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Effect of PH, Salt Saturation, Solvent Ratio, Temperature and Incubation Duration on the Oil Yield in Three-Phase Partitioning Extraction Method from Fig-Leaf Gourd Seeds (Cucurbita ficifolia)

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Abstract

The purpose of this study was to investigate the effect of pH, salt saturation, solvent ratio, incubation duration and temperature in three-phase partitioning method on the yield of oil extracted from fig-leaf gourd seeds (*Cucurbita ficifolia*). The selected range of parameters for study was as followed: pH 4 - 7; 20 - 50% (w/v aqueous) of salt concentration; 0.5: 1 - 2:1 (v/v solvent/aqueous) ratio, incubation duration 30 - 75 minutes and temperature 30 - 60°C. As the results, the highest oil yield of 36.88% was obtained by processing conditions at pH 6, 40% salt concentration, 1.5:1 ratio of solvent/aqueous in room temperature (30°C) within 60 minutes of incubation. For comparison, the yield of oil extracted by Soxhlet method was 45.67%, significantly higher than the yield by three-phase partitioning method. However, the two types of oil were significantly different in several physiochemical properties. In details, total phenolic content and total flavonoid content in the oil extracted by TPP (24.53 mgGAE/100g and 4.91 mgGAE/100g, respectively) was higher than those presented in the oil obtained by Soxhlet method. Likewise, the antioxidant capacity, expressed as DDPH scavenging capacity in the oil by TPP was higher than that in the oil by Soxhlet method.

Keywords: *Cucurbita ficifolia; Oil Yield; Physiochemical Properties; Three Phase Partitioning*

Introduction

Fig-leaf gourd (*Cucurbita ficifolia*) is a climbing plant of the genus *Cucurbita* of the cucumber family *Cucurbitaceae*. It is also called H'mong pumpkin, Thai squash, Chilacayote, and Malabar gourd in different localities. Compared to four species of *Cucurbita* (*C. pepo, C. moschata, C. maxima, and C. mixta*), *C. ficifolia* has most different characteristics, such as black seeds and white flesh [1]. Different parts of *C. ficifolia* are used as food. The flesh of the ripe fruit is used to prepare sweet alcoholic drinks. The fruit is low in beta-carotene, as can be seen from its white flesh, and is relatively low in vitamins and minerals, and moderately high in carbohydrates. The most nutritional part of *Cucurbita ficifolia* is its fat- and protein-rich seeds which are eaten whole, roasted or toasted, and may be ground and used in different stews. These may also be used as vermifuge [2].

The pumpkin seeds are an excellent source of protein (25.2 - 37%) and oil (37.8 - 45.4%). It is estimated that seeds constitute 2.9% in weight of fresh fruit, while in dry basis accounted for 32% [3]. Pumpkin seeds also contain about 4.59% ash and 16.84% crude fiber [4]. The factors influencing pumpkin seed oil content and chemical composition may include the genetic factors, extraction technique, cultivation, climate, production and storage conditions [5]. A range of fatty acids was detected in pumpkin seed oils. The oil from *C. pepo* is rich in poly-unsaturated acids such as oleic and linoleic acid (34.27% and 48.02%, respectively), while stearic acid accounts for much as 16.36% [3]. The oil from fig-leaf gourd seeds is rich in oleic and linoleic acid (14.0 and 60.0%, respectively), while palmitic acid accounts for much as 14.0%, and stearic acid even less [6]. Pumpkin seed oils are rich in bioactive compounds such as carotenoids, phenolics, and tocols [7]. Pumpkin seed oils possess antimicrobial, anti-oxidant, anti-inflammatory, and anti-hypercholesterolemic ef-

fects [8]. It is suitable to be consumed for high blood pressure, breast cancer, diabetic nephropathy, sexual problems [9]. A special health benefit of pumpkin seed oil is preventing the growth and reducing the size of the prostate, improving bladder function and reducing urethral pressure [10].

