Original Research Article

The Effects of Extraction Conditions on Extraction Yield and Syringin Content in Producing standardized *Tinospora crispa* Aqueous Extract with High Antioxidant Activity

Abstract

The herbal plant known as *Tinospora crispa* is reported to have many beneficial effects on health and has great potential in future to be developed as a health product either in the form of traditional medicine, food supplements or in pharmaceutical preparations. However, so far knowledge on processing procedures to produce quality standardized extracts of this plant to be used in product development has still not widely reported. Therefore, the objective of this study was to determine the optimal extraction conditions for producing a standardized *T. crispa* aqueos extract (STCAE) with high extraction yield and high syringin content. Experiments were conducted to determine the effects of various extraction conditions involving temperature (25–100 ° C), extraction time (0.5–6 hours) and liquid (water) to solid ratio (5: 1–25: 1 ml of solvent per g stem dry). Using optimized conditions obtained, the extract was standardized based on syringin and was further investigated on its antioxidant activity through DPPH, FRAP and TBA bioassays. Results revealed that the optimum extraction conditions were found to be 1 h extraction time and 15:1 ml g^{-1} liquid-to-solid ratio. For the extraction temperature, 60 °C was found to be the best. STCAE was produced on the basis of the extract to contain with at least 0.4 wt% of syringin. STCAE was found to possess high antioxidant activities through DPPH, FRAP and TBA bioassays.

Keyword: *Tinospora crispa,* solid-liquid extraction, antioxidant activities

1.0 INTRODUCTION

Tinospora crispa (L.) Hook. f. &Thomson, is a climber that can be found in primary rainforest of South East Asia including Malaysia, Indonesia, Thailand and Vietnam. This tropical liana (woody) with shiny green leaf has several local names including “Patawali” in Malaysia [1], “Andawali” in Indonesia [2], “Makabuhay” in Philippines [3] and “Boraphet” in Thailand [4]. This plant has been traditionally used to reduce hypertension, glucose level in the blood, remedy for various ailments such as fever, asthma, intestinal worms, and skin infections [1], [5], [6], [7].

Scientific studies have shown that crude extracts and isolated compounds of *T.crispa* have various pharmacological activities. It was reported that the crude extracts and isolated compounds of *T.crispa* possessed a broad range of pharmacological activities such as cardioprotective, anti-diabetic, anti-inflammatory, antioxidant, immunomodulatory, cytotoxic and antimalarial activities [8]. Moreover, it was discovered that *T. crispa* possessed an anti-hypercholesterolemic activity and is beneficial in preventing the heart-related diseases [9], [10]. However, it is noted that those above-mentioned studies do not indicate what active ingredient which responsible for the desired effects. Most pharmacological studies were based on crude extracts of the plant and the bioactive compounds responsible for the bioactivities have not been well identified. Further investigations are required to transform the experience based claims on the use of *T .crispa* in traditional medicine practices into evidence-based information.

Nowadays, there are many traditional health products on the market that use herbal extracts as its main ingredient and it is formulated either into capsules, tablets, liquids, pills, etc. [11]. However, to ensure its quality and safety is always guaranteed the products should be standardized based on certain standard of requirements [12]. One of the important methods to standardize herbal products is through phytochemical based standardization. It helps in adjusting the herbal products to a defined content of a constituent/s which have therapeutic activity thus guarantees the quality of the product produced.

Syringin, isolated from stem parts of *T. crispa* has been reported to possess remarkable biological activities such as anti-hypertension [13], [14], [15], anti-diabetic [7] and anti-inflammatory activities [10]. Due to its ability and effects to the abovementioned therapeutic claims, it is essential to use syringin as standard or referral marker in this study for the standardization and future product quality assessment purposes.

Optimization can be referred as an improvement of performance of a system, process, or product to obtain the optimum benefit from it [16]. The yield of extract material, concentration of referral markers and antioxidant activity were strongly dependent on the best extraction condition [17]. However until to date, information regarding the best extraction conditions of *T. crispa* remains limited. Thus, this study was aimed to investigate the effect of different extraction conditions on extraction yield, syringin content and antioxidant activity of standardized *T. crispa* aqueous extract.

2.0 OBJECTIVES

To investigate the effect of extraction conditions on extraction yield, syringin content and antioxidant activity of standardised *T. crispa* aqueous extract.

3.0 MATERIAL AND METHODS

3.1 Collection of Raw Material

About 10kg of fresh stem of *Tinospora crispa* were collected from Forest Research Institute Malaysia (FRIM) at Kepong, Selangor. The plant was authenticated by FRIM botanist (Voucher number: SBID009/15). The stems were cleaned, washed, cut and dried using an oven dryer with operating temperatures of around 55°C. The weight of the sample was monitored daily until a constant weight was obtained. Subsequently, the dried stems of the plant were ground to a particle size of about 1 to 4 mm using a 20 hp pilot scale grinder. The ground sample was kept at room temperature in a closed environment before the extraction process was performed.

3.2 Optimization of Extraction Parameter of *Tinospora crispa.*

Optimization of the aqueous extraction process was carried out by evaluating the effects of different extraction parameters including ratio of solvent to solid, temperature and duration on the extraction yield and concentration of target compound of the extract. Yield was defined as total weight of extract produced per weight dried raw material used. Generally, higher yield is preferred as it means the extraction parameters used is the most cost effective. Whereas syringin was selected as the referral marker due to its ability to reduce cholesterol.

3.2.1 Effect of Solvent to Solid Ratio on Extraction Yield and Syringin Content of *Tinospora crispa* Aqueous Extract (TCAE).

About 2 g dried *T. crispa* stem was placed into three 250ml round bottle flasks and labeled as 1, 2 and 3 which each number represents different ratio of solvent to solid (ml/g) as shown in Table 1:

Table 1: Different ratio of solvent to solid of extraction process

No.	Ratio	Weight of sample & quantity of water required
1	1:5	2 g *T. crispa* + 50ml *RO water
2	1:10	2 g *T. crispa* + 100ml RO water
3	1:15	2 g *T. crispa* + 150ml RO water
4	1:20	2 g *T. crispa* + 200ml RO water
5	1:25	2 g *T. crispa* + 250ml RO water

