Extraction of Phytosterol Concentration in Different Legume Pods by Using Microwave-Assisted Hydrodistillation

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Abstract: *The traditional ways in the extraction of bioactive compounds using conventional methods are disadvantageous from both economic and environmental perspectives. In this, the potential of microwave-assisted hydrodistillation conditions for extraction of phytosterol from legume pods was investigated. Salkowski test performed on the legume pod has shown the reddish brown in all sample which confirmed the presence of phytosterol qualitatively. Liebermann-Burchard procedure and ultraviolet-visible spectroscopy (UV-Vis) apparatus were used to study the concentration of phytosterol at different extraction parameters which are temperature (25–80 °C), solvent concentration (50–100% v/v), irradiation time (1–10 min) and microwave power (400–800 W). The optimal conditions for highest yield of extract (0.219 mg/L) were obtained at a microwave power of 600 W, the irradiation time of 6 min, and ethanol concentration of 75% v/v. Results obtained in this study have shown the capability of microwave-assisted hydrodistillation in the extraction of phytosterol from legume pod. Further works are nevertheless required to provide a deeper understanding of the mechanisms involved to facilitate the development of an optimum system applicable to the industry.*

Keywords: *phytosterol; microwave assisted extraction; one-factor-at-a-time; Salkowski test*

■ INTRODUCTION

Phytosteroid is one of many phytochemicals which is from the big family of phytosterol. Beta-sitosterol is the most abundant phytochemical to be found in the plant structure. In nature, phytosterols can present as the plant sterol that presents in the plant nonsaponifiable oils which has the same structure to cholesterol but with an addition of sterol chain at C24 position [1]. Beta-sitosterol has categorized as the plant-based phytosterol. Study deduce that beta-sitosterol comes with various pharmaceutical properties such as they inhibit the growth of colon and prostate cancer [2-3] and also controlling the carcinogenic property in the colon cancer cell [4]. Besides that, beta-sitosterol also has the properties of antidiabetic and antioxidant that are good in the high sugar blood condition [5].

Fruit peels and pods are usually being discarded, and only the flesh and the seeds of the fruit are consumed. The peels and the pods are considered as a waste as they do not have any beneficial side to the food taste and human health. The pod and peel of the legume usually considered as a waste. But there is a study that indicates that the chemical properties in the pod also has a beneficial effect. There was research present on the studies of the agro-waste that usually not been or fully utilized [6]. Even though the peel and pod are considered as a biodegradable material, but they also can

cause environmental issue [7]. The plant waste such as its legume pod, peel, and skin can be converted into a new product that can be utilized such as adaptable functional components or as natural active component [6].

The extraction process is an essential step to obtain the desired product from the plant. Generally, the extraction of phytosterol was done by using several types of extraction such as Liquid-Liquid Extraction, Solid-Phase Extraction, Soxhlet Extraction, and Microwave-Assisted Extraction [35]. However, several types of extraction process were less efficient in time management, usually used up the high volume of toxic organic solvents and also oppose with numerous step and process that may lead to losses of desired phytochemical [8]. The microwave-assisted extraction process is more efficient to produce a higher yield of phytochemical with less energy and time [9]. Besides that, by using microwave-assisted extraction method also could reduce the use of solvent extraction [10]. However, like the other method, the extraction yield is depending on the extraction parameter such as irradiation duration, temperature, power, solid-liquid ratio and solvent concentration [11].

Thus, it is significant to study the method to extract phytochemical content in legume pod in order to obtain a higher yield in the extraction process. The extraction process that can be used is microwave-assisted extraction (MAE) of phytosterol. Lately, the MAE process gains significant exposure where the extraction process consists of microwave irradiation and organic solvent extraction, where it can efficiently perform the extraction phytochemical with lesser extraction time, a smaller amount of solvent usage and higher extraction yield of desired phytochemical [12].

Therefore, this research studies the usage of microwave-assisted hydrodistillation to extract phytosterol from legume pod. Thus, the aim of this study is to explore the effects of extraction parameter such as irradiation power, temperature, duration, and solvent concentration on the phytosterol concentration yield from the three legume pods.