Solvent extraction is accepted for plant oil production on the industrial scale using hexane as a solvent due to its convenience, solubility, non-corrosive nature and low cost. However, hexane solvent is known as a hazardous and flammable air pollutant [11]. Alternatively, green methods for obtaining oil are under research, such as aqueous extraction, optionally assisted with enzyme, microwave or ultrasounds [12,13]. A novel extraction called three phase partitioning (TPP) method has been explored recently. It was studied for extraction of proteins successfully on the laboratory scale [14]. However, it was considered to be aplicable for oil extraction [15,16]. Tert-butanol (t-butanol) is used in TPP processes preferably to other solvents such as methanol, ethanol, n-propanol, isopropanol, and 1- octanol thanks to higher efficiency [17]. Largely used as a solvent in various applications, t-butanol is also used as a flavoring agent in foods and beverages, in the manufacture of flavors, artificial musk, essences, and in the cosmetics industry. The single-dose systemic toxicity of t-butanol is low, and it has no effects specific for reproduction and also not recognized as a genotoxin [18].

Three phases formed in TPP mainly separate oil in upper organic phase, proteins in the middle precipitate phase and some hydrophilic components in bottom phase simultaneously [15,19]. The three-phase partitioning process includes incubation step and extraction step. Incubation consists of mixing oil-source material with salt solution for a period of time. Extraction includes slow addition of t-butanol, following by phase separation (centrifugation). After centrifugation, oil dissolved in t-butanol forming the upper phase, proteins precipitated with salts in the middle phase, while the bottom phase contains bio-molecules soluble in water, such as carbohydrates, minerals, soluble fibers, etc. [20,21].

Several factors affecting the efficiency of extraction include type of salt and solvent used, their quantity, pH of aqueous phase, temperature, and mixing time. Addition of a weak base like ammonium sulphate promotes separation of the t-butanol phase from aqueous phase and therefore the oil. The salt concentration was most commonly studied in the range of 20 - 50% (w/v), often resulted in 30% as optimum salt addition. The salt concentration at more than 50% may prevent slurry from forming three phases [22]. The ratio of organic solvent to aqueous phase was studied in a range from 0.25:1 to 3: 1. When the ratio is lower, the amount of t-butanol was observed to be insufficient to solubilize the oil present in source. On the other hand, high ratio implied uneconomical use of solvent and it may degenerate proteins and cause trouble in their precipitation [17]. Higher temperature helped in cell disruption, mass transfer of oil and increased solubility of protein; however, a reduction in the yield of oils extracted was observed at higher temperature above 45°C. Appropriate incubation duration is a parameter to be studied and it was considered optimum at 60 minutes in many cases. Long period in TPP extraction may lead to solvent losses due to long time exposure of organic solvents at elevated temperature [20]. The pH also affects on TPP extraction, and the optimum was observed in a slightly acidic medium, in the range of 5.7 and 6.6, as observed from various sources [17].

This study was carried out to extract oil from fig-leaf gourd seeds (*Cucurbita ficifolia*) by TPP extraction. The effect of several process parameters such as salt saturation, solvent ratio, pH, temperature and time incubation was investigated in order to determine the appropriate conditions for high oil yield achievement. Finally, the oil yield and oil quality was evaluated in comparison to that of the oil obtained from standard Soxhlet method.

Materials and Methods

Materials and chemicals

A necessary quantity of fig-leaf gourd seeds (*Cucurbita ficifolia*) was purchased from a local supplier in Tuyen Quang Province, Vietnam. The pumpkins were planted for harvesting seeds only, not for eating flesh. The seeds were treated carefully by removing broken and moldy seeds, dried in oven at low temperature (60°C) to a moisture content of about 5%. The seeds were then shelled and the kernels ground in a food blender (Phillips) into powder, sieved through a sieve size of 1.19 mm (No. 16), and stored in airtight zip-bags for further use.

Folin-Ciocalteu reagent (Merck, Germany), Gallic acid, Rutin hydrate, DPPH (Sigma Aldrich) were purchased from local agents. All other solvents and chemicals such as t-butanol, hexane, methanol, chloroform, ammonium sulphate, sodium carbonate, aluminum chloride, potassium iodide, potassium acetate-(Himedia-India), were of analytical grade and purchased from local chemical distributors in Hochiminh city.