*Reverse osmosis water

The temperature used for this extraction process was 60ºC and the duration of extraction was at 6 hours based on the findings reported by previous study [18]. The extraction was carried out using water bath (Memmert WNB 45 Germany). Hot water bath was used to provide uniform heating during extraction. The extraction process was conducted triplicate. The extracted materials were filtered using filter paper (Whatman No. 1). The filtrates were then freeze-dried to remove water. The freeze- drying process was carried out using laboratory Freeze Dryer (85XL, Millrock, USA). The yield of *Tinospora crispa* aqueous extract (TCAE) was calculated using the following equation [18]:

$$Yield\ (Wt.\ \%) = \frac{W_d}{V_e} \times R_{ss} \times 100$$

Where, W_d is weight of the dried plant (g), V_e is volume of the aqueous extract used for freeze drying (ml) and R_{SS} is solvent to solid ratio (ml/g). Meanwhile, the syringin concentration of the extract was quantified using High Performance Liquid Chromatography (HPLC) analysis. The concentrations of syringin yield were reported in *Wt. %* by using formula [19]:

$$(i)\ Syringin\ yield\ (Wt.\ \%) = \frac{Conc\ (mg/L) \times Volumn\ (L)}{Weight\ (g)} \times 100$$

3.2.2 Effect of Temperature on Extraction Yield and Syringin Content of TCAE.

Twenty grams of dried samples were placed into five 250ml round bottle flasks and labeled as 1, 2, 3, 4 and 5 where each number represents room temperature (25ºC), 40, 60, 80 and 100ºC. Two hundred milliliters of reverse osmosis (RO) water (1:10 g/ml) were added onto each flask and the extraction period was set at 6 h. The temperature was monitored using a thermometer until the targeted temperature was obtained. Extraction was performed using a water bath (Memmert WNB 45 Germany). The extraction process was conducted triplicates. The extracted material was filtered using filter paper (Whatman No. 1) and freeze-dried to remove water. The yield of TCAE syringin content was calculated following the equations in section 3.2.1.

3.2.3 Effect of Duration on Extraction Yield and Syringin Content of TCAE.

Twenty grams of dried samples were weighted into seven 250ml round bottle flasks and labeled as 1, 2, 3, 4, 5, 6 and 7 where each number indicates the extraction period at 30 minutes, 1, 2, 3, 4, 5 & 6 hours, respectively. RO water was used as a solvent for extraction with a solvent to solid ratio at 1:10 (w/v) and the temperature was set at 60ºC. Extraction was performed using water bath (Memmert WNB 45 Germany). The extraction process was conducted triplicates. The extracted material was filtered using filter paper (Whatman No. 1) and freeze-dried. The yield of TCAE syringin content was calculated following the equations in section 3.2.1.

3.3 Determination of Syringin Content from TCAE Using HPLC

After the freeze-drying process, TCAE (20 mg) was diluted with 1 ml of water and sonicated using a sonicator (Hwashin Power Sonic Model 405, Korea) for 10 minutes. Then, the samples were filtered using a syringe filter (Whatman 0.45µm PVDF) before being injected into the HPLC system. The HPLC system consists of a Waters 600 System Controller, Waters 2996 Ultraviolet (UV) detector and was equipped with a Waters 717 Autosampler. The UV Waters 2996 detector detects chemical compounds that pass through the HPLC column and sends the data to the computer for analysis. A Column oven was used to maintain the column temperature during analysis. A Symmetry Waters 5 30 µm C18 column with dimension 250 x 4.6 mm was used as the stationary phase.

The mobile phase was in gradient mode and comprised of 0.1% Orthophosphoric acid, H_3PO_4 and 100% HPLC grade acetonitrile. The mobile phase combinations were selected through optimization for better separation of compounds and shorter time (35 minutes). The injection volume was 10 µl and flow rate was adjusted to 1.0 ml/min. Maximum number of peak presence was observed at wavelength 220 nm. The presence of standard compound syringin in

TCAE was identified by comparing the retention times and UV spectra with the standard. The experiments were conducted in triplicates and the results were presented in ppm (mg/L).

3.3.1 Quantification of Syringin Content

100 µg/ml of standard syringin stock solutions was prepared by dissolving 1 mg of standard in 10 ml of methanol: water (70:30). The solution were stored in a dark glass bottles at a temperature of 4 °C. A working standard solutions, covering concentrations from 5 µg/mL to 200µg/ml were prepared. The quantity of TCAE syringin content was measured based on the calibration curve obtained and converted to wt. % (w/w) follows the equations in section 3.2.1.

3.3.2 Preparation of standardized *Tinospora Crispa* Aqueous Extract (STCAE)

The *T. crispa* aqueous extract (TCAE) was standardized based on a standard compound, syringing. A prototype of STCAE was prepared based on designated optimal extraction parameter obtained. The production process involves extraction, concentration and freeze drying. Evaporation of liquid extract was performed under vacuum below 60^{O}C. The concentrated liquid extract was freeze dried using a laboratory Freeze Dryer (85XL, Millrock, USA) stored at -20^{o}C until used.

3.4 Determination of Antioxidant Activity of STCAE

The quality of STCAE was further verified through its antioxidant activity. The 1,2- diphenyl- 2-picrylhydrazyl (DPPH) assay, Thiobarbituric acid (TBA) Test and Ferric Reducing Antioxidant Power (FRAP) assay were performed, in which Vitamin C and butylated hydroxytoluene (BHT) were acted as standard.

3.4.1 DPPH Radical avenging

The antioxidant activity of STCAE was measured based on its scavenging activity against a stable free radical 1,1- diphenyl-2-picrylhydrazyl (DPPH), as previously described [20]. The activity was compared against the standard antioxidants, namely, BHT and vitamin C. Briefly, for the control, 1 ml of 0.45 mM DPPH was added to 0.5 ml of absolute ethanol. For the sample solution, 1 ml of 0.45 mM DPPH was added to 0.5 ml of extract (5 mg/ml). The step was repeated by replacing the extract with BHT (5 mg/ml) and vitamin C (5 mg/ml). Each sample, was incubated for 30 min and after incubation, absorbance was recorded at 517 nm. The percentage of inhibition representing the sampling activity against DPPH was calculated based on the following equation:

$$\text{(ii) Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance}}{\text{Absorbance of control}} \times 100\% \text{ of test sample}$$

3.4.2 Thiobarbituric Acid Test (TBA)

The TBA values of STCAE wwere determined using the method from previous researcher [21]. One ml of sample from Ferric Thiocyanate method (FTC) was added to two ml of Thrichloroacetic acid (TCA) and two ml of Thiobarbituric acid (TBA). The mixture was then placed in a boiling water bath at 100°C for 10 minutes. After cooling, it was then centrifuged at 3000 rpm for 20 minutes. The absorbance of the supernatant was measured spectrophotometrically at 532 nm and was quantified using the following formulation:

$$\text{(iii) Percentage of inhibition (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

3.4.3 Ferric Reducing Antioxidant Power

The ferric reducing ability of STCAE was evaluated following the method described by previous report [22]. The reagent was freshly prepared by mixing 10 mM 2, 4, 6-tripyridyl triazine (TPTZ) and 20 mM ferric chloride in 0.25M acetate buffer (pH 3.6). Then, 100 µl of extract was added to 300 µl of distilled water, followed by 3 ml of FRAP reagent. The absorbance was recorded at 593 nm spectrophotometrically after 4 min of incubation at room temperature. The reducing ability of the extracts was compared with BHT. The results were expressed as the concentration of antioxidants having ferric reducing ability equivalent to that of 1 mM $FeSO_4$, expressed in milimolar per litre.

