Original Research Article

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

Abstract

Tinospora crispa or known as Patawali was proven to have many beneficial effects and has great potential to be used in traditional medicinal, food supplement and pharmaceutical preparations. Nevertheless, so far the knowledge on its extraction procedures towards producing standardized extract of this plant is not well established. Hence, the objective of this study was to optimise the extraction conditions in achieving high extraction yield and syringin content and to produce standardised *T. cripsa* aqueous extract (STCAE) with high antioxidant properties. Experiments were carried out 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 solvent per g dry stem). Under the optimised conditions, the extract was standardised 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 containing 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

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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 (Noor et al., 1989), “Andawali” in Indonesia (Koay and Amir, 2013), “Makabuhay” in Philippines (Quisumbing, 1951) and “Boraphet” in Thailand (Li et al., 2006). 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 (Najib *et al*., 1999; Zakaria *et al*., 2006; Kongkathip *et al*., 2002; Noor and Ashcroft, 1989).

The promising in traditional applications has led to the chemical and biological studies of this plant throughout modern research. Studies showed 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 (Ahmad et al., 2016). Moreover, it was discovered that *T. crispa* possessed an anti-hypercholesterolemic activity and is beneficial in preventing the heart-related diseases (Zulkhairi, A., et al. 2009; Kamarazaman, I. S., et. al. 2012). 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.

Most of traditional medicine products nowadays were prepared in the form of extract. The kinds of extract were dry extract, viscous extract, and liquid extract that

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produced according to the active constituent and the dosage forms, such as capsule, tablet, liquid, pill, and etc (Harwoko and Nur Amalia, 2016). The extract should be standardized to ensure the quality and safety (Hariyati, 2005). One of the important methods of standardization of herbal products is marker based standardization. It helps in adjusting the herbal products to a defined content of a constituent/s which have therapeutic activity. It was reported that the chemical constituents isolated from stem parts of *T. crispa* contained diterpenoid glycosides including syringin, borapetoside A, borapetoside B, borapetoside C, borapetoside D, borapetol A and B, picroretine and tinotubride (Misak, 1995; Pramana et al., 2011; Ahmad et al., 2016), flavonoids including flavavone-O-glycosides (apigenin), and quaternary alkaloids including berberine, aporphines and palmatine (Umi Kalsom and Noor, 1995; Bisset and Nwaiwu, 1984).

In present study, only syringin was selected as standard marker because of its ability and importance. Syringin, has been reported to possess remarkable biological activities such as anti-hypertension (Rao et al., 2016), free radicals scavenging (Kim, N. Y, et al., 1999; Kim, H. C et al., 2005), anti-diabetic (Noor and Ashcroft, 1989) and anti-inflammatory activities (Kamarazaman et al., 2012). 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 (Araujo *et al.,* 1996). Optimization in analytical chemistry can be carried out using one variable-at-a-time method. This method is carried out by changing one parameter while other parameters are kept at constant level (Bezerra *et al.,* 2008). Yield of extract material, concentration of referral markers and antioxidant activity were strongly dependent on extraction condition. Currently, the information on the optimum extraction of *T. crispa* is still

limited. Thus, this study will provide a good source of information for further up scaling purposes i.e., from bench scale to pilot plant level that can be benefited by the industry.

Oxidative damage can lead to oxidation of cholesterol, the major known factors in the development of heart disease. Oxidation, meaning the addition of oxygen to low-density lipoproteins (LDL or “bad” cholesterol), contributes to the build-up of fatty plaque on artery walls (atherosclerosis), which can eventually slow or block blood flow to the heart. Oxidative damage may be prevented or limited by the intake of dietary antioxidants that exist in herbs and vegetables as vitamins, minerals and other various forms of phytochemicals e.g. carotenoids and polyphenol compounds including ~~flavonoids and~~flavonoids and ~~anthocyanins (~~anthocyanins (Cotelle, 1996; Jantan et al., 2015). ~~Therefore, the~~Therefore, the optimisation of *T. crispa* extraction procedure can be further verified via its antioxidant properties ~~in order to~~to evaluate its effectiveness in defending body cells from oxidative stress and to provide important preliminary data for the use of its potential antioxidant properties for the future studies.

2.0 OBJECTIVES

This study was aimed to investigate the effect of extraction parameters on extraction yield, syringin content and antioxidant activity of standardised *T. crispa* aqueous extract.

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3.0 MATERIAL AND METHODS

3.1 Collection of Raw Material

About 10kg of fresh stem part 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 about 55°C. The weight of the samples was monitored every day until constant weight was obtained. Subsequently, the dried stems of the plant are ground to a particle size of about 1 to about 4 mm by using a 20 hp pilot scale grinder. The ground stem sample is kept at room temperature in a sealed environment prior to the extraction process.

3.2 Optimization of Extraction Parameter of *T. 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 ~~define~~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 Zulkhairi et al. (2008). The extraction was carried out using water bath (Memmert WNB 45 Germany). Hot water

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bath was used to provide uniform heating during extraction. The extraction process ~~were~~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 (Pin et al., 2010):

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

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 ~~sovent~~solvent to solid ratio (ml/g). Meanwhile, the syringin concentration of the extract was quantified using HPLC analysis. The concentrations

for syringin yield were reported in *Wt. %* by using formula (Pin et al., 2010):

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

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

Twenty grams of dried sample was placed into five 250ml round bottle flasks and labeled as 1, 2, 3, 4 and 5 where each number represents on room temperature (25ºC), 40, 60, 80 and 100ºC. Two hundred milliliters of RO water (1:10 gm/ml) were used for each flask and the duration of extraction was set at 6 hours. The temperature was monitored using thermometer until the targeted temperatures is obtained. The extraction was carried out using water bath (Memmert WNB 45 Germany). The extraction process were conducted triplicate. The extracted materials were filtered using filter paper (Whatman No. 1) and freeze-dried to remove water. The yield of TCAE syringin content were calculated following the equations in section 3.2.1.

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

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

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3.3 Determination of Syringin Content from TCAE Using HPLC

After the freeze-dry process, the 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 sample was filtered using a syringe filter (Whatman 0.45µm PVDF) prior to injection into the HPLC system. The HPLC system consists of Waters 600 System Controller, Waters 2996 Ultraviolet (UV) detector and equipped with Waters 717 Autosampler. Waters 2996 UV detector detects chemical compounds that pass through HPLC column and sends the data to the computer for analysis. Column oven was used to maintain the temperature of column during the 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 ~~optimisation~~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. Syringin was selected as the quality indicator for this research. This bioactive compound was reported to be important and mostly contributed to bioactivities as discussed in section 2.11.3.4. The presence of standard compounds' syringin in TCAE was identified by comparison of their retention times and UV spectra with those of standard. The experiments were conducted in triplicate and the results are presented in ppm (mg/l).

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3.3.1 Quantification of Syringin Content

The standard stock solutions of syringin as the standard compound (100 µg/ml) was prepared by dissolving 1 mg of standard in 10 ml of methanol: water (70:30). These solutions were stored in dark glass bottles at 4 °C. Working standard solutions, which spanned a concentration range from 5 µg/mL to 200µg/ml for HPLC analysis were prepared. Quantification of syringin content of TCAE was based on the calibration curve obtained and was converted to the wt. % (w/w) following the equations in section 3.2.1.