TPP extraction procedure

The extraction procedure in TPP method was modified from the literature [15]. The amount of ground seeds (5g) was dispersed in 30 mL distilled water in a falcon tube. The suspension pH was adjusted by using a solution of 0.1N HCl and 0.1N NaOH. The tube was vortexed for 30 seconds. Next, a pre-determined quantity of ammonium sulphate was added to the slurry and stirred gently by vortex for 30 seconds. The process was followed by addition of organic solvents (t-butanol) into the slurry and mixed by vortex for 30 seconds. The slurry was placed in a temperature-controlled water bath to acquire desired temperature for a specified time period to test effect. At every 5 minutes, the slurry was gently stirred by a magnetic stirrer. After a period of incubation completed, the slurry was kept at rest for 60 minutes to facilitate a formation of three phases (upper organic phase, lower aqueous phase and interfacial precipitate layer). The slurry was finally centrifuged at 5000g for 20 minutes. The upper organic layer was collected by a plastic pipette and the solvent was evaporated in vacuum evaporator at 84°C for 5 minutes. The oil was collected for determination of oil yield and for chemical analysis (TPC, TFC and DPPH radical scavenging activity).

Experimental design

As for the effect of PH, the TPP extraction procedure described above was conducted at different pH values of the slurry, such as 4, 5, 6, 7. Other process conditions were fixed as follows: salt saturation (30%), solvent-to-water ratio (1:1), incubation time (45 minutes) and incubation temperature (40°C). The pH with highest oil yield was chosen as a parameter for the following treatments.

The salt concentrations were investigated at 20, 30, 40 and 50% (w/v). The pH value was chosen as above. Other factors were fixed as: solvent-to-water ratio (1:1), incubation time (45 minutes) and incubation temperature (40°C).

The proportion of t-butanol to aqueous phase was studied in different ratios such as 0.5:1, 1:1, 1.5:1, and 2:1. The pH and salt concentrations were selected from results of the preceding experiments. Other factors were fixed as: incubation time (45 minutes) and incubation temperature (40°C).

As for the effect of incubation duration, it was investigated at 30, 45 and 75 minutes. The pH, salt saturation, and t-butanol-to-water ratio were selected from the preceded experiments, while the incubation temperature was fixed at 40°C.

Finally, the TPP procedure for fig-leaf gourd seed slurry was tested at different incubation temperatures, such as 30, 40, 50 and 60°C. All other factors such as pH, salt saturation, solvent-to-water ratio, and incubation time were determined from previous experiments.

Physiochemical analysis

Extraction yield of oil

The yield of oil extracted was calculated by using equation:

$$\%\ oil\ yield = \frac{\text{Weight of oil extracted by TPP in grams}}{\text{Weight of materials in grams}} x100$$

The % yield of oil extracted from TPP was compared to % yield of oil extracted from Soxhlet extraction (standard method).

Total phenolic content in oil

Total phenolic content (TPC) in oil was determined by the method from the previous study [23] with some modification. An amount of oil (1 mL) was first dispersed in 1 mL hexane and extracted with 3 mL of methanol, the methanol phase was collected after centrifuge at 2500 rpm in 15 minutes. The extraction was repeated for two times more with the oil residues. The methanol phase was collected, combined and adjusted to the volume of 10 mL in a volumetric flask. 0.1 mL of methanol phase was pipetted to the test tube (capped and covered with the aluminum foil). 0.5 mL of Folin-Ciocalteu reagent, 1.5 mL of Na_2CO_3 20% and 6 mL of distilled water was added in all to

the tube. It was the incubated in dark for 2h and the absorbance measured at 765 nm using an UV-Vis spectrophotometer. A standard Gallic acid curve was constructed by preparing the dilutions of (2.0, 4.0, 6.0, 8.0 and 10.0 mg/L) in methanol from the stock solution of Gallic acid. The TPC was expressed in a unit of mg GAE/100g oil.