3.5 Statistical Analysis

All data were analyzed using the computer software Statistical Package for Social Sciences (SPSS) version 20.0 and were expressed as mean $\pm$ standard deviation. Comparisons of group means was done by one-way analysis of variance (ANOVA) with a probability less than .05 ($p < 0.05$) taken as indicative of significant difference. The mean value (x) and standard deviation (SD) were calculated for each variable measured. Turkey's pos hoc test was used for multiple group comparison. $P < 0.05$ is considered significant.

4.0 RESULTS

4.1 Optimization of Extraction Parameter of *Tinospora crispa*

4.1.1 Effect of Solid to Solvent Ratio on Extraction Yield and Concentration of Syringin

Figure 1 shows the effect of solid to solvent ratio on the extraction yield of TCAE. The results showed that the extraction yield increased from 9.69% to 16.22% as the solid to solvent ratio increased from 1:10 to 1:25 (g/ml). The highest yield was showed in a ratio of 1:25 (16.25 ± 0.38%), followed by a ratio of 1:20 (16.10 ± 0.23%) and a ratio of 1:15 (15.86 ± 0.55%), respectively. However, no significant changes were observed in the yield of TCAE between them. Meanwhile, the solid to solvent ratio at 1:10 was found to be significantly lower ($p<0.05$) compared to the other groups (11.03 ± 0.40%).

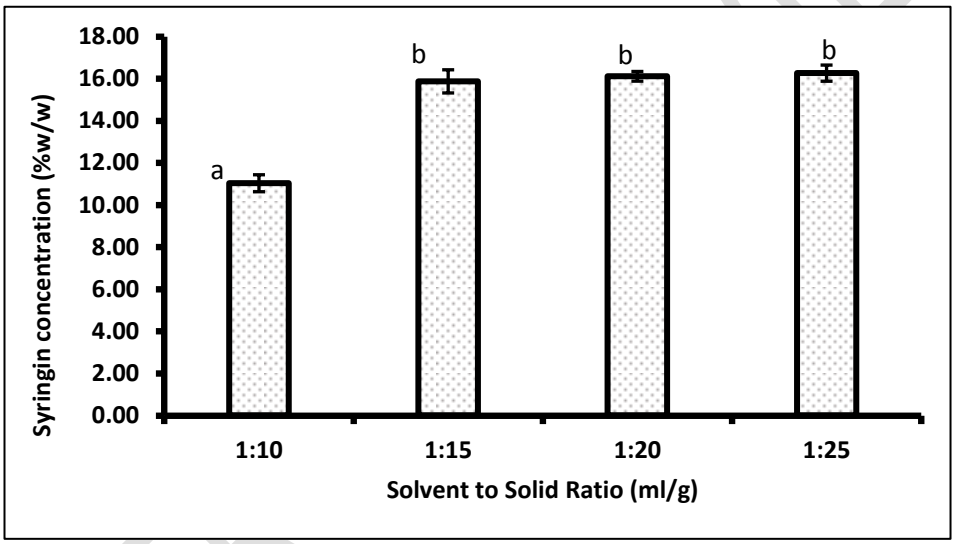

Figure 1. Effect of Different Solvent to Solid Ratio (ml/g) on Extraction Yield of TCAE. Notes: Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.** No significant change observed on the yield of the solvent to solid ratio at 1:15 mg/ml and above.

Figure 2 shows the effect of solid to solvent ratio on the concentration of syringin. The results showed that the syringin concentration increased from 0.44 ± 0.01% to 0.49 ± 0.04 % as the solid to solvent ratio increased from 1:10 to 1:25 (g/ml). The highest syringin concentration was shown in the ratio 1:25 (0.49 ± 0.04%), followed by the ratio 1:20 (0.47 ± 0.02%), the ratio 1:15 (0.46 ± 0.01%) and a ratio of 1:10 (0.44 ± 0.01%), respectively. However, there was no significant change observed on syringin concentrations between all ratios tested. The results indicated that the most suitable and most cost effective solvent to solid ratio to obtain optimal extraction yield and high syringin concentration was at a ratio of 1:15 (g/ml).

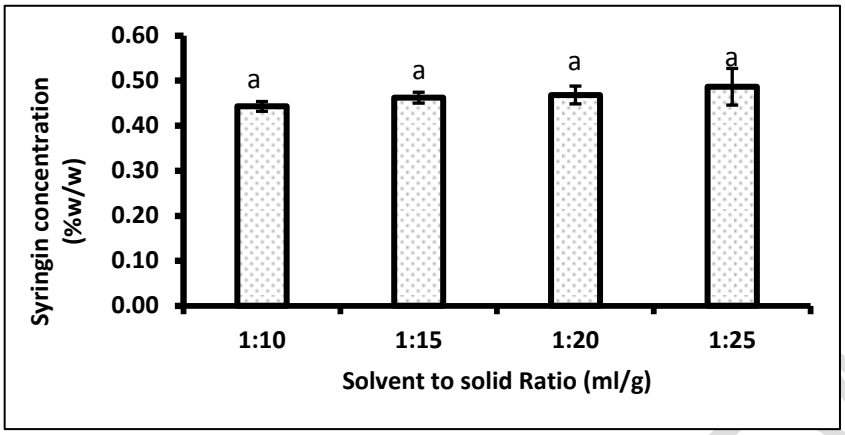

Figure 2. Effect of Different Solvent to Solid Ratio (ml/g) on Syringin Concentration of TCAE. Notes: Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$). Results indicate the most suitable solvent to solid ratio and the most cost effective to obtain optimal extraction yield and syringin concentration of TCAE was at ratio 1:15 (g/ml).

4.1.2 Effects of Temperature on Extraction Yield and Concentration of Syringin

Figure 3 shows the effect of different temperature on the yield of TCAE. The result showed that the extraction yield of TCAE was found to increase with the increase on extraction temperature. Result indicates the most suitable temperature for extraction of *T. crispa* was at 60ºC since there is no significant change on the yield observed at temperature 60ºC (15.95±0.21%), 80ºC (15.87±0.29%) and 100ºC (16.02 ± 0.07%) respectively. The extraction yield of TCAE was significantly lower ($p<0.05$) at 40ºC (14.18 ± 0.32%) followed by 25 ºC (7.98 ± 0.63%) when compared to the other groups.