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

Based on the yield and HPLC analysis, prototype of STCAE was produced using designated optimal extraction parameter obtained. The *T. crispa* aqueous extract (TCAE) was standardized on basis of standard compound, syringin. The production processes involve aqueous extraction, concentration and freeze drying. The concentration of liquid extract was performed below 60^{o}C under vacuum. The concentrated liquid extract was freeze dried using laboratory Freeze Dryer (85XL, Millrock, USA) to produce powdered STCAE. STCAE then was stored at -20^{0}C until used. The quality of STCAE was verified further via 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 BHT acted as the standard.

3.4 Determination of Antioxidant Activity of STCAE

3.4.1 DPPH Radical Scavenging

~~Standardised~~Standardized aqueous extract of *Tinospora crispa* obtained from the earlier optimized extraction process was further evaluated for its antioxidant activity ~~on the basis of~~based on scavenging activity against a stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), as previously described (Yen & Hseih, 1998). 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 absolute ethanol. As for the sample solution, 1 ml of 0.45 mM DPPH was added to 0.5 ml of the extract (5 ml/ml). The step was repeated by replacing the extract with BHT (5 mg/ml) and vitamin C (5 mg/ml). Each samples, were incubated for 30 min and following incubation, the absorbance was recorded at 517 nm. The percentage of inhibition which represents the scavenging activity of the sample against DPPH was calculated as per the following equation:

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

3.4.2 Thiobarbituric Acid Test (TBA)

TBA values of STCAE was determined using the method of Ottolenghi (1959). 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). This 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:

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

3.4.3 Ferric Reducing Antioxidant Power

The ferric reducing ability of the STCAE was evaluated following the method described by Benzie & Strain (1996). 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 are 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 ± 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 *T. 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 obtained. The result indicates that the extraction yield increased from 9.69% to 16.22% as the ratio of solid to solvent increased from 1:10 to 1:25 (g/ml). The highest yield was showed in ratio 1:25 (16.25 ± 0.38%), followed by ratio 1:20 (16.10 ± 0.23%) and ratio 1:15 (15.86 ± 0.55%), respectively. However, there was no significant change observed on the yield of the solid to solvent ratio among them. Meanwhile, the ratio of solid to solvent at the ratio 1:10 was found significantly lower ($p<0.05$) compared to the other groups (11.03 ± 0.40%).

Figure 2 shows the effect of solid to solvent ratio on the syringin concentration of TCAE. The result indicates that the syringin concentration increased from 0.44 ± 0.01% to 0.49 ± 0.04 % as the ratio of solid to solvent increased from 1:10 to 1:25 (g/ml). The highest syringin concentration was shown in ratio 1:25 (0.49 ± 0.04%), followed by ratio 1:20 (0.47 ± 0.02%), ratio 1:15 (0.46 ± 0.01%) and ratio 1:10 (0.44 ± 0.01%), respectively. However, there was no significant change observed on the concentration of syringin between all ratios tested.

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).

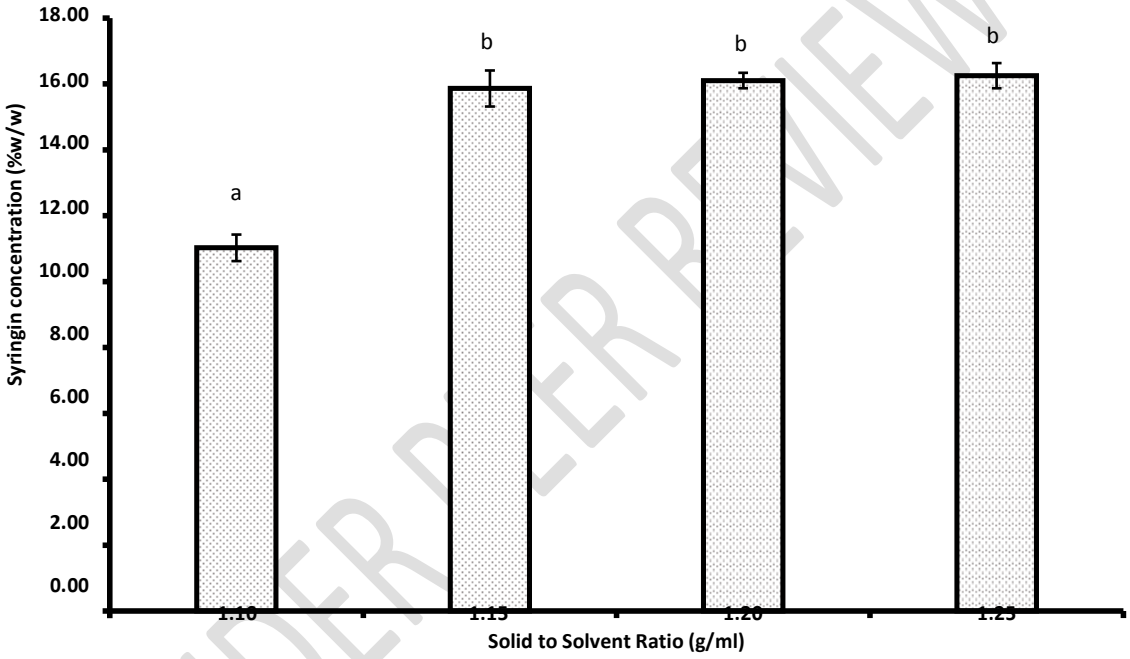

Figure.1: Effect of different solid to solvent ratio (g/ml) on extraction yield of TCAE. Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.**

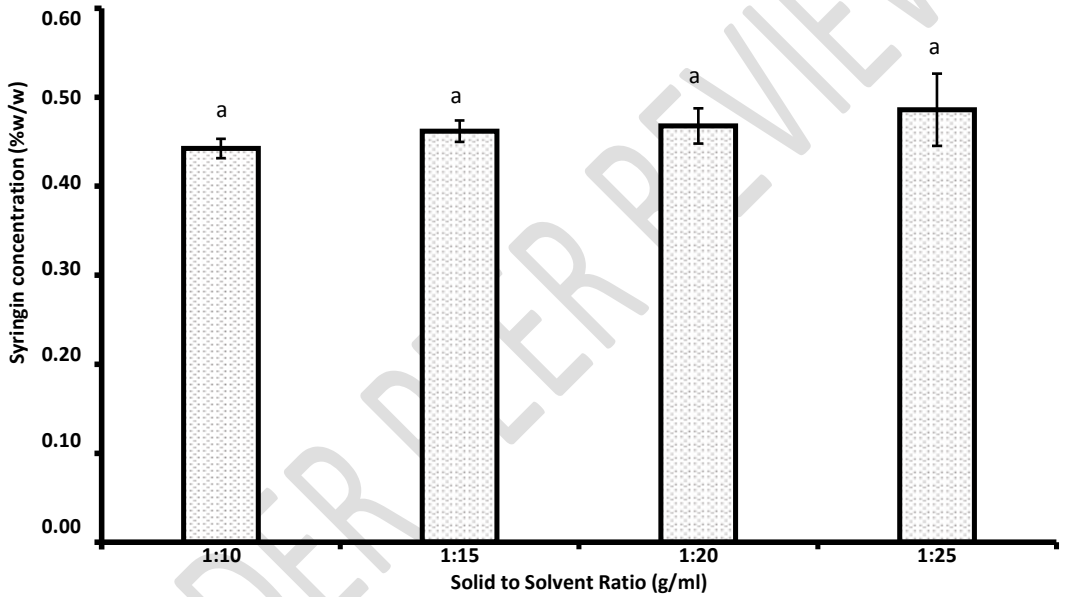

Figure 2: Effect of different solid to solvent ratio (g/ml) on syringin concentration of TCAE. Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.**

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 of TCAE observed between 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 the yield at room temperature (7.98 ± 0.63%) when compared to the other groups.