■ EXPERIMENTAL SECTION

Chemical and Material

The chemicals used were 95% ethanol GmbH, 1M sulphuric acid Merck and 98% acetic anhydride. Methanol HPLC was obtained from Permula Chemical Sdn. Bhd., the ultrapure water was purified by Milli-Q® Advantage A10 Purification System. Standard beta-sitosterol was purchased from Sigma Co., USA. Raw materials used in this study are *Archindendron pauciflorum, Parkia speciosa*, and *Leucaena leucocephala* legume pod. The plant sample obtained from Pekan district area.

Procedure

Plant sample preparation

After the samples were collected, it was prewashed by using distilled water and dried by using the paper towel. Then, the plant was ground by using Panasonic MXAC210SW blender to increase the surface area to contact with the solvent [13]. After that, the plant sample was stored at -20 °C.

Extraction process

The extraction process was conducted by using the microwave-assisted hydrodistillation (MAH) extraction unit. The ratio of plant sample's weight and solvent volume was 1/20 [28]. The extraction process was repeated by using several desired parameters such as extraction solvent concentration, the temperature of the extraction process, duration of the extraction process and the microwave irradiation power. After the extraction process, the liquid sample was reduced its volume to ¼ from the original volume by using rotary evaporator. The sample was then stored at -4 °C for further analysis.

Table 1. Extraction parameter range

Extraction Parameter	Parameter range
Ethanol concentration (%)	45–95
Temperature (°C)	25–80
Time (Duration) (min)	1–10
Power (W)	200–800

Salkowski test

Concentrated extracted sample was measured for 1 mL and was added into 5 mL of chloroform. After that, a few drops of concentrated sulphuric acid was added to the extracted solution. The presence of steroid was indicated by the forming of a reddish brown color in the upper layer solution [14].

Liebermann-Burchard reagent

Fifty milliliters of acetic anhydride was transferred into an amber vial and left in an ice bath for 30 min. After that, 5 mL of sulphuric acid was added carefully into the ice-cold acetic anhydride.

Beta-sitosterol calibration curve

The standard of Beta-sitosterol was diluted at a different concentration range from 0.02 to 0.10 mg/mL to generate the calibration curve. From the data obtained, the calibration curve was plotted where the calibration equation was calibrated. The calibration equation was shown below.

$$y = mx + c \quad (1)$$

where, y = absorbance value; m = s lope of the graph (6.3117); c = constant value (0.0124)

Sample quantitative analysis

After that, 1 mL of reduced volume sample was added with 20 mL of chloroform and added up to 50 mL with the ethanolic water solvent. Then, 5 mL of the sample was transferred into 10 mL volumetric flasks and added with 2 mL of Liebermann-Burchard reagent. The solution volume was adjusted with chloroform. The absorption of the sample was measured by using UV/Vis after 5 min addition of Liebermann-Burchard reagent. Chloroform was used as the measured blank. The method for quantitative analysis was supported by another study by Araujo et al. [15].

■ RESULTS AND DISCUSSION

Salkowski Test

Salkowski test is a type of qualitative analysis to test the presence of steroid in the extracted sample. The steroid was present as the reddish color appeared on the upper layer of chloroform [13]. From all three samples tested, all were positively tested with the steroid.

Fig. 1 shows the presence of steroid in *Archindendron jiringa* legume pod extract. A recent study demonstrates the presence of steroid and triterpenoid in the 70% of ethanolic extract of *A. pauciflorum* legume pod [21]. Besides that, Fig. 2 also detected the presence of steroid in *L. leucocephala* legume pod extract. The *L. leucocephala* plant sample had tested positive with steroid as referring to several other pieces of research [16]. From studies perform by Arun et al. shows the evidence that the slightly present of sterol in hexane and methanol extract of *L. leucocephala* [17]. Lastly, Fig. 3 also

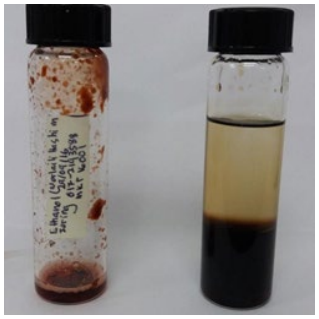

Fig 1. Salkowski test on *A. jiringa*

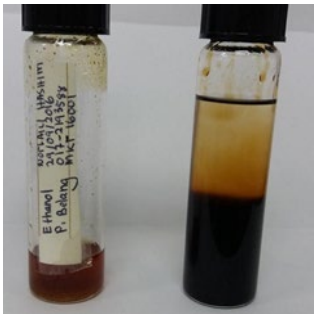

Fig 2. Salkowski test on *L. leucocephala*

Fig 3. Salkowski test on *P. speciose*

generated the present of steroid in *P. speciosa* legume pod extract. Previous studies also derive the presence of steroid in the ethanolic extracted solution from *P. speciosa* [18].