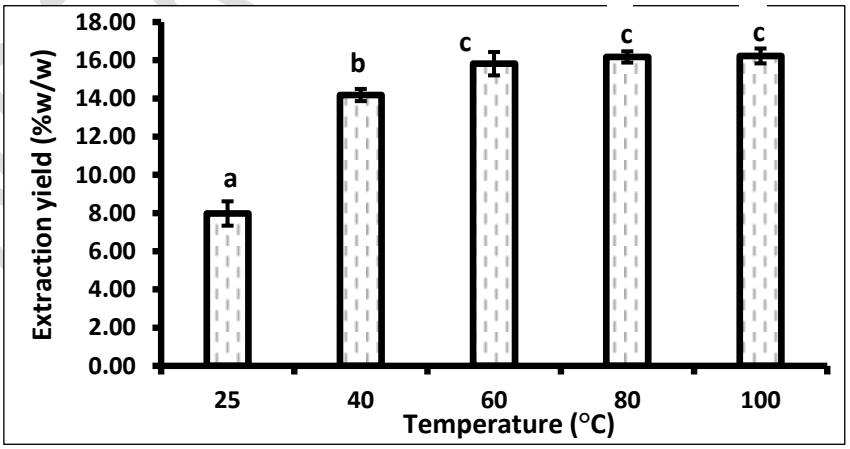

Figure 3. Effect of Varying Temperature (ºC) on Extraction Yield of TCAE. Notes: Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.** Result indicates the most suitable temperature for extraction of *T. crispa* was at 60 ºC since there is no significant change on the yield of TCAE observed at higher temperature.

Figure 4 shows the effect of different temperature on the syringing concentration. The result showed that the syringin concentration was found to increase from a temperature of 25 to 60ºC and began to drop dramatically at temperature 80 and 100ºC. The results indicated that the most suitable temperature for extraction of *T. crispa* was at 60ºC since it has produced significantly ($p<0.05$) the highest concentration of syringin compared to the other groups. Syringin concentrations at 25, 40, and 60ºC were increased by 0.37, 0.38 and 0.45% dry wt and decrease to 0.18 and 0.07% dry wt, at temperature 80 and 100ºC respectively. The results indicate the most suitable temperature and the most cost effective to obtain optimal extraction yield and syringin concentration of TCAE was at 60 ºC.

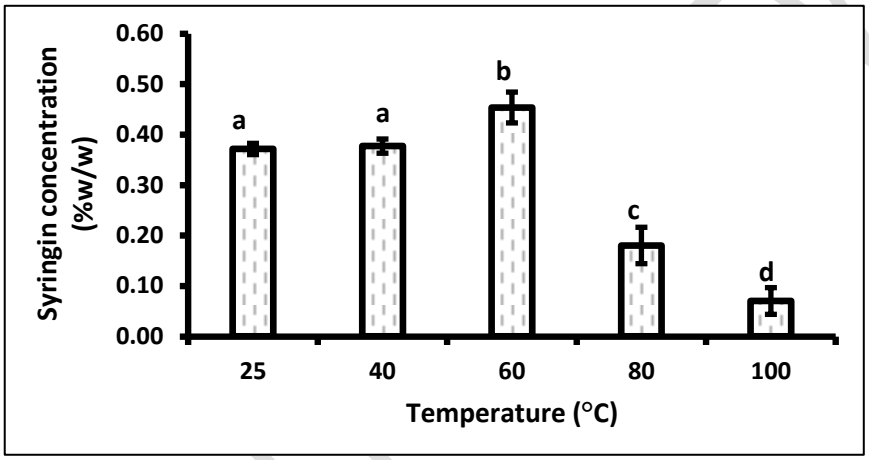

Figure 4. Effect of Varying Temperature (ºc) on the Concentration of Syringin. Notes: Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.** Temperature at 60 ºC produced significantly ($p<0.05$) the highest concentration of syringin compared to the other groups.

4.1.3 Effect of Extraction Time on Extraction Yield and Concentration of Syringin

Figure 5 shows the effect of different extraction time on TCAE yield. The results found that there was no significant change in the TCAE yield after the first hour of extraction. This indicates that the extraction process achieves equilibrium in about 1 hour. The yields of TCAE obtained were found to be 11.10±0.08% (30 minutes), 16.49±0.10% (1 hour), 16.33±0.0.29% (2 hours), 16.48±0.27% (3 hours), 17.02±0.14% (4 hours), 16.52±0.20% (5 hours) and 16.51±0.30% (6 hours), respectively.

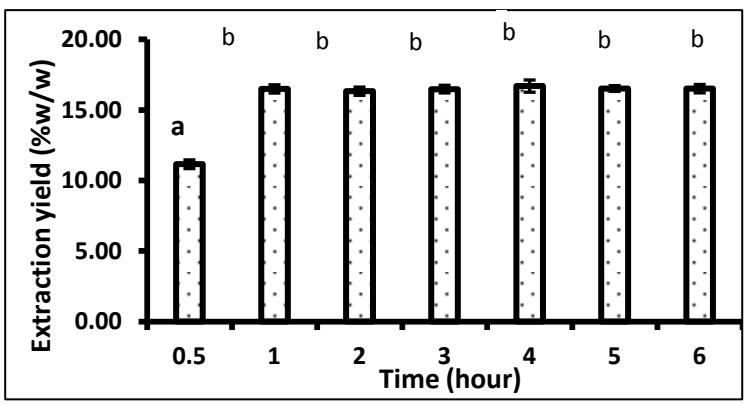

Figure 5. Effect of Different Extraction Time on Extraction Yield of TCAE. Notes: Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.** The result showed no significant change in the extraction yield of TCAE after the first hour of extraction process.

Figure 6 shows the effect of different temperature on syringin concentration. The results showed that the syringin concentration increased from 30 minutes to 1 hour and began to decrease after 1 hour of the extraction process. The degradation could be occurred as a result of heat exposure. The results indicate the most suitable extraction time to obtain the optimal extraction yield and high syringin concentration was at 1 hour.

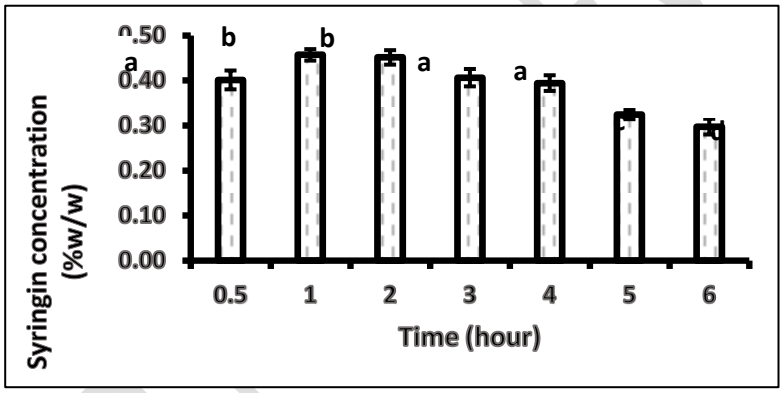

Figure 6. Effect of Varying Extraction Time on the Concentration of Syringin. Notes: Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.** Concentration of syringin increased from 30 minutes to 1 hour of the extraction process and begins to decline after 1 hour.