Figure 4 shows the effect of different temperature on the concentration of syringin. The result showed that the concentration of syringin was found to increase from temperature 25 up to 60ºC and start to drop dramatically at temperature 80 and 100ºC. Result indicates the most suitable temperature for extraction of *T. crispa* was at 60ºC since ~~it's~~it has produced significantly ($p<0.05$) the highest concentration of syringin compared to the other groups. The concentration of syringin at 25, 40, and 60ºC were increased with 0.37, 0.38 and 0.45% dry wt respectively, and decrease to 0.18 and
0.07% dry wt, at temperature 80 and 100ºC respectively

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.

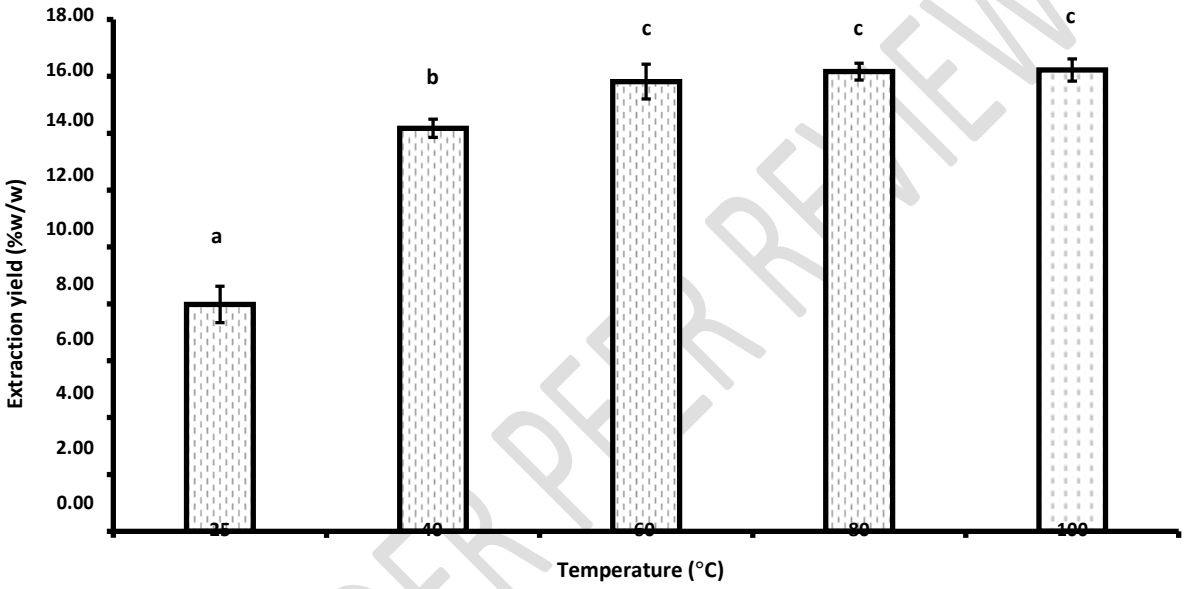

Figure 3: Effect of varying temperature (ºC) on extraction yield of TCAE. Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$).

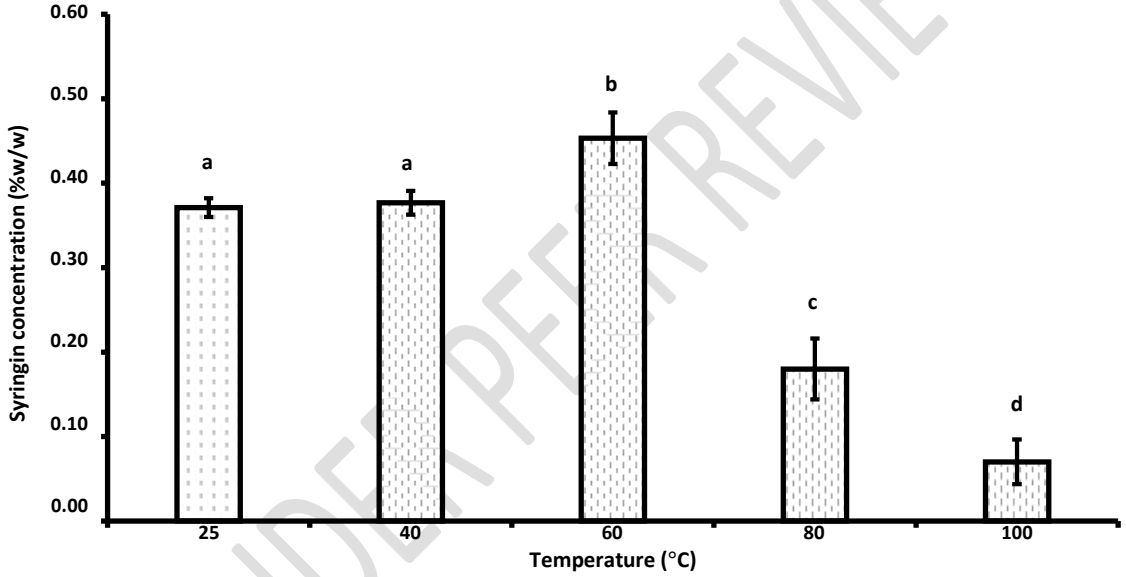

Figure 4: Effect of varying temperature (ºC) on the concentration of syringin. Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$).

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

Figure 5 shows the effects of different extraction time on the yield of TCAE. The result showed that there was no significant change in the extraction yield of TCAE after the first hour. This suggests the extraction process achieved 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 6 shows the effect of different temperature on the concentration of syringin. Result showed that the concentration of syringin was increased from 30 minutes up to 1 hour of the extraction process and begins to show reduction after 1 hour. The degradation could be resulted from the lengthy exposure to heat.

Results ~~indicate the~~indicate the most suitable extraction period to obtain the optimal extraction yield and syringin concentration of TCAE was at 1 hour.