Total flavonoid content in oil

Total flavonoid content (TFC) in the oil samples was determined by aluminum tri-chloride spectrophotometric method reported elsewhere [24] using rutin as a standard. 0.5 mL of oil sample was mixed in 1.5 mL of 95% methanol. Then the solution was added with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and finally adjusted to 5 mL using distilled water before mixing on a vortex machine. The tubes were kept at room temperature for 30 minutes and the absorbance of the reaction mixture was read at 415 nm. A standard curve was made by dissolution of rutin in methanol 80%, the TFC in oil was determined from the standard curve and expressed as mg of Rutin Equivalent per 100 gram oil sample.

DPPH radical scavenging activity of oil

The stable free radical (DPPH) was used for determination of free radical scavenging capacity of the oil extracts according to the method of Parry., *et al* [23]. Briefly, a 0.1 mM solution of DPPH in methanol was prepared and then 1.5 mL of this solution was mixed with 1.5 mL of the methanol extract (described above) at concentrations of 100, 50, 25, 10 mg/L in methanol. After 30 minutes incubation in the dark, the decrease in the solution absorbance was measured at 517 nm by spectrophotometer. The percentage inhibition of DPPH by extracts was calculated by using following formula:

$$\% \text{ inhibition} = \frac{A-B}{A} \; x \; 100$$

A: The absorbance of pure DPPH in oxidized form; B: The absorbance of sample taken after 15 minutes of reaction with DPPH.

Statistical analysis

All the treatments were performed in triplicate and ANOVA test was used for statistical analysis of the data by using standard statistical software SPSS Ver 20.0. Significant differences between the means of parameters were determined by using Tukey's test ($p < 0.05$).

Results and Discussion

Effect of process conditions on total oil yield

Effect of pH on oil yield

Figure 1 illustrated that the effect of pH on oil yield from fig-leaf gourd seeds was significant. The oil yield increased gradually as pH increased from 4 to 5, got it's peak of 22.81% at pH 6.0, before declined as the pH increased to 7.0. It was consistent with the finding in the literature, of which the appropriate solution for TPP treatment was acidic or slightly acidic. For maximum oil yield extracted from sun-heap seeds (*Crotalaria juncea*) using TPP, the pH value for the solution was about 5.8 [22]. As for TPP extraction of oil from flaxseed (*Linum usitatissimum L.*) in assistance of enzymes, the optimal pH value of an enzymatic solution was about 5.0 [11].

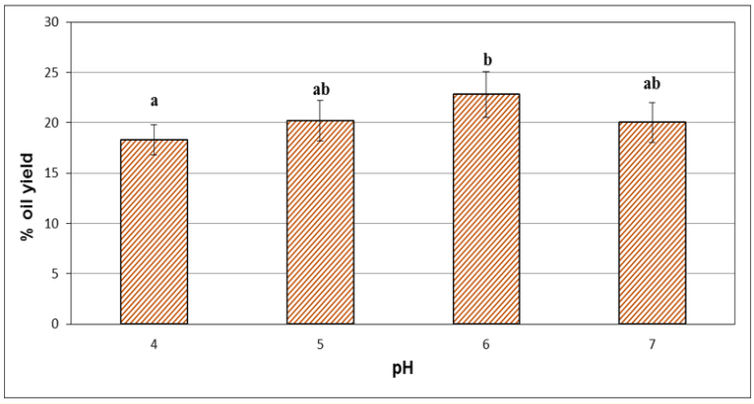

Figure 1: *The effect of pH on oil yield.*

If pH of the solution in TPP process was lower than iso-electric point (pI), proteins were positively charged, bound with cationic parts and precipitated out by TPP. Protein precipitation facilitated the release of oil. Average pI of oil bodies of various sources was observed to be in the range of 5.7 - 6.6 [25].

Effect of salt saturation on oil yield

The effect of salt concentration on oil yield was significant. As showed in figure 2, the oil yield increased as the salt concentration increased from 20% to 40%, and it decreased at higher salt concentration. The salt concentration of 40% was considered the most appropriate, which resulted in oil yield of 23.81%. Results in previous studies concluded that the best salt concentration was in a range of 30 - 50% (w/v). The optimum salt concentration depends on the sources of oil-contained materials. As for soybean and mango kernel, recorded optimum salt concentration was 30% and 50%, respectively [15]. Meanwhile, there was a 40% of salt concentration for the both apricot and rice bran in the study of Sharma and Gupta [13]. The oil is present within lipid bodies and in protein network of seeds. Saturation point of ammonium sulphate characterized for precipitation of protein from the network and thus facilitate better release of oil. Higher salt concentration present supersaturation, causing denaturation of protein and resulting in lower oil yield.