Liebermann-Burchard UV-Vis Analysis

After the addition of chloroform, sulfuric acid, and acetic anhydride into the extracted plant sample, the sample solution was turned to violet-blue then formed green color manifested the present of steroids [13]. The first process occurs in the solution is the protonated of the phytosterol then followed by the loss of H_2O or dehydration process where carbonium ion of 3,5-cholestadiene was formed [19]. Then, the oxidation process occurs where the blue color is formed from the pentacyclic cations [20]. The wavelength to be used in the analysis was set at 625 nm as blue oxidation process was maximum occurs at 625 nm [14]. In Fig. 4 manifested the calibration curve calibrate from the beta-sitosterol standard. The result of the beta-sitosterol concentration present in the plant sample was shown in Fig. 5 to 8.

Effect of Solvent Concentration

In this research, ethanol was used as the extraction solvent. There was plenty of reason for the use of ethanol as it is toxic free, safe to the environment and mostly use extraction solvent for extracting natural based product [21-22]. Besides that, ethanol also can dissolve the desired compound and also has a great polarity for the microwave to absorb the desired compound [23]. The ethanol mixed with water was used. Ethanol has a lower polarity so that allowing the solubility of beta-sitosterol to increase in the extraction solvent [24]. The mixture of ethanol with water allows the heating process of the microwave to be improved because of water high dielectric constant [25]. Besides that, water also enhances the penetrability of plant matrices to increase the mass transfer process and the diffusion of desired phytochemical [26]. Another condition was fixed at the temperature of 75 °C, three cycles at a duration of 6 min per cycle and irradiation power of 600 W.

Different concentration of ethanol gave different result in the phytosteroid extracted concentration content. The concentration of beta-sitosterol in *L. leucocephala* has the highest value followed by *A. pauciflorum* and then *P. speciosa* with the lowest value of 0.2191, 0.0313, and 0.1101 mg/mL, respectively. The yield produced increased as the increase of the concentration of the ethanol and gained the maximum yield at the concentration of 75% and drop after the concentration above 75% in all plant legume sample.

Phytosteroid has the properties of hydrophobic. The fat-soluble phytosteroid is favorably extracted in the high ethanol concentration. But, water-soluble phytosteroid is more beneficially extracted in low ethanol concentration. So, based on the research result, the most suitable ethanol concentration was 75%. This was supported by the result obtained from the extraction