4.2 HPLC profile of TCAE Containing Syringin

Figure 7 shows the HPLC chromatogram of the syringin referral standard while Figure 8 represents the HPLC chromatogram of TCAE containing syringin at 25°C. The reversed-phase HPLC chromatogram of aqueous extract of TCAE exhibited peaks of syringin corresponding to retention times at 16.22 ± 0.057 minutes. Syringin was determined by comparing the HPLC chromatograms of the extracts as well as by spiking the extracts with the syringin standard. The same approach was performed to determine the contents of syringin at subsequent temperatures.

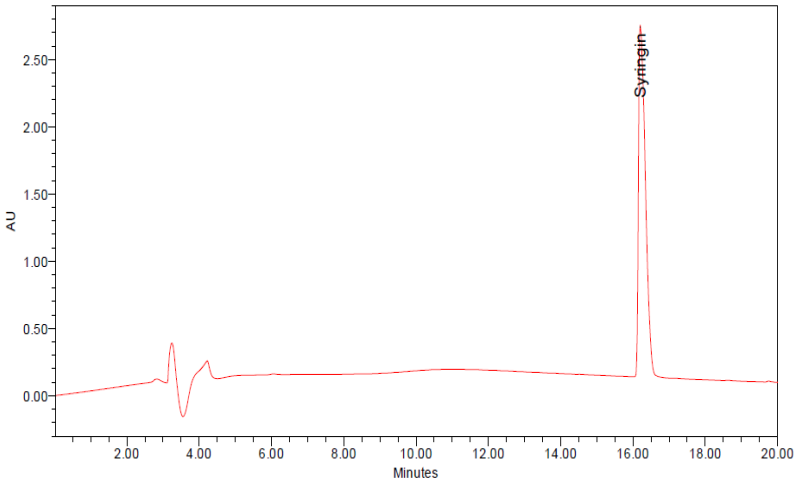

Figure 7: HPLC chromatogram of syringin standard compound.

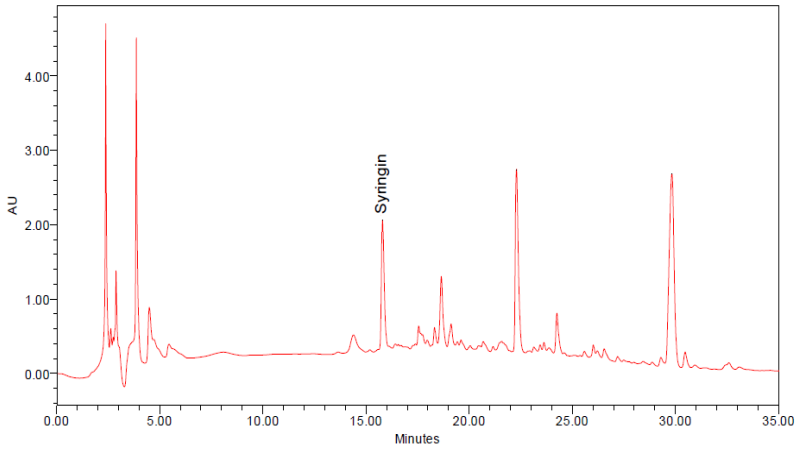

Figure 8: HPLC chromatogram of TCAE containing syringing at 25°C

4.2.1 Standardization of TCAE

The TCAE was standardized based on the referral compound syringin. The quantitative determination of syringin by HPLC indicated that syringin corresponding to retention times at 16.22 ± 0.057 minutes. The calibration curve plotted for the standard solution of syringin over the concentration range of 20-1000 µg/mL showed a correlation coefficient (*r*2) of 0.98. From the calculation, it was found that STCAE is defined to contain at least 0.4 wt% of syringin of total extract.

4.3 Antioxidant Activity of STCAE in *vitro*

Using the optimal extraction parameters obtained, *T. crispa* standardized extract was prepared and evaluated on its antioxidant activity through DPPH, TBA and FRAP bioassays and the results were shown in Figure 9, 10 and 11.

4.3.1 DPPH Radical Scavenging

The DPPH assay was utilized to evaluate the ability of antioxidants to scavenge free radicals. The scavenging activities of STCAE, vitamin C and Butylated hydroxytoluene (BHT) against DPPH radicals were compared and shown in Figure 9. Results showed that STCAE exhibited high scavenging activity with the percentage of inhibition of 82.31±0.37%. However, result showed that the inhibition power of STCAE is significantly lower ($p<0.05$) when compared to vitamin C and BHT. Vitamin C exhibited the highest inhibition activity with 97.33±0.50% followed by BHT 95.98±0.41% respectively.

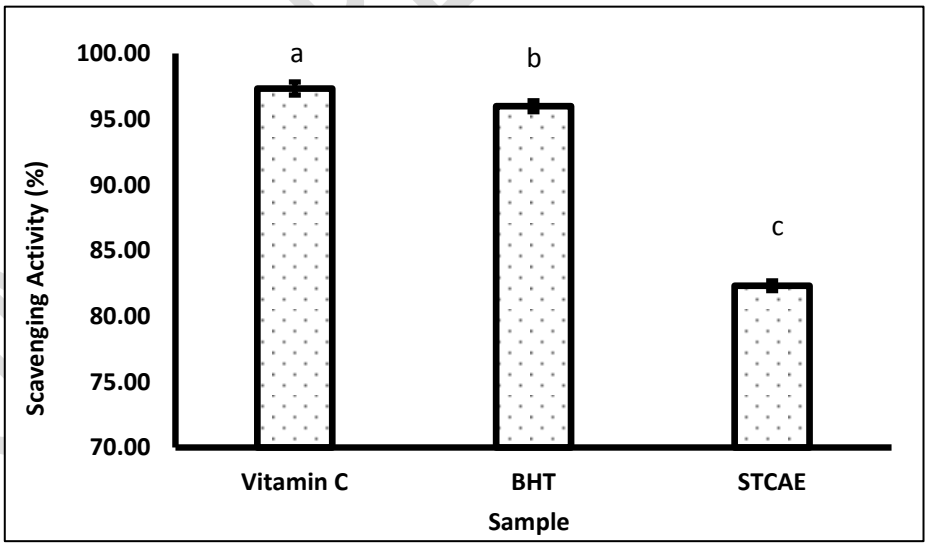

***Figure 9*:** DPPH free radical scavenging of standardized *Tinospora crispa* aqueous extract compared to vitamin C and butylated hdroxytoluene (BHT). Data expressed as mean ± SD. Bars with different alphabets are significantly different ($p>0.05$).

