C for further analysis.

Phytochemical Screening

The purpose of the phytochemical screening was to carry out a preliminary validation for the presence of triterpene glycosides right after the extraction process. The procedure is accessible and gives quick results. For this test, 2 mL of the sample was pipetted and transferred into a test tube, followed by 2 mL of chloroform. Then, 2 mL of concentrated sulphuric acid was added slowly to the wall of the test tube and left for two minutes, and a change in the colour of the solution was observed. Formation of a reddish-brown colour indicated the presence of a glycoside.

Table 1. Parameters of UAEE method for different enzyme volumes.

Constant parameter	Sonication time	40 min
	Temperature	55 °C
	Duty cycle percentage	50 %
	Solvent concentration	50 % ethanol (v/v)
Manipulated parameter	Enzyme Volume	0.1 mL, 0.2 mL and 0.3 mL

Table 2. Parameters of UAEE method for different solvent volumes.

Constant parameter	Sonication time	40 min
	Temperature	55 °C
	Duty cycle percentage	50 %
	Enzyme Volume	0.2 mL
Manipulated parameter	Solvent concentration	50 %, 70 %, 90 % ethanol (v/v)

Analysis

The quantification of asiaticoside was performed by HPLC analysis. The HPLC system consisted of a controller, photodiode array (PDA) detector, computer with Empower software, and an injector. The column used was Excil ODS 5 µm (C18) (150 mm x 4.6 mm). The wavelength for detection was set at 205 nm with the mobile phase, 0.01 % phosphoric acid (solvent B) and 100 % acetonitrile (solvent A) with elution of 30:70 v/v, with 5 minutes running time. The injection volume was 10 µL for the standard and 30 µL for the sample at a flow rate of 1 mL/ min. The standard stock solution for asiaticoside was prepared by dissolving in methanol-water and then filtered before being subjected to the HPLC instrument for the standard curve.

Fourier Transform infrared spectroscopy (FTIR) was applied to study chemical bonds and characterise the compounds present in the sample. The analysed samples were the pure dried samples, dried samples with solvent before UAEE treatment, and 0% enzyme dried samples after UAE. The analysis was performed by ATR on the FTIR spectrophotometer (model Thermo Scientific Nicolet 380), and the spectral range was set between 4000-500 cm^{-1} [16].

Scanning electron microscopy (SEM) analysis was employed to assess the surface morphology of the *C. asiatica* extracts before and after extraction, for comparative purposes. The samples analysed under SEM included the pure dried samples and ultrasonic enzymatic assisted extraction dried samples. The magnification scale used in this study was 500x.

RESULTS AND DISCUSSION

Phytochemical Screening Analysis

The phytochemical screening used chloroform and sulphuric acid as reagents to test the presence of triterpenoid glycosides from *C. asiatica* after UAAE extraction. Based on the theory of Salkowski's test on cholesterol, chloroform dissolved the sample extract, making it easier for a dehydration reaction to occur. The addition of concentrated sulphuric acid removed two functional groups from the bioactive compounds in the sample extract, producing bi-sulphonic acid that produced a dark red colour [17]. Table 3 shows the presence of glycosides through the production of a brick-red precipitate in the UAEE extraction process with different enzyme volumes.

Based on Table 3, 0.2 mL of the enzyme produced the highest amount of glycoside because of the intensity colour of the red brick precipitate produced. The intensity of the brick red precipitate increased with enzyme volume and decreased at 0.3 mL. Thus, it can be concluded that 0.1 mL and 0.3 mL of enzyme were inappropriate for optimising glycoside production because these amounts were inadequate and excessive, respectively.

Table 3. Phytochemical test results for different enzyme volumes.

Concentration of Enzyme (%)	Presence of Bio-compound
0.1 mL	++
0.2 mL	+++
0.3 mL	++

Note: + indicates present
(-): Absence of turbidity/flocculation/precipitation
(+): Slight opacity
(++): Reactive product and no turbidity/flocculation
(+++): Present of precipitate/heavy flocculation

Table 4. Phytochemical test results for different solvent concentrations.

Solvent Concentration (%)	Presence of Bio-compound
50	++
70	+
90	++

Note: + indicates
(-): Absence of turbidity/flocculation/precipitation
(+): Slight opacity
(++): Reactive product and no turbidity/flocculation
(+++): Presence of precipitate/heavy flocculation

The phytochemical test was also carried out for the solvent concentration parameter as tabulated in Table 4. The intensities of the red brick precipitate with 50% ethanol and 90% ethanol were very similar. Our preliminary assumption was that the 50% and 90% ethanol solutions were able to dissolve similar levels of bioactive compounds, while the 70% ethanol solution was able to dissolve the most water-insoluble bioactive compounds.