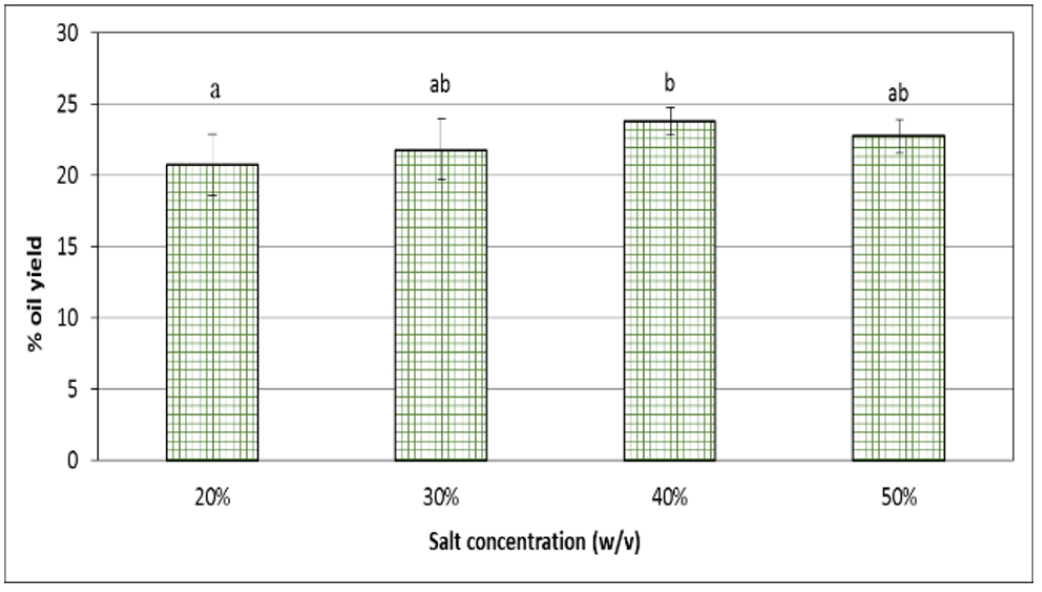

Figure 2: *The effect of salt concentration on oil yield.*

Effect of solvent to aqueous ratio on oil yield

Figure 3 indicated that the effect of solvent ratio on the oil yield was significant. An increase in proportion of t-butanol relative to the water phase from 50% to 150% brought in a sharp surge of the oil yield from 23.29 to 31.12%. As more solvent was added, the oil yield fell down to 27.29%. As the result, 1.5:1 of the solvent-to-water ratio of 1.5:1 was considered as a best choice for TPP extraction. This ratio may depend on the source of materials, it found to be of 1:1 and 2:1 for rice bran and soybean, respectively [15]. However, solvent-to-water ratio of 2:1 was found to be optimum for apricot [13]. In general, the appropriate ratio is in a range from 1:1 to 2:1. At lower proportion of t-butanol, it is insufficient to synergize with ammonium sulfate. On the other hand, at high proportion of t-butanol, it is prone to cause protein denaturation and hinder protein precipitation. Furthermore, t-butanol contributes to increase in the slurry viscosity, so overdosing t-butanol may result in decease in mass transfer rate of oil.

