Optimization of Ultrasonic-Assisted Enzymatic Extraction of Tannic Acid from Chromolaena Odorata sp.

AAA Kamal¹, M Mohamad¹, KA Sulaiman¹, NA Mohidem², NF Shoparwe¹, PT Teo¹, MN Masri¹ and AH Yusoff¹
¹Faculty of Bioengineering and Technology, Universiti Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia.
²Advance Materials and Process Engineering (AMPEN) Laboratory, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia.
Email: mardawani.m@umk.edu.my

Abstract. Previous study only implemented the time consuming and low amount of yield technique for extraction from Chromolaena odorata which is conventional method. Nonconventional extraction method with short extraction time and high amount of yield was applied in this study by applying ultrasound-assisted enzymatic extraction (UAEE). UAEE was used to extract tannic acid from Chromolaena odorata. The extraction parameters involved were enzyme concentration, sonication time and duty cycle at constant temperature of 50°C, solid to liquid ratio of 1:10 and sonication power at 60% amplitude. The optimum extraction process was found at cellulase enzyme concentration of 4%, sonication time of 60 minutes and duty cycle of 50% with the obtained concentration of tannic acid at 1.6152 mg/mL. The study showed that the UAEE could be employed to enhance yield of tannic acid, reduce the extraction time and ensuring green extraction method were applied in the study.

1. Introduction
The area of study that is approached by this research is herbalism which is a traditional medicinal or folk medicine practice based on the use of plant or plant extracts. Enzyme-assisted extraction of natural functional compounds from plants is widely studied in the recent years for its advantages in easy operation, high efficiency, and environment friendly feature [1]. Chromolaena is a plant genus of the family Asteraceae. There are several species under Chromolaena gene such as Chromolaena Odorata and C. hirsuta. Chromolaena sp is being used traditionally for its many medicinal properties. Previous studies have demonstrated that the leaf extract has antioxidant, anti-inflammatory, analgesic, antimicrobial, cytoprotective and many other medicinally significant properties [2]. The genus Chromolaena produces phenolic and other substances with important biological activities.

Previous studies of the leaves and stems of Chromolaena odorata revealed the presence of essential oils, steroids, triterpenes, alkaloid, and flavonoids [3]. This study explores further under the flavonoid class. In this study, the main significant chemical compound that needs to be extracted is tannic acid. Tannic acids are water-soluble polyphenols that are present in many plant foods that have variety of biological actions that includes anti-oxidative, anti- microbial activities and anti-inflammatory activities. Tannic acid's anti-carcinogenic and anti-mutagenic potential may be related to its antioxidant properties, which are important for the protection of cellular oxidative damage, including the peroxidation of lipids [4].

In previous research which used the conventional chemical methods, when solvent-based extraction method to extract the Chromolaena odorata. Chemical methods for biologically active extraction are
widely used because they are well established and easy to perform. However, solvent-based extraction of *Chromolaena odorota* sp often suffers from requires long extraction times with low extraction yields, and the final product often contains traces of organic solvents that decrease the product quality [5]. The solvent that used will also affect the environment such as there have the potential to produce toxic during extraction and it need to undergo treatment after extraction.

In this study, the method used is green extraction process whereas used to obtain various plant extracts with minimum impact on the environment. It will reduce energy consumption, allow use of alternative solvents and renewable natural products, and ensure a safe and high-quality extract. Ultrasonic-assisted enzymatic extraction (UAEE) is one of green extraction method. Besides, UAEE is well known with properties of faster extraction, higher yield recovery, reduced solvent usage and lower energy consumption when compared to non-enzymatic methods [6]. These properties will also enhance the release of tannic acid from *Chromolaena odorata*. Hence, the aim of this paper is to study the extraction of tannic acid from *Chromolaena odorata* using ultrasound-assisted enzymatic extraction (UAEE). In this study, phytochemical screening as a qualitative method was performed in order to determine the presence of tannic acid. HPLC was used for the determination of the concentration of tannic acid in the extracts.

