Retention of Bioactive Compound in the Concentration of *Centella Asiatica* Extract through Progressive Freezing

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Abstract. Phenolics and flavonoids were the two major bioactive compounds in *C. asiatica* that contributes to its therapeutic values. However, concentration by evaporation resulted in denaturation of the compounds due to their heat sensitivity nature. Therefore, this paper aims to introduce progressive freezing (PF) process as a new concentration method for the *C. asiatica*. PF separates two liquid compounds based on their freezing point difference. One with a higher freezing point would solidify first while another one would remain as a liquid. Low temperature involved in this process could minimize the heat abuse to the volatile compounds of *C. asiatica*. In this study, maceration was done to get the *C. asiatica* extract by preparing 1:80 ratio of solute to solvent and stirred at 1200 rpm for 24 hours. On the other hand, the PF was conducted in a cylindrical stirred crystallizer. For PF process, two operating parameters which are coolant temperature ranging from -6°C to -12°C, and stirring rate ranging from 100 rpm to 250 rpm have been tested for their effects towards the process performance. Two determinant parameters which were effective partition constant, \( K \) and retention efficiency (Reff) of bioactive compounds (flavonoid and phenolic) in the concentrated solution had been used to portray the process efficiency. On completing the experimental work, it was found that lower coolant temperature, and a moderate stirring rate resulted in the higher concentration efficiency of the bioactive compounds. The highest concentration efficiency of 69% and the smallest effective partition constant (K) of 0.51 were achieved at -10°C, 150 rpm and 30 minutes.

Introduction

*Centella Asiatica* (*C. asiatica*) plant or familiar to be known as Pegaga or Gotu Kola belong to *Apiceae Sp.*, which is enriched with therapeutic potential in treating a broad range of diseases such as diarrhea, hepatitis and inhibit the growth of tumor cells (Zahara, Bibi, & Shaista, 2014). It is widely found in Asian countries and is used as source of food and herbs to cure several ailments. A recent study by Hamidpour et al. (2015) had proven that doses of *C. asiatica* influenced the cell nerves of the pyramidal cells in the prefrontal cortex, the hippocampus, and other areas, in newborn and adult mice and rats.

The major bioactive components found in *C. asiatica* are madecassic acid, asiacic acid, triterpenes with its derivatives such as madecassoside, asiasicoside, and triterpene ester glycosides. These compounds are the core ingredients of numerous compound formulations used for curing gastrointestinal disorders and for revitalizing brain cells (Zahara, Bibi, & Shaista, 2014). To be precise, flavonoids and two other components which are saponosides and diosmin in *C. asiatica* have been assessed for treating chronic venous insufficiency (CVI) in which blood faced difficulties to return to the heart due to abnormal condition of valves in the leg veins (Hamidpour et al., 2015). Flavonoid is an essential compound in a plant for producing the colors required to attract pollinating insects. It can protect the plant against an abiotic and biotic strike, building a defense against fungal and responsible for interaction between the plant and its surroundings. Research has been made on the effects on human health and proven to be useful as anti-cancer agents and inhibition of key enzymes involved in tumor growth (Sak, 2014).

On the other hand, a higher amount of phenolic content was also found in *C. asiatica* leaves, as compared to the different parts of the plant (Gohil, Patel, & Gajjar, 2010). The phenolic compound is said to be the chief contributor of antioxidant activities which benefits human skin from aging. Besides,
free radicals from the environment can cause risk to human health as it may promote uncontrolled cell growth which leads to aging as well as cancer. Due to its high anti-oxidant activity, C. asiatica may be a useful ingredient in skin ointment that protects the human cell from damage caused by reactive oxygen species (Gohil, Patel, & Gajjar, 2010). Despite all of this richness with bioactive compounds that are beneficial for human health, C. asiatica is said to be one of the threatened plants due to its overexploitation listed by International Union for Conservation of Nature and Natural Resources (IUCN).

Commercializing the use of C. asiatica in therapeutic or health care industry requires prior processing steps of extraction and concentration. According to Azwanida (2015), the extraction process aims to separate the soluble plant metabolites, leaving behind the insoluble cellular residue. Several conventional extraction techniques for plant matrix have been introduced, which include maceration, Soxhlet, or continuous hot extraction, ultrasound-assisted, and microwave-assisted extraction. On the other hands, the concentration process is needed to remove the solvent used from the plant extract to reduce further its mass and volume, which in turn leaves behind a concentrated extract. This step is beneficial in preparing the products for subsequent process operations such as mixing, drying, crystallization, spoilage prevention, water activity reduction, shelf life-lengthening, and others. By concentrating the solution, stability and handling of the product can also be improved (Amran & Jusoh, 2016). The conventional concentration method is through evaporation. In this method, solvent is removed from the extract as vapor, leaving concentrated juice. However, during the solvent removal, a portion of bioactive compounds value could be vaporized together as they are the most volatile compound. Therefore, browning and degradation in taste and food value may occur. Due to that reason, evaporation must be performed in a short time as possible and minimal temperature to save heat-sensitive substances.