Effect of Enzyme Volume on Asiaticoside Yield

The yields of asiaticoside from the UAEE extraction method with different enzyme volumes of 0.1 mL, 0.2 mL, and 0.3 mL are presented in Fig 1. The controlled parameters are as tabulated in Table 1: 40 min sonication time, temperature under 55 °C, frequency of 60 W, solvent concentration of 50 % (v/v) and with 50 % duty cycle percentage. During the extraction period, 0.1 mL, 0.2 mL, and 0.3 mL of the enzyme were used to determine the appropriate amount of enzyme to be applied with ultrasound as an integrated method for asiaticoside extraction. The retention time of asiaticoside by HPLC analysis was 2.603 minutes out of 5 minutes running time. The retention peak of asiaticoside and the amount of asiaticoside obtained was determined using a calibration curve of standard asiaticoside in the range of 0.001 mg/ml to 0.005 mg/ml. The highest yield of asiaticoside was achieved with 0.2 mL of enzyme (0.796 mg/mL) followed by 0.1 mL (0.755 mg/mL) and 0.3 mL (0.704 mg/mL). 0.3 ml was the highest volume of enzyme used, but this produced the lowest amount of asiaticoside. The HPLC results in Figure 1 correspond to the results of the phytochemical screening for different enzyme volumes in Table 3.

The results showed that as the enzyme concentration increased from 0.1 mL to 0.2 mL, there was a significant jump in the amount of bioactive compounds obtained. When the enzyme concentration was 0.1 mL, it needed to bind to the overwhelming amount of substrate. As the enzymes were specific, only one substrate at a time could be catalysed for cell wall degradation. Hence, most cell walls that did not undergo enzyme-substrate reaction stayed intact and could protect their content. When the enzyme concentration was increased to 0.2 mL, the amount of enzyme available for the enzyme-substrate reaction also increased, and more of the β-1,4 glycosidic bonds that made up the cell walls were able to be broken down, exposing the bioactive compounds within the cells [18]. However, 0.3 mL produced the lowest amount of asiaticoside due to substrate limitation factors.

After the initial increase in reaction rate, once all substrates were occupied, no more enzyme-substrate reactions could be carried out. Triterpene glycosides are amphiphilic with a sugar molecule that could be mono- or oligosaccharides linked to a functional group of terpenes with a glycosidic bond [19]. Hence, the high concentration of enzyme may break down the glycosidic bonds of the triterpene glycosides, resulting in a lower yield. The effect of higher enzyme concentrations would be less noteworthy after the cell walls were highly degraded [20].

Effect of Solvent Concentration on Asiaticoside Yield

The yields of asiaticoside from the UAEE extraction method with different concentrations of solvent (50 %, 70 % and 90 % (v/v)) was presented in Fig 2, while the controlled parameters are as tabulated in Table 2: 40 min sonication time, temperature under 55 °C, frequency of 60 W, 50 % duty cycle percentage and 0.2 ml of enzyme. During the extraction period, 50 %, 70 % and 90 % solvent solutions were used in the samples to assist in leaching out bioactive compounds. As shown in Figure 2, the 50 % (v/v) solvent solution generated the highest amount of asiaticoside (0.796 mg/mL) compared to the 90 % (v/v) (0.760 mg/mL) and 70 % (v/v) (0.626 mg/mL) solutions. This also corresponds to the phytochemical screening results in Table 4, in which the 50 % solvent solution showed the darkest brick red precipitate while 70 % produced the lightest.