2. Materials and Methods

2.1 Materials and chemicals

*Chromolaena odorata* was collected from Agropark Universiti Malaysia Kelantan (UMK) Bukit Kudang, Jeli, Kelantan and Lata Keding, Bukit Kudung, Jeli. The whole plant (leaves, flower, stem and root) was used in extraction process. One kilogram of the plant was collected for this study. The plant that had been collected include leaves that matured and the size of leave is about 10cm. The plant is mostly 1-meter height. The cellulase from *Aspergillus niger* aqueous solution 700 units/gram was purchased from Sigma Aldrich chemical company while the tannic acid was purchased from Merck Sdn Bhd. Besides that, the chemicals that used were ethanol, hydrochloric acid (HCl), ammonia, sodium acetate, acetic acid, methanol (HPLC grade), HPLC water and acetonitrile (HPLC grade).

2.2. Sample preparation

The whole *Chromolaena odorata* plant (leaves, flower, stem and root) was washed with running water. After washing, the plant was undergoing air dry for two weeks. The dry plant was blended and milled to powder form before extraction process. After that, the powder was undergoing sieving machine to constant the mesh size (425 μm). The samples were stored in polyethylene bags at ambient temperature without sunlight.

2.3. Solution preparation for extraction

5.0 g *Chromolaena odorata* powder was added to 100mL of distilled water due to whereas ratio for solid to liquid is 1:10. Acetate buffer then was added to adjust the pH until pH 5. Next, the solution was immersed to shaken water bath at 50°C to preheat for 30 minutes. In this study, there are three parameters for the UAEE extraction, which were concentration of enzyme (cellulase), sonication time and duty cycle.

2.4. Extraction process

For parameter concentration of enzyme, the following process was conducted. The enzyme that used in this study was cellulase. The cellulase solutions were prepared with four different concentrations of 0%, 2%, 4% and 6%. After preheat for 30 minutes, the pectinase was added into the solution. The solution then was immersed to shaken water bath at 50°C for one hours. Next, the solution had undergone cool down process in 5 minutes. 15 mL of ethanol was added after the cooling process and the mixture then subjected to ultrasonic treatment. The sample had undergone ultrasonic extraction
with 40 minutes sonication time, 50% duty cycle and 60% amplitude which referred to the sonication power set up for entire studies.

For parameter of sonication time, the following method was employed. After preheat 30 minutes of solution, the 4% of cellulase concentration (optimum parameter from previous study for the effect of enzyme concentration) then was added to the solution. The solution then immersed to shaken water bath at 50°C for one hour. Next, the solution undergone cool down for 5 minutes. 15mL of ethanol was added after cooling process and the mixture then proceed to ultrasonic treatment. The sample had undergone ultrasonic extraction of 50 % duty cycle and 60% amplitude in different time of sonication which were 0, 20, 40 and 60 minutes.

For parameter of duty cycle, the following method was performed. After preheat 30 minutes of the solution, 4% of cellulase was added to the solution. The solution then immersed to shaken water bath at 50°C for one hour. Next, the solution was undergoes cool down for 5 minutes. 15 mL of ethanol then was added after the cooling process and the mixture was subjected to the ultrasonic treatment. The sample undergoes ultrasonic extraction of 60% amplitude in 60 minutes sonication time (optimum parameter obtained from previous study for the effect of sonication time) with different duty cycle at 25%, 50%, and 75%.

After extraction, the sample first was filtered to separate solid sample of *Chromolaena odorota* sp. powder to ensure that the crude extract at last is ready for analysis. The sample solution then undergone centrifuged at 5800 rpm for 15 minutes to collect the supernatant. All the supernatant was filtered by using filter paper.

2.5. Phytochemical screening

2 mL of extract was measured. Then, a few drops of 5% ferric chloride solution were added to the measured extract. Formation of blue, green or violet colour indicated the present of the tannin [7]. Modification for the test was developed according to the sample’s analysis.

2.6. High Performance Liquid Chromatography (HPLC)

For standard preparation, 100 mg of the standard tannic acid was weighted and added into 10 mL of deionized water (HPLC grade) as a stock solution. The standard solution was diluted from the stock solution to 1,2,4,6 and 8 mg/mL and diluted with deionized HPLC water up to 10 mL. Calibration curve was constructed for tannic acid by plotting the peak areas against the concentrations.