4.3.2 Thiobarbituric Acid Test (TBA)

The percentage of inhibition of STCAE evaluated in TBA test is shown in

Figure 10. The result showed that, STCAE exhibited moderate lipid peroxidation inhibition activity with MDA value 50.46±0.25%. Result indicates that the percentage of inhibition of STCAE was significantly lower ($p<0.05$) when compared to vitamin C and Butylated hydroxytoluene (BHT). BHT exhibited the strongest antioxidant activity with MDA value 77.41 ± 0.34% followed by vitamin C with MDA value 72.74 ± 0.25% respectively.

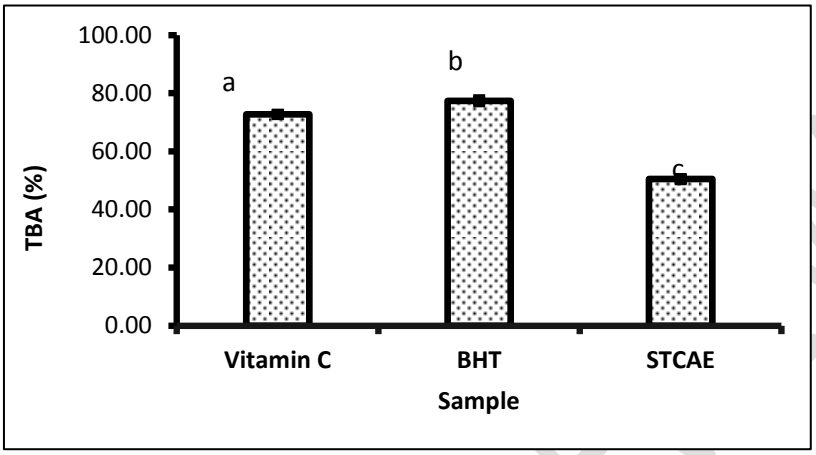

Figure 10: Thiobarbituric Acid Test (TBA) of standardized *Tinospora crispa* aqueous extract compared to vitamin C and butylated hdroxytoluene (BHT). Data expressed as mean ± SD. Bars with different alphabets are significantly different ($p>0.05$).

4.3.3 Ferric Reducing Antioxidant Power

The reducing ability of STCAE against the ferric ion which act as oxidant agent is shown in Figure 11. The results showed that, STCAE exhibited high antioxidant activity with FRAP value of 0.89+0.07 mmol/L. However, the FRAP value of STCAE is not comparable with both Butylated hydroxytoluene (BHT) and vitamin C which again exhibited the strongest antioxidant activity with the value of 1.28±0.02mmol/L and 1.03± 0.01mmol/L respectively.

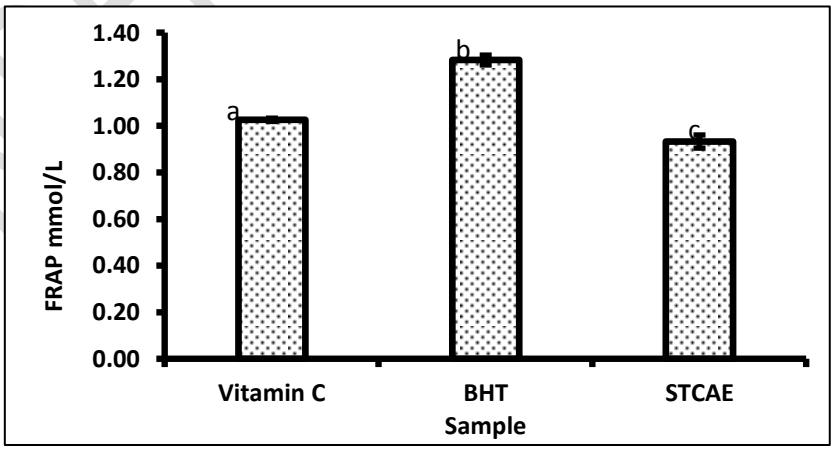

Figure 11**:** Feric reducing antioxidant power (FRAP) assay of standardized *Tinospora crispa* aqueous extract compared to vitamin C and butylated hdroxytoluene (BHT). Data expressed as mean ± SD. Bars with different alphabets are significantly different ($p>0.05$).

5.0 DISCUSSION

Optimization of extraction process is essentially required to obtain the optimum concentration of phytochemical constituents and to maintain their activities [23]. Optimization is also referred as an improvement of performance of a system, process or product to obtain the optimum benefit from it i.e., high yield of extraction [20]. Higher extraction yield is preferred as the extraction parameters applied are the most cost effective and the most preferable by the industry players. The aim of an extraction process should be to provide for the maximum yield of substances and of the highest quality which consist of high concentration of target compounds and therapeutic effect of the extracts [24].

There are two most commonly methods used in optimization studies, namely the classical single factor experiments and the response-surface methodology (RSM). The classical single factor experiments are a one-factor-at-a-time approach, in which only one factor is varying at a time while all others are kept constant. Present study used the single-factor experiments, despite being having some drawbacks, such as time-consuming, expensive, possible interaction effects between variables cannot be evaluated and misleading conclusions may be drawn [25], [21]. However, single factor experiments can provide fundamental information on the ranges for significant extraction parameters on the extraction of targeted compounds from plant materials.

The quantity of solvent during the extraction process is one of the important factors to obtain high extraction yield [26]. Result from present study revealed that, the extraction yield of TCAE was found to increase with the increase in ratio of solvent to solid. These results were consistent with the mass transfer principle where the driving force for mass transfer is according to the concentration gradient between solid and solvent. A high solid-to-solvent ratio could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows for greater solid extraction by the solvent [27], [28].

The results also showed a marked increase in syringin yield when the ratio of solvent to solid increased proportionally from 10 to 25 ml/g. However, there was no obvious change in syringin yield, as the ratio continued to increase. Therefore, to avoid wastage of solvent use, 15 ml/g was selected as the optimal ratio of solvent to solid. Moreover, the 15 ml/g ratio also showed the highest syringin concentration compared to other ratios studied.

Temperature plays an important role in the extraction of bioactive compounds from plant material. The Results of this study have shown that, the most suitable temperature for obtaining high extraction yield and high concentration of syringin were at 60ºC. The similar trend was observed from previous finding who reported that optimum condition to obtain higher yield of syringin from the bark of *Ilex rotunda* was 50 °C in which the yield gradually decreased when extraction temperatures increased up to 80 °C [29].