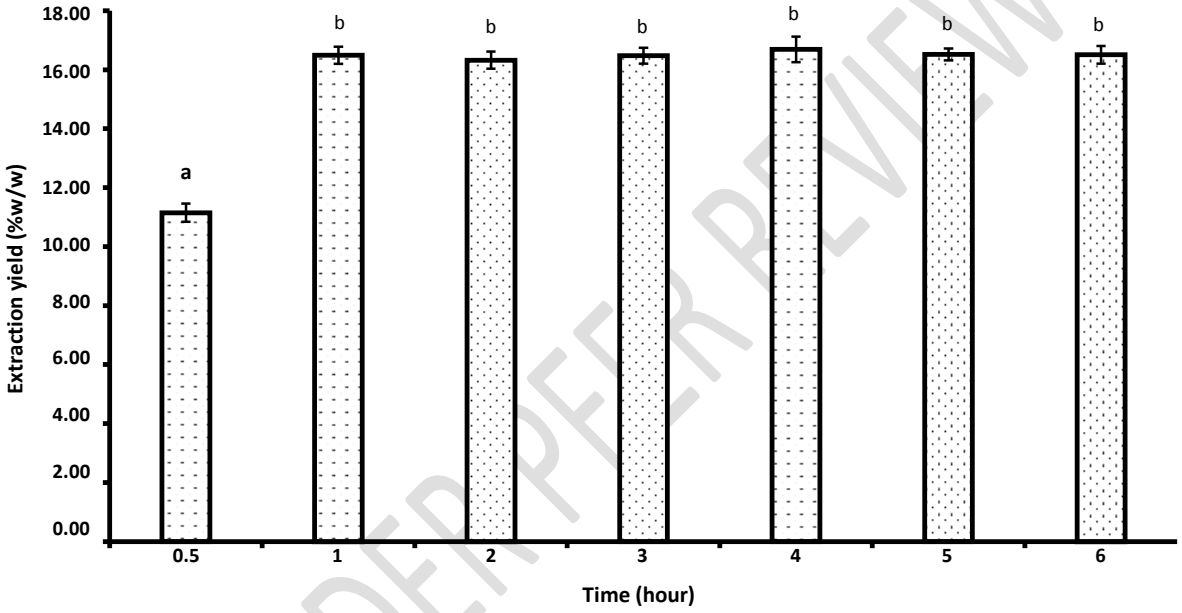

Figure 5: Effect of different extraction time on extraction yield of TCAE. Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.**

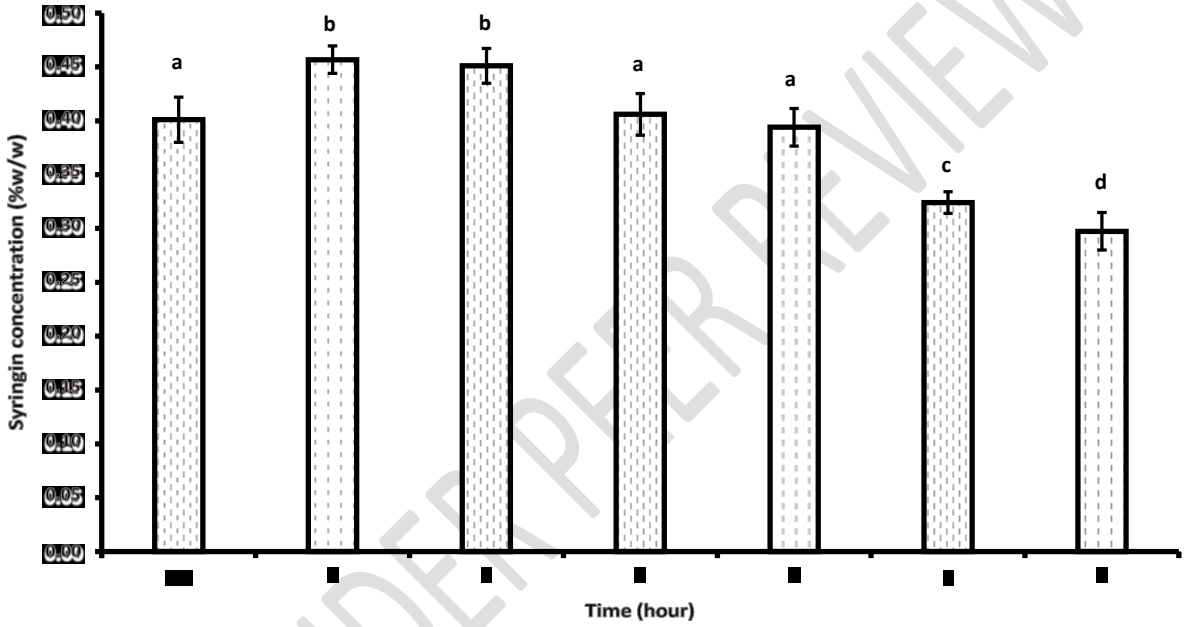

Figure 6: Effect of varying extraction time on the concentration of syringin. Each value represents the mean ± SD. Bars with different alphabet are significantly different ($p<0.05$)**.**

4.2 HPLC profile of TCAE Containing Syringin

Figure 7 shows the HPLC chromatogram of referral standard syringin while Figure 8 to 12 represent the HPLC chromatograms of TCAE containing syringin at the temperatures of 25°C, 40°C, 60°C, 80°C and 100°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.

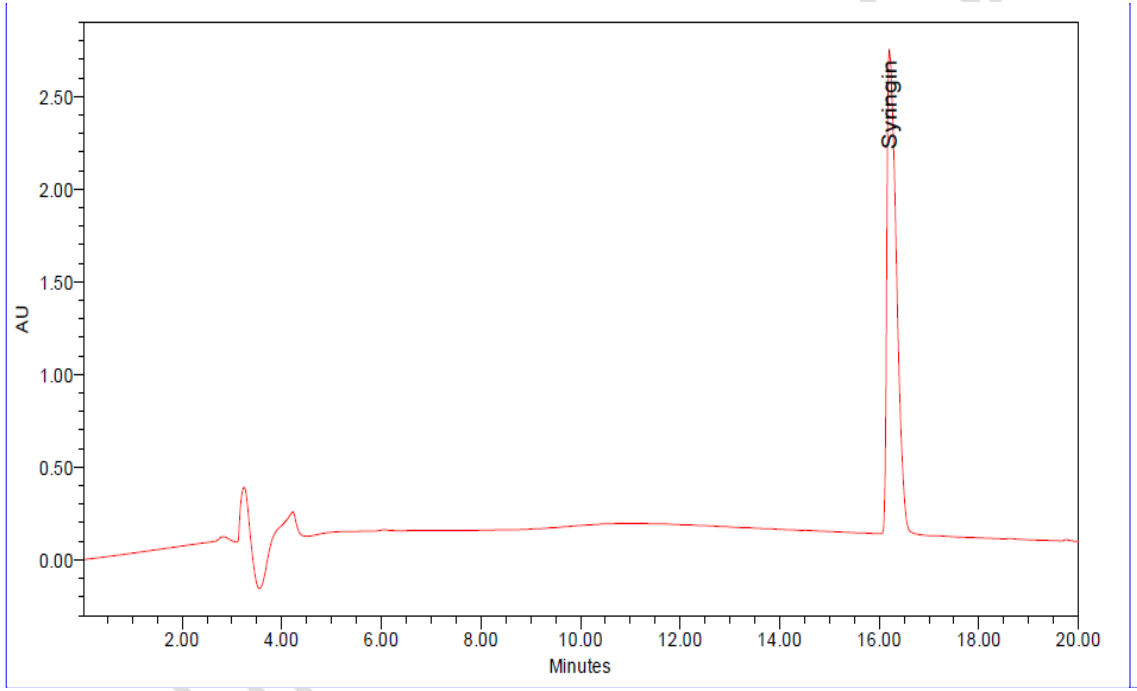

Figure 7: HPLC chromatogram of syringin standard compound.