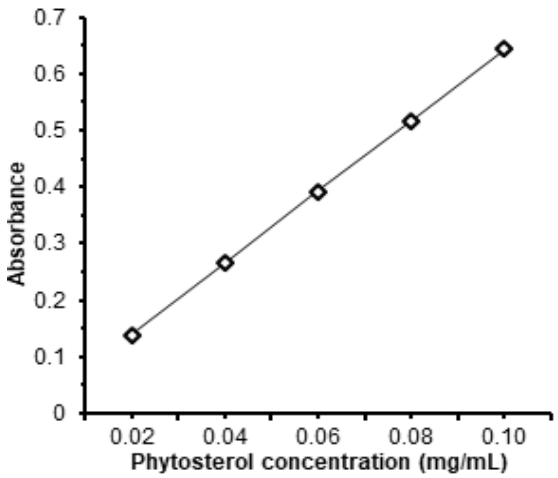

Fig 4. Calibration curve of beta-sitosterol (phytosterol) standard absorption

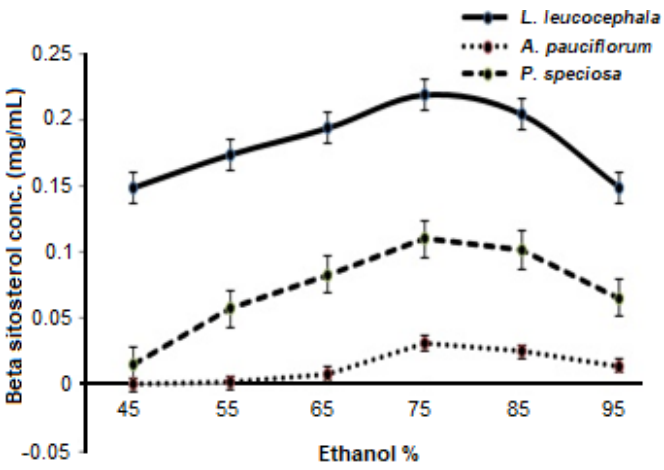

Fig 5. The concentration of beta sitosterol present at a different ethanol concentration

of steroid saponin from *Dioscorea zingiberensis* Wright [27]. Fig. 5 shows the legumes concentration at different ethanol extraction solvent concentration.

The Temperature of the Extraction Process

The temperature of the extraction process varied from 25 to 80 °C. Another condition was fixed at three cycles at a duration of 6 min per cycle, ethanol concentration at 75% and irradiation power of 600 W. In common, the suitable temperature will result in better solute solubility in the extraction solution. Temperature also a factor that is correlated with irradiation power where it controls the amount of energy transformed into heat in the dielectric material [28]. The concentration of beta-sitosterol in *L. leucocephala* was the highest among the other two plant legume, *A. pauciflorum* and *P. speciose,* which was 0.2067, 0.0828, and 0.1091 mg/mL, respectively. As for the result of the phytosteroid yield, the yield increase as the temperature increase from 50 to 75 °C. This was because of the increase of the diffusion coefficient of the extraction solvent when the viscosity of the solvent decreased within the temperature range that would assist the efficiency of the microwave-assisted extraction process. As the temperature increase above the optimum temperature of 75 °C, the beta-sitosterol concentration decreased might be caused of the extraction of the non-phytosterol component that may occur and increase in volume making a dilution of beta-sitosterol in the extracted solution [29]. This may because the high temperature partially causes the phytosteroid to damage and reduced. So, the most suitable temperature to be set for the microwave-assisted extraction process was 75 °C.

Theoretically, the plant tissue will be softened at high temperature and then alter the cell membrane of the plant cell [30] that is resulting in the extraction process of the phenolic compound to be done easier [31]. But, a longer time in the extraction process may cause the oxidation and degradation of the desired product from the plant [32]. Studies assumed that phenolic compound is very sensitive to any heat process and can easily be oxidized. This deduces that the maximum temperature of the extraction process must be controlled at all-time [33].

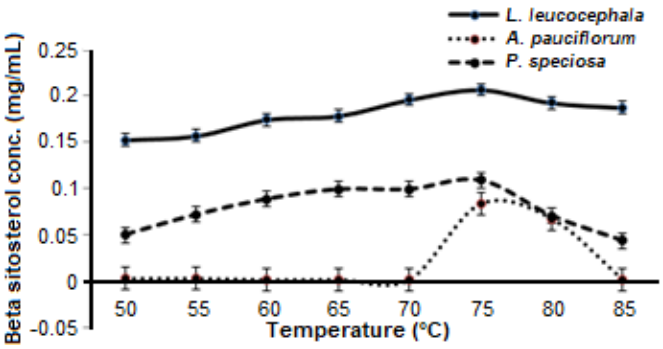

Fig 6. represents the legumes concentration at different microwave irradiation temperature.

Duration of the Extraction Process

As for the microwave-assisted extraction process, it saves a lot of time as compared to another conventional method. As time has been reduced, the cost in product process will also be reduced. The longer the duration of the extraction, the longer time of compound to be exposed to the irradiation power. The excess exposure to the irradiation power may lead to the loss of desired phytochemical [28]. Besides that, the longer duration of the extraction process may lead to another unwanted reaction such as enzymatic degradation and oxidation and may cause the desired yield compound to reduce [33]. The extraction process is mainly depended on time and temperature if the irradiation. As the temperature and duration of the extraction process are increased, the product yield of the desired compound also increase, but the elongated duration of extraction process may result in the deficit thermolabile bioactive compounds [34]. The duration of the extraction process varied from 1 to 10 min. Another condition was fixed at the temperature of 75 °C, ethanol concentration at 75% and irradiation power of 600 W.