Therefore, this study introduces a new concentration technology in concentrating the C. asiatica extract, namely progressive freezing concentration (PFC). PFC is a process of concentrating the liquid product by freezing the solvent molecule as a solid or crystal layer on a cooled surface until it forms a single large crystal/solid block (Amran & Jusoh, 2016). Since there is only one block of solid/crystal formed, the collection of the solid from the liquid becomes much more manageable (Zanariah, Norzita, Anwar, Yamani, & Mazura, 2017). PFC has proven to be an efficient separation technique in the food industry. For instance, this technology has provided an opportunity for the dairy processing industry to produce enhanced functional and organoleptic qualities. PFC has proven to minimize the damage of protein and flavors of the dairy product caused by the high temperature in the conventional evaporation process. By PFC, the whole milk has been concentrated up to 44 wt% total solids (TS) and skim milk up to 40 wt% TS (Auleda, Raventós, Sánchez, & Hernández, 2011).

In this study, the retention of bioactive compound (phenolic and flavonoid) that exist in the C. asiatica after the PFC process was analyzed at different readings of coolant temperature and stirring rate. The efficiency of the process was then evaluated and portrayed by two determinant parameters which are effective partition constant, $K$, and retention efficiency ($R_{eff}$) of the two bioactive compounds in the concentrated solution.

Methodology

**Raw Materials Preparation**

The raw material used in this study is C. asiatica plant. It was freshly purchased from the local market in Perak, Malaysia. The plant material was washed under running water to remove any dirt. Prior placed it in the oven, the wet plant material was dried under the sunlight for 1-2 hours to prevent the leaves from wilt and turned yellowish. Then, the plant was placed in the oven at 50°C for 24 hours. Once dried, the plant material was ground to powder form by using a dry blender. Lastly, the plant powder was kept in a closed container for future use. On the other hand, a 50:50 volume ratio of ethylene glycol and water was mixed and used as a coolant for the freezing process. Ethylene glycol is commonly used as a coolant in many commercial and industrial applications as it can transfer heat at a very low temperature (Amran & Jusoh, 2016).

**Maceration Process**
The extraction of the plant juice was started by adding 10 g of ground plant material to 800 mL of distilled water in a beaker. The solution was then mixed with a magnetic stirrer at a speed of 350 rpm for 24 hours. The surface of the beaker was covered with aluminum foil and kept away from sunlight. After 24 hours, the extract was vacuum-filtered and stored in a cold room.

**Experimental Setup for PFC**

The apparatus and device involved in the PFC process are cylindrical freeze crystallizer (RnR Tool & Machining, Malaysia), a digital stirrer (IKA, Malaysia), two retort stands (Benua Sains Sdn. Bhd., Malaysia) and a refrigerated water bath (Protech, Malaysia). The plant extract was placed in the crystallizer, while the coolant was stored in the refrigerated water bath where the temperature is being controlled at the desired values. The retort stand was used to hold the crystallizer while it was immersed into the refrigerated water bath. The digital stirrer was used to stir the sample so that the separation can take place more efficiently. Figure 1 shows the experimental setup of the PF process.

![Figure 1: Experimental setup for the PFC process](image)

**Experimental Procedure**

After the coolant has been prepared at the desired volume ratio of ethylene glycol and distilled water, the temperature of the coolant was then set at the desired value (ranged from -6 °C to -12°C). After the temperature was reached, 500 ml of the plant extract was poured into the crystallizer. The speed of the digital stirrer was then set at the desired stirring rate (ranged from 100 – 250 rpm). Next, the crystallizer was immersed in the coolant, and the sample was stirred by the digital stirrer to let the crystallization to take place. After 30 minutes, the crystallizer was taken out of the water bath. A volume of the formed solid, and the concentrated sample in liquid form was transferred into a sample bottle to measure the bioactive compound content by using a UV-Vis spectrophotometer. The total phenolic content (TPC) and total flavonoid content (TFC) of the extract were determined by the Folin–Ciocalteu method and the aluminum chloride colorimetric method, respectively (Baba & Malik, 2015). The analytical analysis were done at wavelengths of 370 nm (TFC) and 750 nm (TPC). The experimental procedure was repeated at different coolant temperature and stirring rate.

**Calculation of Effective Partition Constant (K)**

Effective partition constant, $K$ evaluates the efficiency of the system, which is related to the quality of the ice produced based on the concentration and volume of target solution and concentrated sample as Equation (1):
(1-K) log (Vo/Vl) = log (Cl/Co)     (1)

where Vl and Vo indicate the volume of concentrates and initial sample in mL, respectively. On the other hand, Cl and Co indicate the concentration of bioactive compounds in the concentrate and initial sample in mg GA/g and mg Quercetin/g, respectively. The retention of the bioactive compound in percentage is calculated using the Equation (2).

Reff (%) = [(Cl – Co) / Co] x 100     (2)

where the Cl and Co indicate the final and initial solute concentration in the solution.