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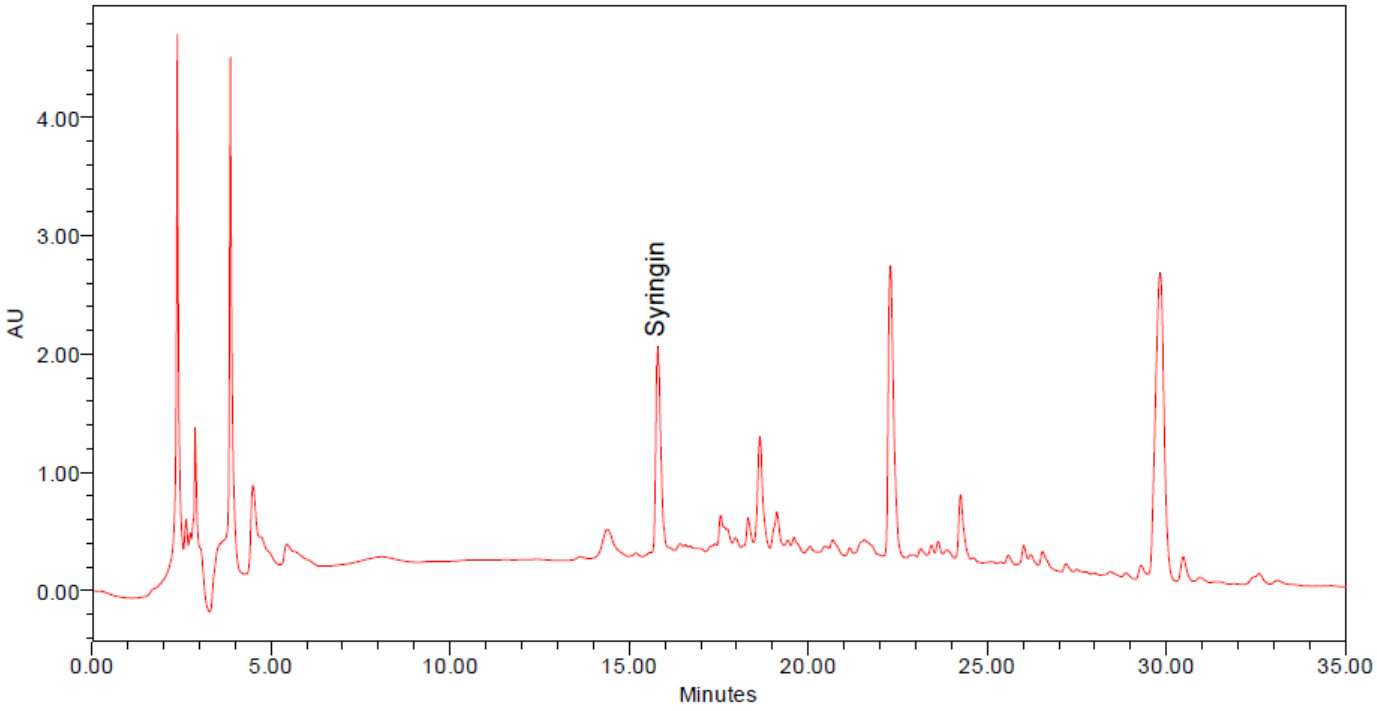