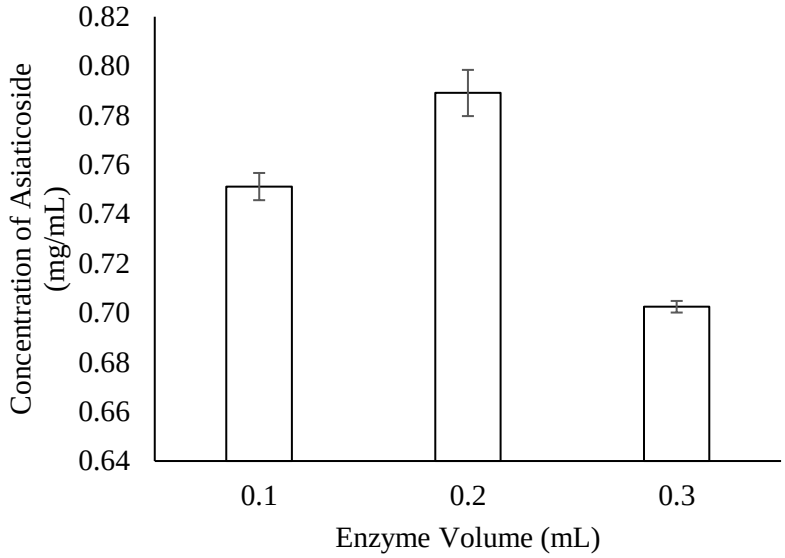

Figure 1. Quantification of asiaticoside against enzyme concentration.

The concentration of ethanol and the amount of water play a pivotal role in determining the ability of the solvent to leach out bioactive compounds from samples. The topological polar surface area of asiaticoside reported from PubChem is 335 Å², which is highly polar. However, non-polar groups such as alkyl and alkene are abundant in asiaticoside [16]. The presence of non-polar ethyl groups in ethanol helped to dissolve these non-polar groups as well as the polar groups. In this study, when the presence of water and ethanol were balanced in the 50 % ethanol solution, the yield of asiaticoside was the highest as the ethanol-water interaction was the highest, increasing the freedom of movement as both molecules formed water-ethanol attractions [21]. As a result, more carbonyl and hydroxyl groups could leach out and be dissolved in the 50 % ethanol [22]. The result for 90 % ethanol showed a slight decline in the yield of asiaticoside as the high concentration of ethanol and low concentration of water could not form the same number of interactions with each other, lowering the freedom of movement. Previous research also found that a 50 % ethanol solution produced the highest total amount of phenolic compounds and flavonoid compounds compared to 70 % and 90 % ethanol solution [23].

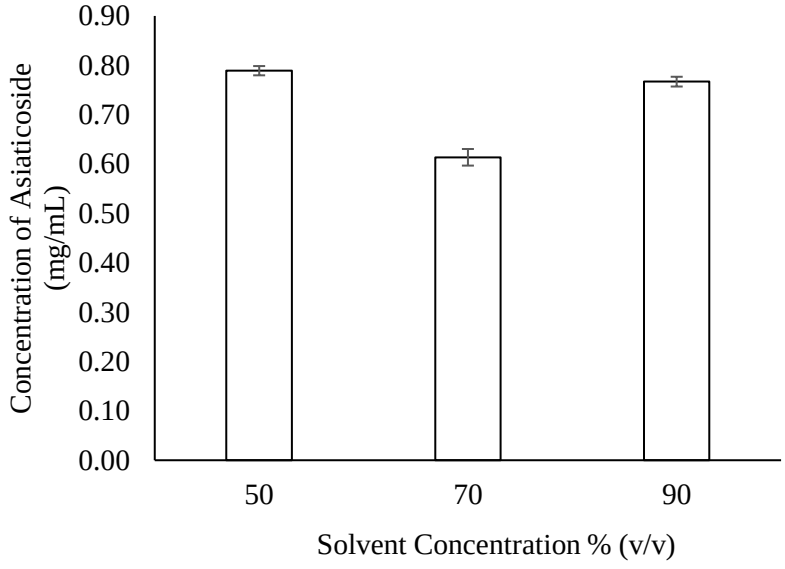

Figure 2. Quantification of asiaticoside against solvent concentration.

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform infrared spectroscopy (FTIR) was used to predict the organic compounds present within the sample by absorption of infrared light. The functional groups present in the ample were characterized by analysing the peaks obtained. For the detection of asiaticoside within the sample, an asiaticoside standard from a previous study was used as a reference. In this study, the supernatant obtained after extraction was utilized as a sample for FTIR analysis as follows: Pure dried sample, dried sample undergoing conventional solvent extraction of 50% (v/v) ethanol for 40 minutes and finally the optimum sample, dried sample with 0.2 mL enzyme undergoing 40 minutes of UAE with 50% (v/v) ethanol. The purpose of analysing these samples was to study the effects of the solvent and UAEE on the sample.