Higher temperature increased the solubility and diffusion coefficient of the solute, allowing higher yield and extraction rate [30]. However, elevating the temperatures up to a certain level might affect decomposition of antioxidants which were already mobilized at lower temperatures [31]. Other than that,

denaturation of membranes as well as degradation of the polyphenolic compounds may influence quantification of the bioactive compounds [32]. Moreover, extraction costs are expected to increase with increasing of the extraction temperature.

Meanwhile, the result also suggested that, the most suitable extraction time and the most cost effective for obtaining high yield of TCAE was at 1 hour. Result obtained was dissimilar to previous studies who mentioned that the optimum extraction time was at 6 hours [24]. These phenomena could be explained by the Fick's second law of diffusion, predicting that a final equilibrium between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) might be reached after a certain time, leading to deceleration in the extraction yield [33]. Moreover, prolonged extraction time increases the chance of decomposition and oxidation of phenolics due to their long exposure to unfavorable environmental factors like temperature, light and oxygen [34]. On the other hand, the increased extraction time is uneconomical and time consuming from the industrialization point of view.

Beside extraction conditions, there are several factors that might affect the differences in percentage of secondary metabolite present in herbal plants. First, different geographical locations of the plant species as sampling locations of the plants were varied from island population, coastal population, and hill forest population. [35], [3 6] .

The standardized *T. crispa* aqueos extract obtained by following the optimized extraction procedures was further evaluated for its antioxidant properties. The free radical scavenging ability of STCAE was assessed by the discoloration of DPPH solution and was compared to vitamin C and BHT. Results showed that STCAE able to scavenge DPPH radical with the percentage of inhibition of 87.06±0.23%. which in accordance with the previous reports who mentioned that crude aqueous extract of *T. crispa* extracted at 60°C for 6 hours exhibited 85.95±0.52% of scavenging activity against DPPH radical [24].

The result also indicates that, STCAE exhibits significantly lower ($p<0.05$) antioxidant activity (50.46±0.25%) when compared to controls. Interestingly, antioxidant activity showed in this study was higher when compared to previous study [18] who found that crude water *T. crispa* extract obtained from 60°C and 6 hours of extraction parameters exhibited 39.20±2.97% antioxidant activity. It was reported that, prolonged extraction time increases the chance of decomposition and oxidation of phenolics due to their long exposure to unfavorable environmental factors like temperature, light and oxygen [37].

In the FRAP assay, the difference of FRAP value between STCAE with vitamin C and BHT were about 0.09 mmol/L and 0.35 mmol/L respectively. The result clearly showed that STCAE has high antioxidant activity due to the small difference of FRAP value between STCAE and controls. Antioxidant compounds in some herbs are likely to be heat labile [38]. The processes of steaming, flaking, and boiling of plants have been reported to decrease their biological compounds [39]. Short extraction period and low extraction temperature conducted in present study might preserved the constituents of biological compounds thus contribute to a high antioxidant reading.

High antioxidant activity of STCAE is most probably due to the presence of syringin. It was reported that, syringin isolated from *F. rhynchophylla,* exhibited a strong radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and a potent inhibitory effect against nitric oxide (NO) synthesis, respectively. Syringin also demonstrated the peroxyl radical scavenging capacity comparable to that of glutathione [18].

6.0 CONCLUSION

Based on the findings of this study, it can be concluded that to obtain high extraction yields and high syringin concentrations of *T. crispa*, 60°C was selected as the appropriate temperature. The best extraction time was at 1 hour while the optimum solvent to solid ratio was 1:15 g/ml. STCAE contains at least 0.4 wt% of syringin. STCAE was found to possess high antioxidant activity through DPPH, FRAP and TBA bioassays. The results indicate that *T. crispa* can be used as an easily accessible source of natural antioxidants and can be used further as a possible health supplement in the pharmaceutical industry.

7.0 REFERENCES

[1] Najib, N. A. R., Furuta, T., Kojima, S., Takane, K., Ali, M. M. (1999). Antimalarial activity of extracts of Malaysian medicinal plants. *J. Ethnopharmacol. 64,* 249-254.

[2] Koay, Y. C., & Amir, F. (2013). A review of the secondary metabolites and biological activities of *Tinospora crispa (*Menispermaceae). *Trop. J. Pharm. Res.* 12, 641–649. doi:10.4314/tjpr.v12i4.30

[3] Quisumbing, E. (1951). Medicinal Plants of the Philippines. Quezon: Katha Publishing Co Inc. Rackova, L., Majekova, M., Kostalova, D. & Stefek, M. (2004). Antiradical and antioxidant activities of alkaloids isolated from *Mahonia aquifolium*. Structural aspects. *Bioorganic & Medicinal Chemistry 12(17):* 4709-4715.

[4] Li, S., Long, C., Liu, F., Lee, S., Guo, Q., Li, R. (2006). Herbs for medicinal baths among the traditional Yaocommunities of China. *J. Ethnopharmacol. 108,* 59–67. 10.1016/j.jep.2006.04.014.

[5] Zakaria, Z.A., Mat Jais, A.M., Somchit, M.N., Sulaiman, M.R., Faizal, F.O. (2006). The *in vitro* antibacterial activity of *Tinospora crispa* extracts. *J. Biol. Sci., 6 (2),* 398-401.

[6] Kongkathip, N., Dhumma-upakorn, P., Kongkathip, B., Chawananoraset, K., Sangchomkaeo, P., Hatthakitpanichakul, S. (2002). Study on cardiac contractility of cycloeucalenol and cyloeucalenone isolated from *Tinospora crispa. J. Ethnopharmacol., 83*, 95-99.

[7] Noor, H., Ashcroft, S.J.H. (1989). Antidiabetic effects of Tinospora *crispa* in rats. *J. Ethnopharmacol. 27,* 149-161.

[8] Ahmad, W., Jantan, I., & Bukhari, S.N.A. (2016). *Tinospora crispa* (L.) Hook.f. & Thomson: A review of its ethnobotanical, phytochemical, and pharmacological aspects**.** *Frontiers in pharmacology*, 7(59), 1-19.