Figure 8: HPLC chromatogram of TCAE containing syringin at 25°C

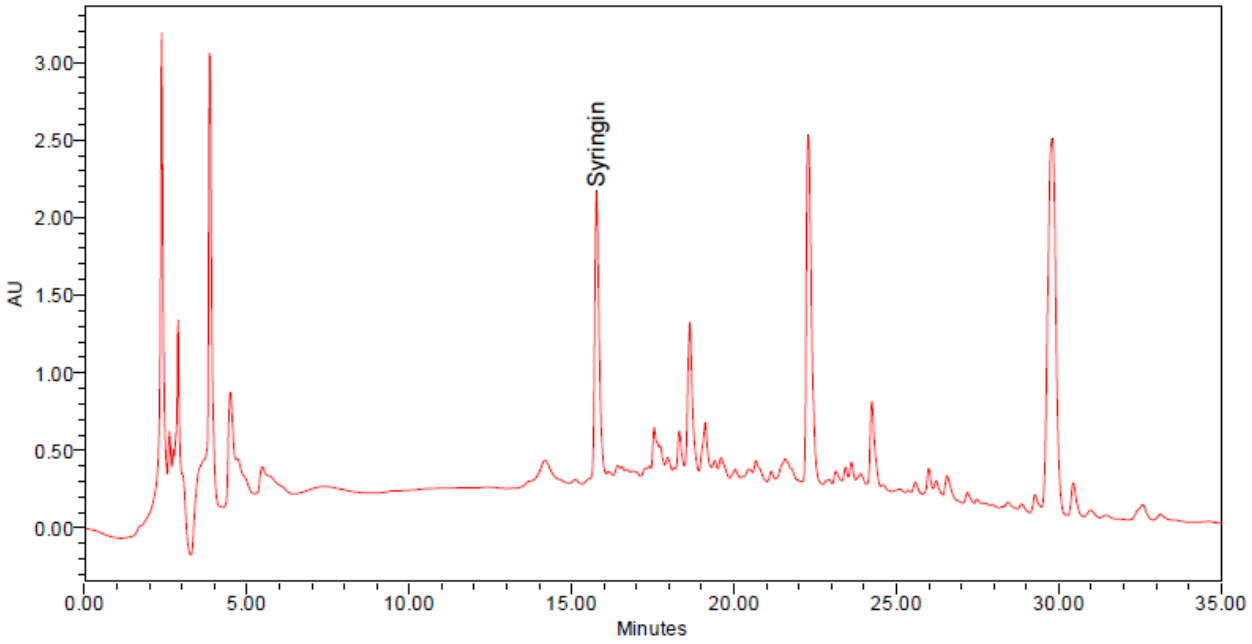

***Figure 9*:** HPLC chromatogram of TCAE containing syringin at 40°C

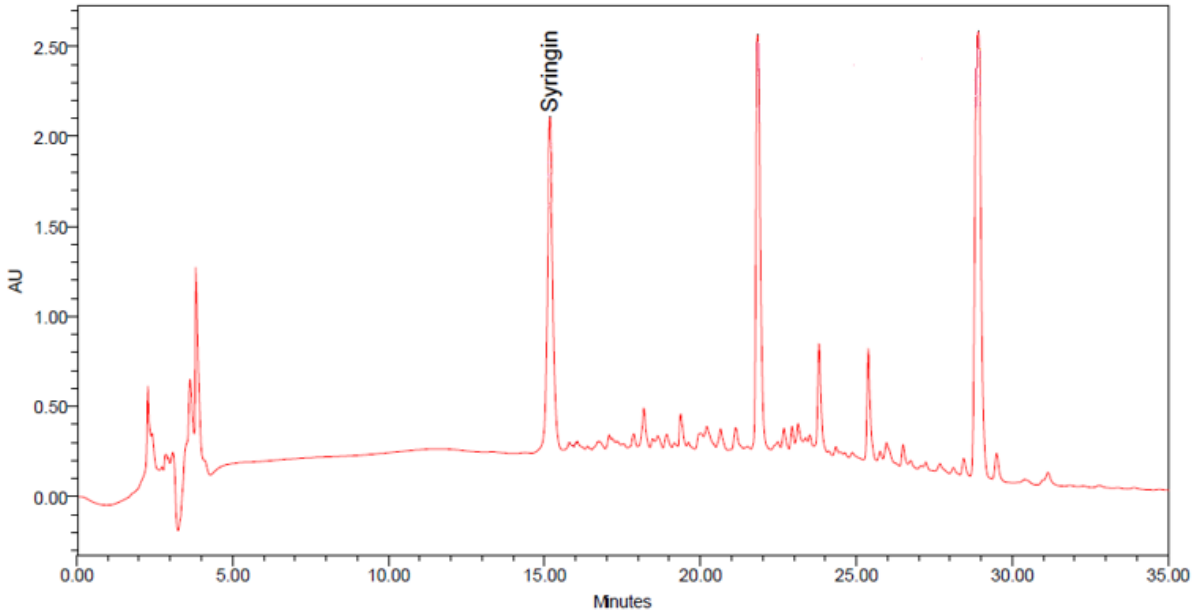

Figure 10: HPLC chromatogram of TCAE containing syringin at 60°C

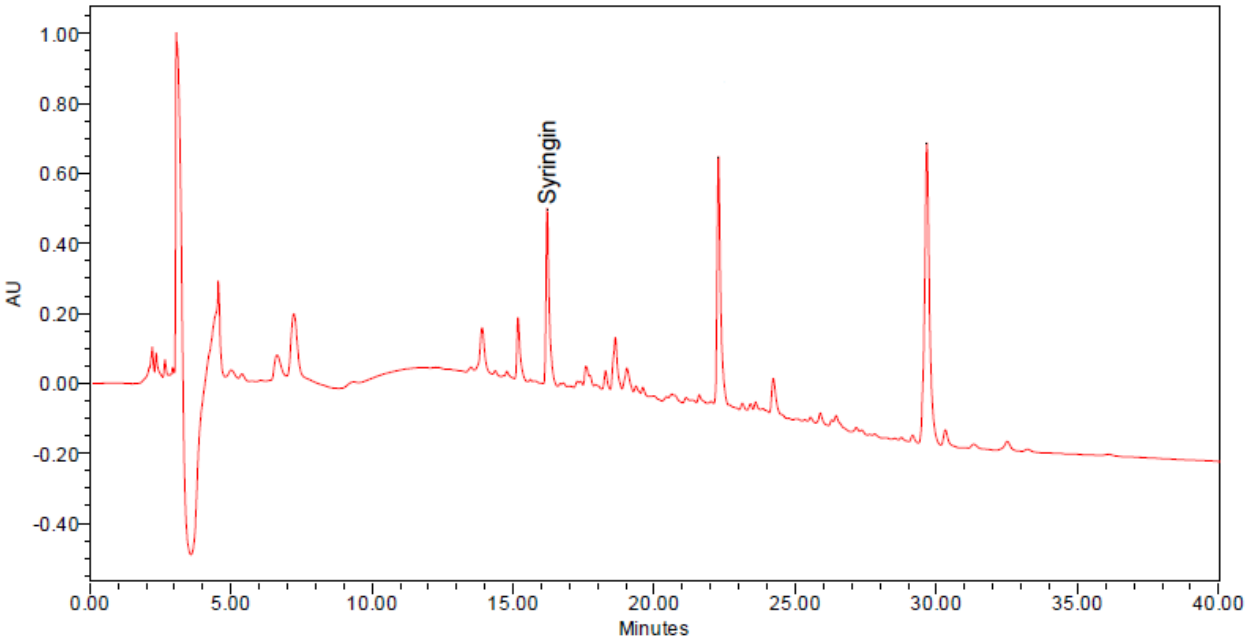

Figure 11: HPLC chromatogram of TCAE containing syringin at 80°C

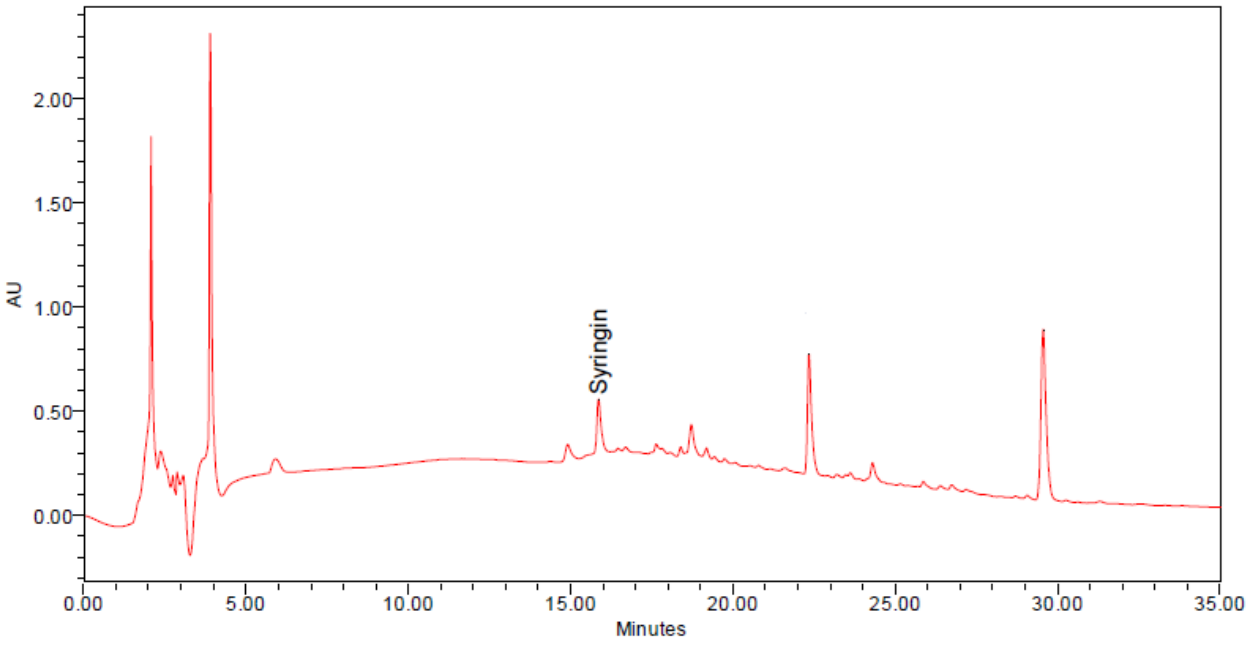

***Figure 12*:** HPLC chromatogram of TCAE containing syringin at 100°C

4.2.1 Standardisation of TCAE

The TCAE was ~~standardised~~standardized ~~on the basis of~~based on the marker compound syringin. The selection of syringin was based on its ability to reduce cholesterol (Chaosheng Li et al., 2011). The quantitative determination of marker compound by HPLC indicated that TCAE exhibited peaks of 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, as shown in Figure 13. From the calculation, it was found that STCAE is defined to

contain at least 0.4 wt% of syringin of total extract.