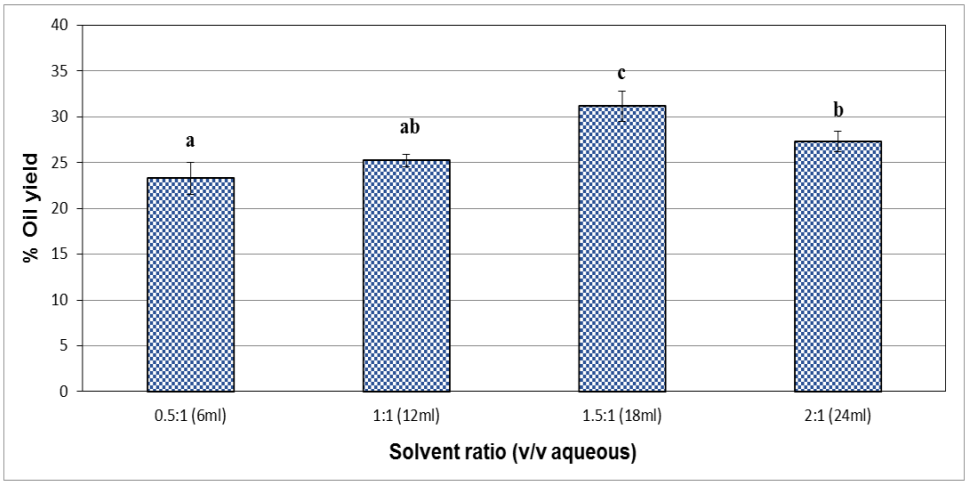

Figure 3: *The effect of solvent to aqueous ratio on oil yield.*

Effect of incubation time on oil yield

The incubation time is always a parameter to consider in a TPP process. There was a significant increase in oil yield, from 32.73 to 35.05%, when the incubation time increased from 30 to 60 minutes (Figure 4). However, the incubation time could be chosen as 45 minutes, as the yield was not significant different from 60 minutes. In fact, the range of incubation duration varied from 30 to 120 minutes, depending on material sources and the form of pre-treatment. In TPP extraction for sun heap seeds, the slurry was incubated for 60 minutes because it was suitable contact time for solid and solvent along with efficient mechanism for protein precipitation [22]. Furthermore, for both rice bran and soybean it was the same optimum of incubation time of 60 minutes [15]. Extraction for longer period may cause solvent loss and subsequent decreased in oil yield.

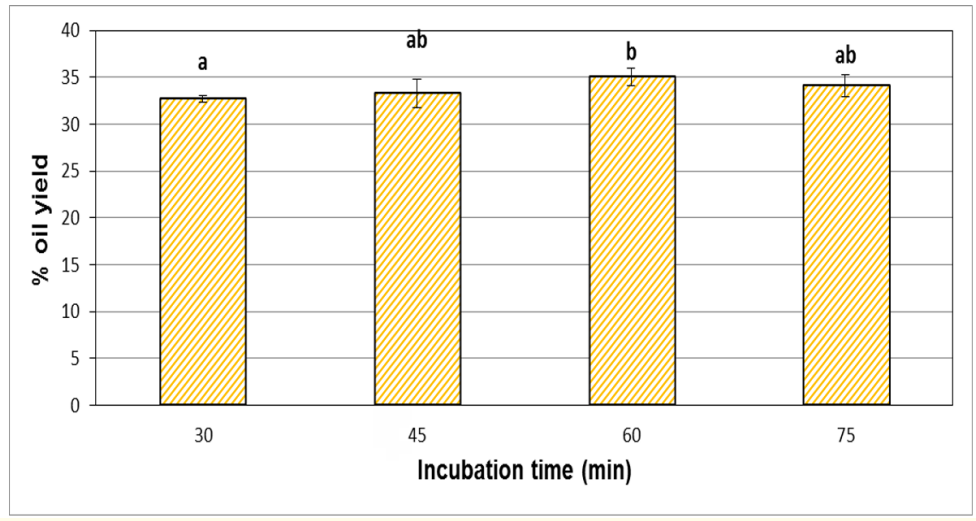

Figure 4: *The effect of incubation time on oil yield.*

Effect of incubation temperature on oil yield

The effect of incubation temperature on oil yield in TPP extraction is presented in figure 5. The results indicated that the temperature should not exceed 40°C. As the temperature increased from 40 to 50°C, the extraction yield decreased from 35.05 to 32.11%, respectively. As the temperature reached 60°C, even more dramatic decrease in the yield was observed. Likewise, Dutta., *et al*. [22] found that the oil yield from sun hemp seeds was highest at temperature of about 37°C and it also decreased when the incubation temperature exceeded above 40°C. At the temperature above 40°C, t-butanol becomes more volatile, leading to a certain degree of solvent loss. It resulted in a reduced synergistic effect with ammonium sulfate, thus producing a lower extraction yield. Temperature appeared to have an important influence on the performance of TPP processes. As a common practice, majority of TPP processes are operated at low temperatures, thus, ensuring minimal protein denaturation and saving energy as well.