The concentration of beta-sitosterol was found highest in *L. leucocephala*, followed by *A. pauciflorum*, then *P. speciosa* which was 0.2037, 0.0858, and 0.1075 mg/mL, respectively. As the extraction process time increased to 6 min, the yield of phytosteroid also increased. But, as the time increased to longer duration, the yield reduced. Elongated duration exposed to the

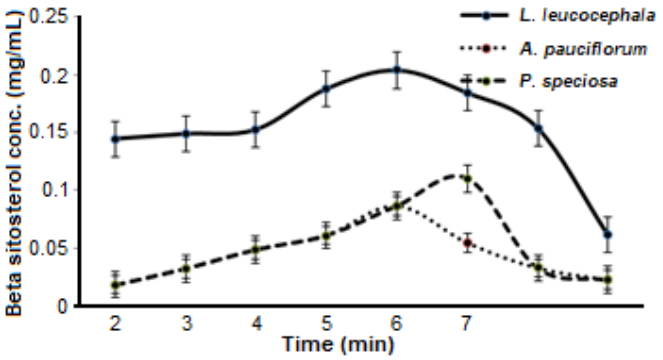

Fig 7. The concentration of beta-sitosterol present at different extraction duration

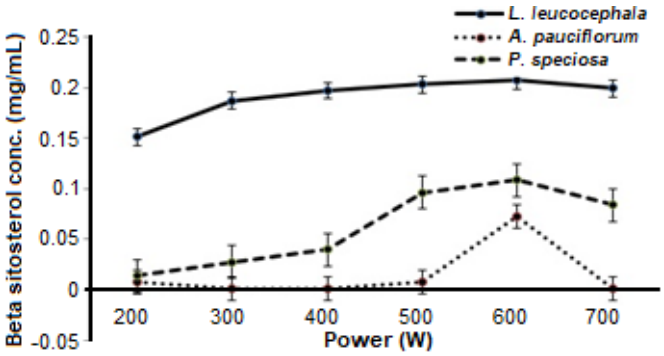

Fig 8. The concentration of beta-sitosterol present at different extraction power

microwave irradiation may cause the phytosteroid degraded into another substance other than phytosteroid as the exposure duration to oxygen is longer [32]. Therefore, the most suitable duration for the extraction process was 6 min. Fig. 7 generated the legumes concentration at different microwave irradiation duration.

Microwave Irradiation Power

In the extraction process, the irradiation power also plays a part in extracting the desired phytochemical from the plant structure by breaking the cell wall and also altering equilibrium and mass transfer condition in the extraction process [35]. The time and power of the radiation are two factors, but they were interconnected to each other. The most suitable approach for power and time is as a combination of lower power with longer radiation time expose to the extracted material [31]. The irradiation power of the extraction process varied from 100 to 800 W. Another condition was fixed at the temperature of 75 °C, ethanol concentration at 75% and three cycles at a duration of 6 min per cycle. Based on the result, it evidences that the phytosteroid yield increased as the power of irradiation increased. The concentration of beta-sitosterol in *L. leucocephala* was detected as the highest value, while *A. pauciflorum* had the second highest value followed by *P. speciosa* which had the lowest beta-sitosterol concentration for 0.2070, 0.0725, and 0.1085 mg/mL, respectively. As the irradiation power increased, the molecular interaction between the substance in the sample would distress leading to the reduction of the phytosteroid yield. It may also damage the phytosteroid content in the sample. So, the most suitable irradiation power for the extraction process was 600 W. Fig. 8 surfaced the legumes concentration at different microwave irradiation power.

■ CONCLUSION

Based from the solvent concentration studies, the concentration of phytosterol contain in the *L. leucocephala* (0.2191 mg/mL) was the highest compared to the content of phytosteroid in *A. pauciflaurum* (0.0313 mg/mL) and *P. speciosa* (0.1101 mg/mL). From this study, it showed that the most optimum condition for the extraction process of beta-sitosterol from legume pod was 6 min of irradiation duration using 600 W of irradiation power, 75% of solvent concentration and 75 °C irradiation temperature. The present of phytosteroid can be analyzed using the spectrometric principle, first done with the application of the Liebermann-Burchard test and then followed by the UV-Vis spectrometer analysis.

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