Next, for sample preparation, the concentration of tannic acid in extract was identified using HPLC methods. For the preparation of sample, 1mL sample was filtered through 0.45μm syringe filter into the HPLC vial before being injected into HPLC system. The sample was analysed by Meta-Chem C18-A (250 × 4.6 mm, 5 μm) column. The mobile phase that was used in this study is HPLC water with acetic acid (99:1 v/v) in solvent A and methanol (HPLC grade) in solvent B using gradient elution mode. The flow rate was fixed at 1 mL/min and the injection volume fixed at 5 μL. Running time of each sample is 8 minutes. The sample then analysed under 270nm wavelength. Table 3.1 shows the conditions for HPLC analysis of *Chromolaena* plant extract for the determination of tannic acid concentration.

3. Results and Discussion

3.1. Phytochemical screening of tannic acid

As for the phytochemical screening of tannic acid by adding 5% of ferric chloride into 2ml of aqueous extract. The presences of soluble green, blue and violet precipitate or colouration indicate the presence of tannin in the sample [7]. Tannic acid is also known as a hydrolysable tannin. This shows that the phytochemical screening is also applicable for this compound. Moreover, tannic acid is bound to mucins in formalin-fixed and formalin-free fixed tissues in aqueous solution and its presence can be detected as a dark grey, blue-black to black complex with ferric chloride [8]. Table 1 shows the results
for the phytochemical screening for the effect of enzyme concentration at 50% duty cycle and 40 minutes of sonication time.

Table 1. Phytochemical screening for effect of enzyme concentration at 40 minutes sonication time and 50% duty cycle.

| Sample | Enzyme concentration (%) | Tannic acid (blue-black colouration) |
|--------|---------------------------|-------------------------------------|
| P 1.1  | 0                         | +                                   |
| P 1.2  | 2                         | +                                   |
| P 1.3  | 4                         | +                                   |
| P 1.4  | 6                         | +                                   |
| P 1.1.2| 0                         | +                                   |
| P 1.2.2| 2                         | +                                   |
| P 1.3.2| 4                         | +                                   |
| P 1.4.2| 6                         | +                                   |

From the table shows ++ is for densely present colouration/precipitate, + is for mildly present coloration and - is absent of the precipitate. Table 2 shows the phytochemical screening of samples from effect of sonication time towards enzyme concentration at 4% and 50% duty cycle.

Table 2. Phytochemical screening on effect of sonication time towards tannic acid UAEE at enzyme concentration 4% and 50% duty cycle

| Sample | Sonication time (min) | Tannic acid |
|--------|-----------------------|-------------|
| P 2.1  | 0                     | +           |
| P 2.2  | 20                    | +           |
| P 2.3  | 40                    | +           |
| P 2.4  | 60                    | ++          |
| P 2.1.2| 0                     | +           |
| P 2.2.2| 20                    | +           |
| P 2.3.2| 40                    | +           |
| P 2.4.2| 60                    | ++          |

From the results, other than sonication time at 60 minutes producing the mildly present of blue-black precipitate while at the 60 minutes produce an intense blue-black colouration from the sample. This could indicate that higher intensity of the colouration, the concentration of tannin (tannic acid) is also higher compared to the rest of the sample. The mildly present sample shows that the precipitate (colouration) is mostly at the bottom of the test tube while the densely present, the precipitate (colouration) is not settling at the bottom of the test tube. Table 3 shows the phytochemical screening for parameter duty cycle at enzyme concentration of 4% and sonication time of 60 minutes.