Table 5 shows the absorptions and peaks of the asiaticoside standard as a reference. The absorption peaks obtained from FTIR analysis of the samples are shown in Figure 3, which depicts the fingerprint and functional group regions of these samples.

Table 5. FTIR analysis of standard asiaticoside (Source: [16]).

Wavenumber (cm^{-1})	Absorption of Energy (Bond)
3275-3550	C-H
2906	R-OH
1732	C=O
1062	C-O
653-680	$(CH_2)n$

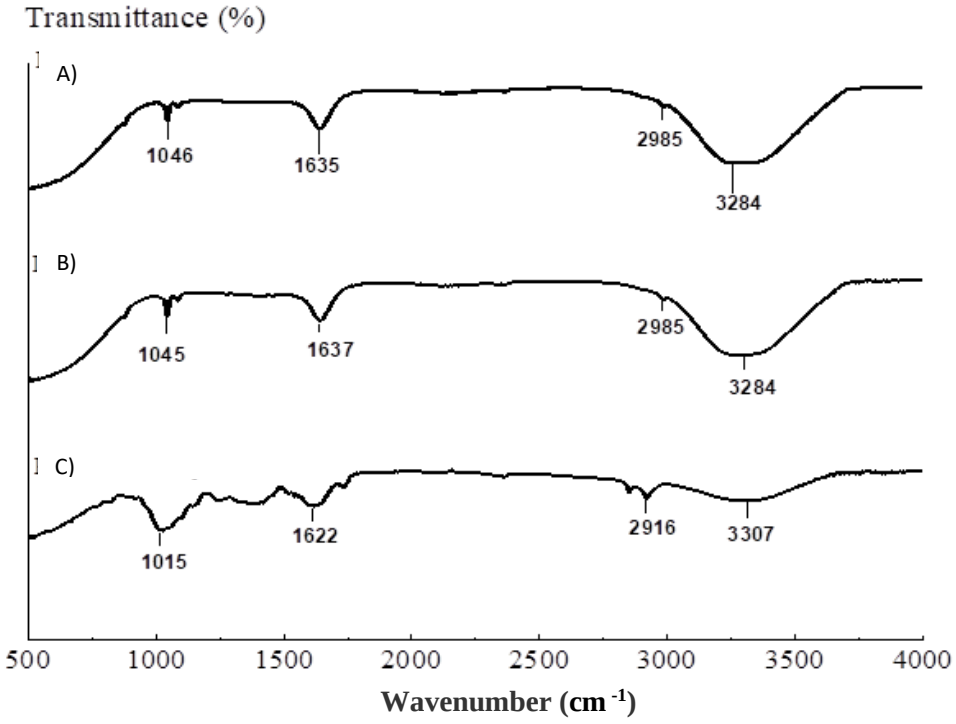

Figure 3. FTIR spectra of *Centella asiatica sp*. extracts: (A) pure dried sample, (B) dried sample with 40 minutes conventional extraction, (C), Optimum sample after UAEE.

In the fingerprint region (<1500 cm^{-1}), sample (C) showed a peak at 1015 cm^{-1}, while both samples (B) and (A) showed almost similar peaks at 1045 cm^{-1} and 1046 cm^{-1}. Based on the asiaticoside reference standard in Table 5, this can be attributed to the presence of a C-O bond which shifted from a broad peak at 1015 cm^{-1} in the pure dried sample to sharp and narrow peaks at 1045 and 1046 cm^{-1} once water and solvent were added for both solvent extraction and UAEE [24]. This trend shows a decrease in absorption intensity, indicating a more microstructured chemical bond [25].

Samples (B) and (C) produced sharp and slightly broader peaks at 1637 cm^{-1} and 1635 cm^{-1}, respectively. Based on the asiaticoside reference standard, this absorption could be attributed to the presence of C=O stretching vibrations [24]. There was an increase in the energy absorption intensity, indicating the presence of a C=O bond. Meanwhile, sample (A) showed energy absorption at 2916 cm^{-1}, while both (B) and (C) showed very narrow and sharp peaks at 2985 cm^{-1}. These peaks indicate the presence of CH bonds, and the intensity was decreased in both samples (B) and (C) compared to sample (A). Thus, once the sample underwent an extraction process, the aliphatic bonds were lost [24].