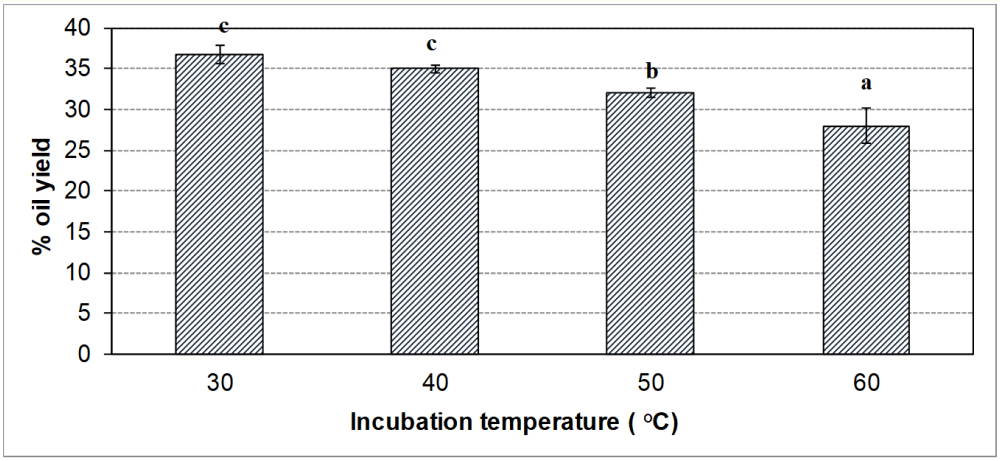

Figure 5: *The effect of incubation temperature on oil yield.*

In conclusion, the best conditions in TPP process for oil extraction from fig-leaf gourd seeds were pH 6, 40% of salt concentration, 1.5:1 solvent-to-aqueous ratio, 60 min of incubation duration and 30 - 40°C of incubation temperature. For comparison, Sharma and Gupta [13] carried out TPP extraction for rice bran with similar conditions selected, such as salt concentration (40%), incubation duration (60 min) and incubation temperature (30 - 35°C), but difference in solvent-to-aqueous ratio (1:1). In another study, Gaur., *et al*. [15] worked at larger solvent-to-aqueous ratio (2:1) for TPP extraction of oil from soybeans. Other conditions were chosen as salt concentration (30%), incubation duration (60 min) and incubation temperature (37°C). Beside, Dutta., *et al*. [22] reported that pH 5.7, salt concentration (50%), solvent-to-aqueous ratio (1:1), incubation duration (60 minutes) and incubation temperature (37°C) were as the best choice for TPP extraction of oil from sun hemp seeds.

Extraction yield and physico-chemical properties of oils by different methods

The yield of oil obtained by Soxhlet extraction using hexane as a solvent was 45.55%, significantly higher than that by TPP method, which was 36.53% (Table 1). The oil quantity by Soxhlet method is considered as the intrinsic content of oil in the dried fig-leaf seeds, so it can be seen that the TPP extraction method (without assistance enzymes, ultrasound or microwave) could bring up to 80.2% of total oil recovery. In comparison, Gaur., *et al*. [15] reported 98 and 86% recovery rate of oil from soybeans and rice bran, respectively by TPP with assistance of proteases. Likewise, 83% recovery rate of oil from *Jatropha curcas* L seed kernels was achieved in TPP without enzyme, while in enzyme-assisted TPP, the rate was 97% [26]. It suggested that there is an open opportunity to improve the oil yield in TPP processes, with assistance of additional factors or pre-treatment.