Table 3 shows the phytochemical screening for parameter duty cycle at enzyme concentration of 4% and sonication time of 60 minutes.

| Sample | Sonication time (min) | Tannic acid |
|--------|-----------------------|-------------|
| P 2.1  | 0                     | +           |
| P 2.2  | 20                    | +           |
| P 2.3  | 40                    | +           |
| P 2.4  | 60                    | ++          |
| P 2.1.2| 0                     | +           |
| P 2.2.2| 20                    | +           |
| P 2.3.2| 40                    | +           |
| P 2.4.2| 60                    | ++          |

According to Table 3, the blue-black colouration is densely present in every sample. This indicates that high concentration of tannic acid (tannin) is present in the sample. Based on Table 1, Table 2 and Table 3 that shows the concentration of actual tannic acid in the extract shows that when the concentration of tannic acid is 1.1 mg/mL and above, the result for phytochemical screening will significantly show densely present blue black coloration thus conclude in higher amount of tannic acid (tannin) is found in the aqueous extract.
Table 3. Phytochemical screening for effect of duty cycle at 4% enzyme concentration and 60 minutes sonication time.

| Sample | Duty cycle (%) | Tannic acid (blue-black colouration) |
|--------|----------------|-------------------------------------|
| P 3.1  | 25             | ++                                  |
| P 3.2  | 50             | ++                                  |
| P 3.3  | 75             | ++                                  |
| P 3.1.2| 25             | ++                                  |
| P 3.2.2| 50             | ++                                  |
| P 3.3.2| 75             | ++                                  |

3.2. Effect of Cellulase Enzyme Concentration

Figure 1 shows the effect of enzyme concentration towards concentration of tannic Acid at duty cycle 50% and sonication time of 40 minutes. The tannic acid concentration increased from 0.3191 mg/mL at 0% and 0.3573 at 2% to significant value of 1.1006 mg/mL when the enzyme concentration increases to 4% which show the effective enzyme hydrolysis occurred during the extraction process. However, by increasing the enzyme concentration to 6% resulted to the decreasing yield of tannic acid to half from the highest one which is 0.6911. It was found that by implicating the effective specific enzyme concentration will allow to significantly increase the yield of compound desired. The dramatic decrease in overall rates of enzymatic hydrolysis and rates of adsorbed enzymes per quantity is responsible in the low yield of tannic acid at 6% enzyme concentration. Synergetic effect is produced when applying pure enzymes such as cellulase. The degradation of the cell wall depends on the sequential hydrolysis of the structural polysaccharides as known the pectic substances cover the cellulose microfibers that attached to the hemicellullolitic polymers, their hydrolysis is partially repressed. The addition of enzyme and the consequent degradation of pectic polymers enables higher exposure of cellulose and hemicellulose to cellulase and hemicellulase enzymatic action [9] that cause increasing effect of enzymatic hydrolysis towards the Chromolaena odorota cellulose and hemicellulose layer thus lead toward release higher concentration of (polyphenol) tannic acid or any phenolic content. The results were obtained by ensuring to keep constant few parameters. The water substrate ratio does not give significantly impact of the yield concentration. The enzyme concentration at 4% shows the best result with 1.0006 mg/mL compound extractability. Therefore, extraction time of 40 minutes and substrate water ratio of 1:20 were used in the UAEE for studying the effect of enzyme concentration on enhancing extraction efficiency.

The experimental design was employed to optimize operating conditions of enzymatic assisted extraction. The pre-treatment temperatures and solvent pH values ranging from 49.9°C to 51°C and from 5 to 5.5, respectively, were selected taking into consideration the optimum working temperature and pH range of the cellulase enzyme [10]. The pre-treatment phase for the extraction also actually activates the enzyme activity to enhance the extraction efficiency. From previous studies, enzyme such as cellulase have been proofed significantly increase the overall phenolic compound content of raspberry under the experimental conditions applied in the study possibly due to the presence of secondary activities such as different class of cellulase such as β-glucosidase or β-galactosidase that mode of action is different from the other [9]. Compared to non-enzymatic assisted extraction, the biocatalysts tested and their mixtures in equal proportions increased the soluble solids content. The hydrolytic capacity of enzymes capable of degrading the main components of the cell wall, releasing sugars from macromolecules such as cellulose, hemicellulose and lignin, could explain the increase of the yield [9].
3.3. Effect of Sonication Time

From Figure 2, it was found that the highest concentration of tannic acid obtained at sonication time 60 minutes with average of 1.2350 mg/mL. Supposedly from the trend, the longer sonication time, higher yield produced. This is because the longer cavitation effect is applied to breakdown the cell wall of the *Chromolaena odorata*, higher yield would be release from the cell. However, from the obtained result it was observed that at 0 min towards 20 mins shows increase of the yield however at 40 mins, the concentration is slightly decrease. This could happen due to inconsistency of parameter during handling the parameter.