A broad final peak was present at 3307 cm^{-1} for sample (A) but shifted to 3284 cm^{-1} for (B) and (C). These peaks indicate the presence of hydrogen bonds as confirmation of the existence of water in the pure dried sample [24]. The peaks became narrower and sharper in both (B) and (C) as water and 50% (v/v) ethanol were added, showing an increase in the presence of hydrogen bonds. Hence, the increase in energy absorption was consistent with more hydrogen bonds.

Based on these findings, particularly in the fingerprint regions of the spectra, there was a high possibility that the chemical bonds and functional groups that belong to asiaticoside were present. The peaks at 1046 cm^{-1} and 1045 cm^{-1} in samples (B) and (C) were within the range of a C-O bond [26]. The position of the C-O bond may vary from 1000 cm^{-1} to 1300 cm^{-1} depending on the type of sample in which it is found [27].

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to determine the surface structure of the air-dried samples of *C. asiatica*. Figure 4 below illustrates the surface morphology of the sample before and after the extraction process. Figure 4(A) displays the image of the pure dried sample, while Figure 4(B) shows the image of the sample after the extraction process.

SEM analysis was used to study the surface morphology of the sample before and after the extraction process. The aim was to analyse the physical changes on the sample's surface after being treated with UAEE. As we can see in Figure 4(B), after the extraction process, the cell walls on the surface of the sample were ruptured and exposed, compared to 4(A), in which the surface structure was still intact and compact. The role of cellulase in this extraction was to degrade the cell walls, by mainly attacking the polysaccharide chains, especially the beta β-1,4-glycosidic bonds [28]. Hence, most of the cell walls were degraded during preliminary treatment. Ultrasonics plays a vital role in the extraction process where the ruptures resulted from the increased depth and velocity of liquid brought along by cavitation bubbles under high frequency during sonication [29]. The bubbles maintain their spherical shape during implosion in pure liquids because the surroundings are uniform. When a bubble collides with a solid surface, such as the sample surface, it collapses asymmetrically, resulting in high-speed jets of solvent toward the cell walls [30]. The images prove that the UAEE can degrade cell walls and allow penetration of organic compounds into the cell matrix.

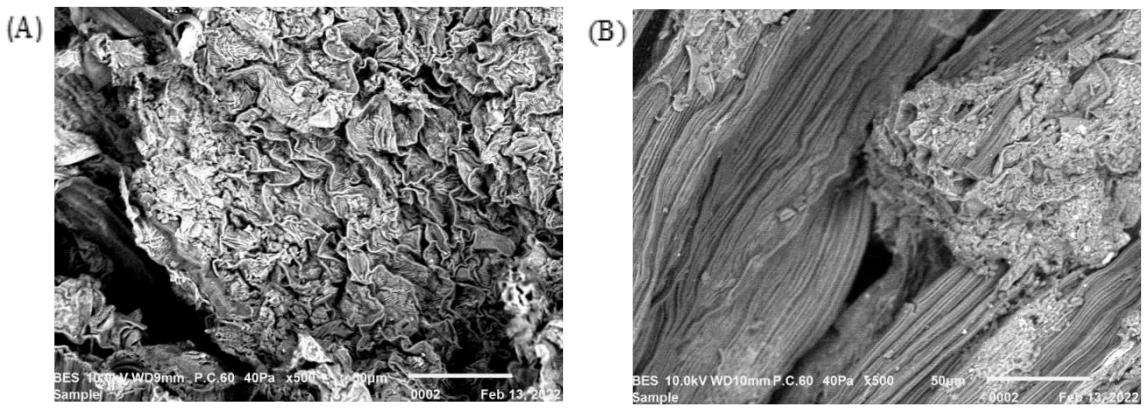

Figure 4. SEM analysis of *C. asiatica sp*. extract: (A) pure dried sample, (B) after ultrasonic enzymatic assisted extraction.

CONCLUSION

In this study, triterpene glycosides in the form of asiaticoside were extracted from *Centella asiatica* using ultrasonic-assisted enzyme extraction (UAEE) using different enzyme volumes and solvent concentrations. Thus, the enzyme and UAE integration method for asiaticoside extraction is a new approach to the process that showed a positive response. It was proven through HPLC analysis that the target compound, asiaticoside, was present in each sample and the sample associated with 0.2 mL of cellulase and 50 % ethanol provided the optimum yield of asiaticoside. These findings could provide beneficial information for further studies on the extraction of triterpenoid glycoside compounds. A comparative study on different methods of triterpenoid glycoside extraction from *C. asiatica* should be conducted to finetune the extraction process.

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