[9] Zulkhairi, A., Hasnah, B., Sakinah, I., Nur Amalina, I., Zamree, M.S., Mohd Shahidan, A. (2009). Nutritional composition, antioxidant ability and flavonoid

content of *Tinospora crispa* stem. *Adv. Nat. Appl. Sci.* 3, 88–94.
[10] Kamarazaman, I.S., Amom, Z., & Ali, R.M. (2012a). Inhibitory properties of *Tinospora crispa* extracts on TNF-α induced inflammation on human umbilical vein endothelial cells (HUVECS). *Int. J. Trop. Med. 7*, 24–29. doi:10.3923/ijtmed.2012.24.
[11] Harwoko, & Choironi, N. A. (2016). Quality standardization
[12] Hariyati, S. (2005). Standardization extracts of Indonesian medicinal plant, one of the important stages in the development of Indonesia traditional medicine. *Info POM. 6 (4),* 1-5.
[13] Rao, U. S. M., Zin, T. and Sundaram, C. S. (2016). The effect of syringin on the expression of TNF-α, iNOS, ICAM-1 and its' mRNA in the heart, brain and kidneys of spontaneously hypertensive rats, *Der Pharmacia Lettre,* 8 (3):53-61
[14] Kim, N.Y., Pae, H.O., Ko, Y.S, Yoo, J.C., Choi, B.M., & Jun, C.D. (1999). *In vitro* inducible nitric oxide synthesis inhibitory active constituents from *Fraxinus rhynchophylla*. *Planta Med., 65*, 656-658.
[15] Kim, H.C., An, R.B., Jeong, G.S., Oh, S.H., Kim, Y.C. (2005). 1,1-Diphenyl-2- picrylhydrazyl radical scavenging compounds of Fraxini Cortex. *Nat. Prod. Sci., 11,* 150-154.
[16] Araujo, A. L., Mota Soares, C. M., & Moreira De Freitas, M. J. (1996). Characterization of material parameters of composite plate specimens using optimization and experimental vibrational data. *Composite part B: Engineering, 27*, 185 – 191
[17] Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76,* 965 – 977.
[18] Zulkhairi, A., Abdah, M. A., Kamal, N. H. M, Nursakinah, I, Moklas, M. A. M, Hasnah, B., et al. (2008). Biological Properties of *Tinospora crispa* (Akar Patawali) andits antiproliferative activities on selected human cancer cell lines. *Mal. J. Nutr., 14,* 173-187.
[19] Pin, K. Y., Chuah, T. G., Abdul Rashih, A., Law, C. L., Rasadah, M. A & Choong, T. S. Y. (2009). Drying of Betel Leaves (*Piper betle* L.): Quality and drying kinetics, drying technology, *27:1,* 149-155, DOI: 10.1080/07373930802566077.
[20] Yen, G.C., and and Hsieh, C.L. (1998). Antioxidant Activity of Extracts from Du-zhong (Eucommia ulmoides) toward Various Lipid Peroxidation Models in Vitro, *Journal of Agricultural and Food Chemistry*, *46* (10), 3952-3957, DOI: 10.1021/jf9800458
[21] Otto, C., Geiss, H. C., Empen, K., Parhofer, K. G. (2004). Long-term reduction of C-reactive protein concentration by regular LDL apheresis. *Atherosclerosis*, *174 (*1), 151-156.
[22] Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239,* 70-76.
[23] Aziz, R. A., Sarmidi, M. R., Kumaresan, S., (2003). Phytocehemical processing: the next emerging field in chemical engineering aspects and opportunities. *J. Kejurut. Kim. Malay. 3,* 45–60.
[24] Spigno, G., De, & F. D. M., (2009). Microwave -assisted extraction of tea phenols: a phenomenological study. *J. Food Eng. 93,* 210–217.
[25] Bas, D., & Boyaci, I. H. (2007). Modeling and optimization 1: Usability of response surface methodology. *Journal of Food Engeenering, 78:* 836-845.
[26] Virot, M., Tomao, V., Le Bourvellec, C., Renard, C. M., Chemat, F., Ultrason

Sonochem., (2010). 17, 1066-107.
[27] Al-Farsi, M.A. & Chang Y. L. (2007). Optimization of phenolics and dietary fibre extraction from date seeds. *Food Chemistry, 108(3):* 977-985.
[28] Tan, P. W., Tan, C. P. and Ho, C. W. (2011). Antioxidant properties: Effect of solid-to-solvent ratio on antioxidant compounds and capacities of Pegaga *(Centella asiatica). International Food Research Journal 18:* 553-558.
[29] Rao, U. S. M., Zin, T., Abdurrazak, M., Ahmad, B. A. (2015). Chemistry and pharmacology of syringin, a novel bioglycoside: A review. *Asian J Pharm Clin Res.*, *8, (3),* 20-25.
[30] Zhao, L. C., He, Y., Deng, X., Xia, X. H., Liang, J., Yang, G. L., et al. (2012). Ultrasound- Assisted Extraction of Syringin from the Bark of *Ilex rotunda* Thumb Using Response Surface Methodology. I*nt. J. Mol. Sci.*, *13*(6), 7607-7616; doi:10.3390/ijms13067607
[31] Silva, E. M., Souza, J. N. S., Rogez, H., Rees, J. F. and Larondelle, Y. (2007). Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region. *Food Chemistry, 101:* 1012-1018.
[32] Liyana-Pathirana, C. M. & Shahidi, F. (2005). Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chemistry 93:* 47–56.
[33] Abad-Garcia, B., Berrueta, L. A., Marquez, D. M. L., Ferrer, I. C., Gallo, B., & Vicente, F. (2007). Optimization and validation of methodology based on solvent extraction and liquid chromatography for the simultaneous determination of several polyphenolic families in fruit juices. *Journal of Chromatography A, 1154:* 87-96.
[34] Naczk, M. & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A, 1054*: 95-111.
[35] Ullah, N., Khurram, M., Amin, M. U., Khan, T. A., Khayyam, S. U., Khan, F. A., et al. (2012). Impact of geographical locations on Mentha spicataantibacterial activities. *Journal of Medicinal Plants Research Vol. 6(7*), 1201-1206,
[36] Dong, J., Ma, X., Wei, Q., Peng, S. & Zhang, S. (2011). Effects of growing location on the contents of secondary metabolites in the leaves of four *selected superior clones of Eucommia ulmoides. Industrial* Crops and Products, 34: 1607-1614.
[37] David, G. S., George, E. I., Diejun, C., Atanu, B., Fred, J. E., Roque, L. E. (2007). Phenolic content and antioxidant capacity of supercritical carbon dioxide-treated and air- classified oat bran concentrate microwave-irradiated in water or ethanol at varying temperature. *J Food Chem, 108:* 23-30.
[38] Bryngelsson, S., Mannerstedt-Fogelfors, B., Kamal-Eldin, A., Anderson, R., Dimbergs, L. H. (2002). Lipids and antioxidant in groats and hulls of Swedish oats (*Avena sativa* L). *J Sci Food Agric, 82:* 606-614.
[39] Rasheed, A., Roja, C. (2012). Formulation, standardization and pharmacological evaluation of a poly herbal traditional remedy-Ashwagandharishtam. *Oriental Pharmacy and Experimental medicine*, 51-58.