In this study, cavitation phenomena are adversely affected by high surface tension and vapor pressure which both is temperature related. By increasing the sonication time thus will increase cavitation phenomena towards affecting the surface tension of solvent and increase the pressure towards the rate of cell lysis to occur. When this ensues, the tannic acid release from the plant cell will increase. By subjecting the sonication causes the effect of micro-streaming and can enhance the mass transfer generated by the collapse of the cavitation bubble. This in turn results in the destruction of the cell wall, thus providing better contact and solvent interactions in and out of plant materials. Sonication treatment has also been reported to improve the extractability of solvents from materials at low temperatures [11].

Act of soaking of the dried *Chromolaena odorata* sp. to replenish the moisture that was originally present when it was fresh, soak the dried herbal material in the extraction solvent. This causes the plant material to swell and the release of the compounds into the extraction solvent from the swollen material. The driving force for this is the concentration gradient of target compounds that is greater than in the solvent inside the swollen plant material. However, ultrasonic force will give greater driving towards the cavitation. Cavitation could be defined is when the intensity of the sound in the liquid increases, tiny bubbles arise, grow, and collapse. The collapse of the bubble will lead to high temperature, high pressure, and high pressure from bubbles then increase velocity of liquids near the bubbles. This phenomenon is known as ultrasonic cavitation [12]. From the results, it was found that at 60 minutes of sonication time, the yield of tannic acid is the highest .With this, to further the studies towards effect of duty cycle towards the extraction of tannic acid by UAEE, 60 minutes sonication time and 4% enzyme concentration were used as the constant parameters.
3.4. Effect of Duty Cycle

From Figure 3 shows, at duty cycle 50% give out the highest concentration of tannic acid produce. However, the effect is not adversely impacting the yield of tannic acid compared to duty cycle of 25% and 75%. As known by implying the pulsed ultrasonic is more efficient than continuous run of sonication effect. Supposedly the higher percentage duty cycle, the higher cavitation effect towards the cell lysis thus lead to higher extraction yield. In this case of UARM that using horn, to avoid damage, the horn must be operated in pulse mode. It is also expected that the extraction yield obtained using pulse mode is greater than that obtained using continuous mode, which can occur during pulse mode operation due to the generation of non-steady state condition. Therefore, the use of ultrasonic probes in pulse mode offers two benefits, higher extraction output and lower energy consumption with better operational life. Nevertheless, by increasing duty cycle to 75% which ratio at 7.5s:2.5s, slightly decreased the yield produce. This occurs possibly due to nearly continuous sonication implied that could lower the efficiency of the cavitation. Thus, the implosion of the extract is maximized and overwhelmed the limit of compound thus destroying part of the compound that lead to the decreasing concentration of tannic acid at duty cycle of 75%. Thus, from the study of duty cycle it was found that by implying 50% of duty cycle towards the UARM, thus give out highest concentration of tannic acid produced. The 50% duty cycle which allows the horn of the ultrasonic reduces the interfacial area between plant material and solvent phase more effectively thus leading to producing the highest yield. Through a process called cavitation, which involves the formation, growth and implosive collapse of bubbles in a liquid, ultrasound energy is transferred to the polymer chains during ultrasonic treatment. In the cavitation bubbles and the immediate surrounding area, violent shock waves are produced, and these can be used to isolate the compound from cell of the plant [12].
4. Conclusion
In conclusion, it was found that the optimum extraction parameters for the extraction of tannic acid from *Chromolaena odorota* sp. at 4% cellulose enzyme concentration, 60 minutes of sonication time and 50% duty cycle resulted to highest concentration of tannic acid at 1.6152 mg/mL that was obtained from UAEE.

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