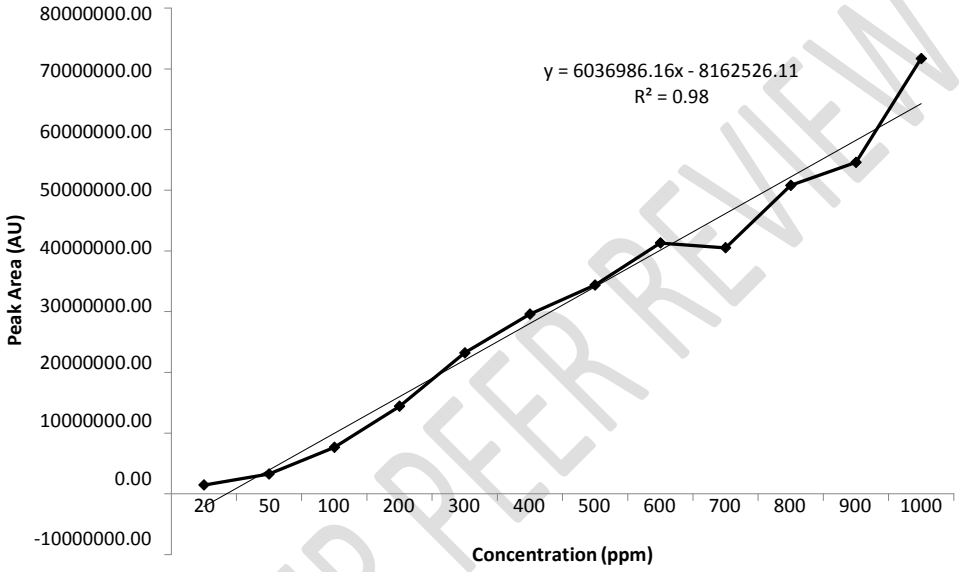

***Figure 13*:** Standard curve of Syringin.

4.3 Antioxidant Activity of STCAE in *vitro*

T. crispa ~~standardised~~standardized extract obtained from the optimized extraction parameter was evaluated on its antioxidant activity through DPPH, TBA and FRAP bioassays and the results were shown in Figure 14, 15 and 16.

4.3.1 DPPH Radical Scavenging

The DPPH assay was utilized to evaluate the ability of antioxidants to scavenge ~~free radicals~~free radicals. ~~The scavenging~~The scavenging activities of STCAE, vitamin C and Butylated hydroxytoluene (BHT) against DPPH radicals were compared and shown in Figure 14. Results showed that STCAE exhibited high scavenging activity with the percentage of inhibition of 82.31±0.37%. ~~However~~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.

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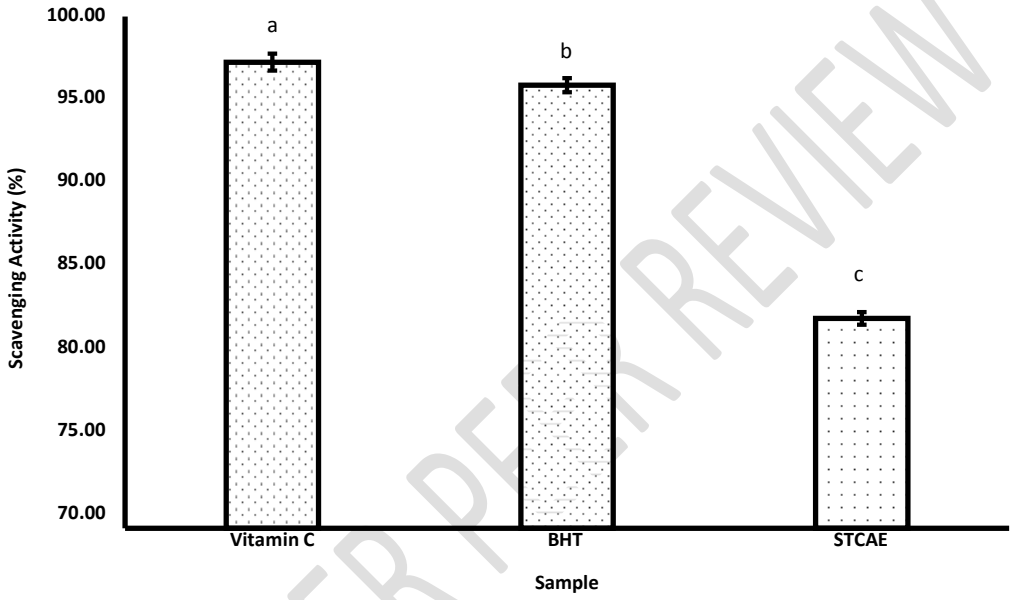

***Figure 14*:** DPPH free radical scavenging of ~~standardised~~standardized *Tinospora crispa* aqueous extract compared to vitamin C and butylated hdroxytoluene (BHT). Data expressed as mean ± SD. Bars with different alphabets are significantly different (p>0.05).

4.3.2 Thiobarbituric Acid Test (TBA)

The percentage of inhibition of STCAE evaluated in TBA test is shown in Figure 15. 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.

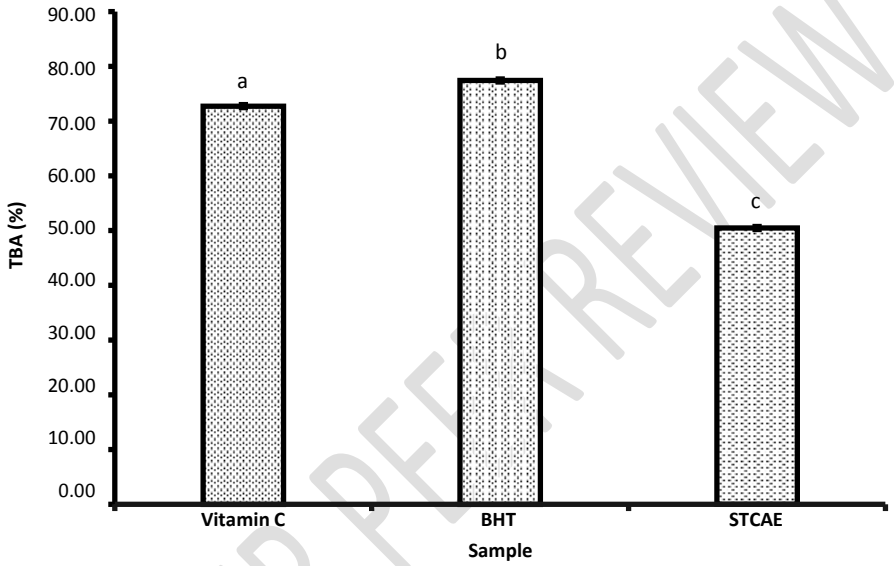

Figure 15: Thiobarbituric Acid Test (TBA) of ~~standardised~~standardized *Tinospora crispa* aqueous extract compared to vitamin C and butylated hdroxytoluene (BHT). Data expressed as mean ± SD. Bars with different alphabets are significantly different

(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 16. The results showed that, STCAE exhibited high antioxidant activity with FRAP value of 0.89+0.07 mmol/L. ~~However~~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.