	Oil yield (%, w/w)	TPC (mg GAE/100g)	TFC (mgRE/g)	Antioxidant activity (% inhibition)
Soxhlet	45.55[a]	21.29[a]	14.28[a]	35.41[a]
TPP	36.53[b]	24.53[b]	19.19[b]	44.94[b]

Table 1: *Oil yield and physico-chemical properties of oils by two methods.*
(Note: The means in a column with the same letter are not significantly different).

The TPC in the oil by TPP extraction (24.53 mgGAE/100g) was higher than that in the oil by Soxhlet extraction (21.29 mgGAE/100g) (Table 1). The data for TPC in plant oils were reported in the literature. The TPC in oil extracted from *C. maxima* pumpkin seeds using hexane was 23.32 mg GAE/kg oil, in order lower than the result in this study. As reported previously, the TPC in *C. pepo* cold-pressed seed oil was 3.96 mgGAE/100g [5], while other authors [27] reported the TPC in *C. pepo* cold-pressed oil from roasted seed was 19.6 mgGAE/kg. Teh and Birch [28] reported the TPC data for oils from Hemp seed, flax seed and canola seed (cold-pressing) as 188.23; 136.93; and 59.17 mg GAE/100g, respectively. Difference in material sources, methods of pre-treatment and extraction using different solvents may lead to the difference in TPC data.

The TFC in oils by TPP and Soxhlet extraction was 19.19 and 14.28 mgRE/100g, respectively (Table 1). The flavonoids in pumpkin seed oils are negligible. The TFC in fig-leaf gourd seed oil were much lower than in olive oils, which was in a range of 156 - 361 mgRE/100g [29]. Dang and Nguyen [30] reported the TFC data for crude cashew nut oil from cold-pressing and soxhlet as 17.39 and 10.72 mgQE/100g, respectively (quercetin as a standard). In the research of Teh and Birch [28], it was reported that the TFC in hemp seed, flax seed and canola seed oil (cold-pressed) was 19.5, 18.75, and 16.41 mgLUE/100g, respectively (luteolin as standard).

The antioxidant capacity of oils was assessed by using DPPH scavenging test, expressed as percentage of DPPH inhibition. The capacity to inhibit stable DPPH radicals of oils extracted by TPP and Soxhlet method were 44.94 and 35.41%, respectively. Antioxidant activity of oil is attributed by a range of compounds, especially the phenolics present in oil. The antioxidant capacity was correlated to the trends of TPC and TFC in oil as presented in table 1. The content and profile of phenolic compounds in oil may depend on the process conditions and the type of solvent for extraction. As for *C. maxima* pumpkin seed, the oil extracted with chloroform/methanol (rich in phenolics) was more effective in DPPH scavenging capacity than the poor phenolic oils extracted with hexane or pentane [31]. Among the 9 types of oil, hemp and pumpkin seed oil expressed highest DPPH scavenging capacity, and the capacity in turn was closely correlated to the TPC values in oils [32]. Parry., *et al*. [23] reported that cold-pressed oil from pumpkin seeds exhibited 36% DPPH inhibition, much lower than that of parsley seed oil (87 - 91%). Comparing the DPPH scavenging capacity of walnut kernel oils obtained by cold pressing and Soxhlet extraction, the latter was more effective, since solvent can bring more compounds from seed matrix into the extract [33].

Conclusion

TPP method for oil extraction is considered as a greener technique than a standard Soxhlet method using organic solvents, however, the oil yield is often lower. It stipulates further steps for improving the oil yield, which may include application of different types of enzymes, ultrasonic power or microwave treatment. There is a range of advantages of TPP processes. The process required short running time, usually within 2h and low operation temperature (ambient temperature or at most 40°C). In general, t-butanol used in TPP system possessed good solubility for oils, but low toxicity. It is relatively safe for having higher boiling point, lower vapor pressure than hexane. Moreover, separation of t-butanol from oil by simple vacuum evaporation technique at 50°C can be completed without difficulty. Finally, the effect of TPP separation process on physicochemical properties of the oil was considerable. The oil from TTP process possessed higher TPC, TFC and better DPPH scavenging capacity than that from Soxhlet method. TPP is an economically and technically feasible process to obtain oil containing bioactive compounds from fig-leaf gourd seeds.

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