Results and discussion

Effect of Coolant Temperature

As stated before, the coolant temperature was varied at -6 °C to -12°C while keeping both stirring rates and freezing time constant at 150 rpm and 30 minutes, respectively. From the UV-VIS analysis, the initial reading of TFC and TPC are 12.27 mg GAE/g and 13.47 mg Quercetin/g, respectively. Figure 2 illustrates the changes in TFC and TPC at different coolant temperature, while Figure 3 shows the changes in K and Reff values at different coolant temperature.

As mentioned by Ramos et al. (2005), heat transfer within the wall of crystallizer and the coolant for the concentration process through ice crystallization could be enhanced by low temperature of the coolant. In other words, low surface temperature could provide the needed initial supercooling for the ice nucleation. From the figure, the highest TFC (20.43 mg GAE/g) and TPC (22.53 mg Quercetin/g) concentrations were recorded at the lowest coolant temperature of -12°C. The phenomenon proves that more water has been frozen and separated from the C.asiatica extract at a lower temperature. The result obtained is in line with the theory where the ice growth rate increases with decreasing temperature which means the colder the condition is, the higher the ice growth rate will be (Amran, 2015). Ice growth accelerates with increasing difference between the temperature of entering solution which is C.asiatica extract and surface temperature which is the coolant (Ab. Hamid et al., 2015). However, as the temperature gets lower beyond its optimum point, the purity of ice could be affected as more solute will eventually trap in the ice due to higher ice growth rate.
Based on Figure 3, the K value decreases with temperature, and the lowest values obtained were 0.55 for TFC and 0.56 for TPC at -12°C. The lower K value is preferable as the concentration efficiency increased. The result concludes that more flavonoid and phenolic compounds retained in the concentrate and higher purity of ice crystals obtained. The obtained concentration efficiency was quite high, with 67% for both TFC and TPC. Higher retention of bioactive compounds in a solution might be due to its unique molecular structure that helps water retention by integrating between molecules easily, hence ease the ice growth (Safiei, Ngadi, Johari, Zakaria, & Jusoh, 2017).

Effect of Stirring rate
The stirring rate was varied from 100 to 250 rpm while both coolant temperature and freezing time were kept constant at -10°C and 30 minutes, respectively. Figure 4 illustrates the changes in TFC and TPC at a different stirring rate, while Figure 5 shows the changes in K and Reff values at different stirring rate.

According to Fukui and Maeda (2002), the fluid flow structure of the solution controls the transfer phenomena of heat and mass in the PFC process. A high or sufficient solution stirring is needed to remove the trapped solutes from the crystal so that a highly concentrated solution can be obtained (Shirai et al., 1998). Theoretically, an increase in the stirring rate promotes higher heat transfer rate for ice crystallization (Wakisaka et al., 2001). At this condition, formation of ice crystals in a planar form from the cooling wall. From Figure 4, it was found that the highest bioactive compound concentration was at 150 rpm, and further increase in the stirring rate would reduce the bioactive compound concentration. The result implies that increasing the stirring rate beyond the optimum point will result higher solute trapped in the ice and reduce its purity, thus less concentrated solution produced. Theoretically, higher stirring rate reduces the solidification rate that leads to less solute trap in ice (Jusoh, Yahya, Ab. Hamid, & Safiei, 2014). It is in line with a model developed by Miyawaki where reduction of solute accumulation at interface of ice-liquid with higher stirring rate has achieved (Miyawaki, Kato, & Watabe, 2012). However, the speed rate has always a limit for an optimum result as shown in Figure 5 where it represents the K and Reff values at different stirring speed.
The lowest $K$ values were obtained at 150 rpm, which represents an excellent progressive freezing performance with $R_{eff}$ of 48% and 58% for flavonoid and phenolic content respectively, as shown in Figure 5. However, as the speed increased, the partition constants were no longer showing an increase in the retention efficiency. Stirring speed plays a vital role in ice purification where less solute is expected in the ice formed. Increase in stirring rate means building up the kinetic energy that leads to higher shear force of the fluid. As a result, the force will carry away the solute that is entrapped between the dendritic structures of ice and leaves the unfrozen solution at saturation level (Md Zamani, Yahya, Zakaria, & Jusoh, 2015). However, increasing the stirring rate beyond the optimum point might have the potential to erode the ice layer formed thus less concentrated solution produced.
Conclusion

Although there are several methods in concentrating extract, progressive freezing is one of the techniques that suit in preserving therapeutic benefits, especially in fruits and herbs plant, since the low temperature involved in the process is advantageous in preserving the heat sensitive components.

Maceration with water as the solvent was conducted at room temperature with 1200 rpm for 4 hours since the solute size is already ground into powder-based. Once maceration has completed, quantification of bio-active compounds shall be run using the UV-Vis Spectrophotometer. A standard calibration curve is needed prior analyzing the concentration of both compounds.

Progressive freezing in overall was a success in producing concentrated juice based on color observation of the ice and concentrate samples. Justifying all the three parameters involved proved that PFC has successfully retained the bioactive compounds provided that the optimum condition is applicable. The highest phenolic and flavonoid concentration was at -12°C with 150 rpm for 30 minutes. Almost half of the bioactive compounds were able to be retained, as shown by the K value and concentration efficiency results. All in all, the objectives listed for this project has been successfully achieved.

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