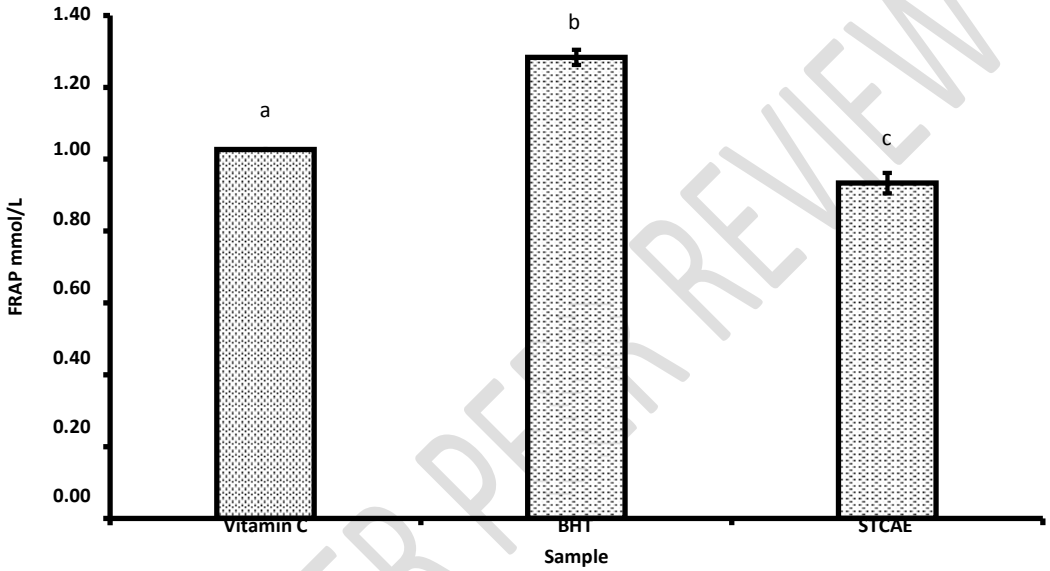

***Figure 16*:** Feric reducing antioxidant power (FRAP) assay of ~~standardised~~standardized *Tinospora crispa* aqueous extract compared to vitamin C and butylated hdroxytoluene (BHT). Data expressed as mean ± SD. Bars with different alphabets are
significantly different (p>0.05).

5.0 DISCUSSION

Optimization of extraction process is essentially required to obtain the optimum concentration of phytochemical constituents ~~and also~~and to maintain their activities (Aziz et al.,
2003). Optimization is also referred as an improvement of performance of a system, process or product to obtain the optimum benefit from it ~~i.e.~~i.e., high yield of extraction (Araujo *et al.,* 1996). Higher extraction yield is preferred as the extraction parameters applied are the most cost effective and the most preferable by the industry players for further up scaling purposes. ~~Thus~~Thus, optimization of extraction process will contribute to the maximum quantity of products (high extraction yield) of highest quality (~~eg.~~e.g. activity) at the lowest possible cost. 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 (Spigno *et al*., 2007).

There are two most commonly used in optimization studies, the classical single factor experiments and the response-surface methodology (RSM). The classical single factor experiments ~~is~~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 (Bas and Boyaci, 2007; Bezerra *et al.*, 2008). However, single factor experiments ~~are able to~~can provide fundamental information on the ranges for significant extraction parameters on the extraction of targeted compounds from plant materials.

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The solvent quantity is among important factor to the yield of extraction (Virot et al., 2010). Result from present study revealed that, the extraction yield of TCAE was found to increase with the increase in ratio of solvent to solid *(Figure 1).* These results were consistent with mass transfer principle where the driving force for mass transfer ~~is considered to be~~is the concentration gradient between the solid and the solvent. A high solid-to-solvent ratio

could

promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by solvent (Al-Farsi and Chang, 2007; Tan et al., 2011).

Results also indicated an obvious increase of extraction yields of syringin, when the solvent to solid ratio was proportionally increased from 10 to 25 ml/g, but there was no obvious change in the yields of syringin, as the ratio continued to increase. To avoid the wasting consumption of solvents, 15 ml/g was chosen as the optimum ratio of the solvent to solid. Moreover, ratio 15 ml/g also exhibited the highest syringin concentration compared to other ratios studied. Thus, it was found that ratio 1:15 g/ml was the most suitable solid to solvent ratio in obtaining high quality of *T. crispa* aqueous extract.

Temperature plays an important role in the extraction of bioactive compounds from plant materials. Result revealed that, the most suitable temperature for obtaining high extraction yield and high concentration of syringin were at 60ºC. A similar finding was reported by Mohd Farhan et al., (2015) who discovered 60ºC was the most suitable extraction temperature for obtaining high yield of *Orthosiphon stamineus* extract. Rao et al., (2015) demonstrated that, the optimum condition to obtain the highest yield of syringin from 9 medicinal plants were at 30 minutes extraction time and 40ºC temperature. Similar finding was reported by Zhao et al., (2012), who mentioned the highest extraction yield of syringin extracted from the bark of *Ilex rotunda* could be achieved when using 50 °C as the yield gradually decreased when extraction temperatures increased up to 80 °C.

Silva *et al.* (2007) reported that temperature was the most important parameter in extraction of *Inga edulis* leaves because higher temperature increased the solubility and diffusion coefficient of the solute, allowing higher yield and extraction rate. This principle is also applicable in the extraction of *T. crispa.* However, elevating the temperatures up to a certain level might be ~~effected~~affected to the decomposition of antioxidants which were already mobilized at lower temperatures (Liyana-Pathirana and Shahidi, 2005). Other than that, denaturation of membranes and a possible degradation of polyphenolic compounds caused may happen and influence quantification of bioactive compounds (Abad-Garcia et al., 2007).

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~~Moreover, extraction~~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 (*Figure 3).* Result obtained was dissimilar to previous finding by Zulkhairi et al., (2008) who found out that the optimum extraction time was at 6 hours. 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 (Silva et al., 2007). Moreover, prolonged extraction time increases the chance of decomposition and oxidation of phenolics due to their long exposure to ~~unfavourable~~unfavorable environmental factors like temperature, light and oxygen (Naczk and Shahidi, 2004). On the other hand, the increased extraction time is uneconomical and time consuming from the ~~industrialisation~~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~~population, and hill forest population. This is supported by previous study conducted on *Mentha spicatha* by Ullah *et al*. (2012) confirms that variations in phytochemical content are related to geographical location. According to the report, the impact of different altitudes, moisture and temperature of different locations are the factors contributing to changes in secondary metabolites. Another study by Dong *et al*. (2011) on secondary metabolites content in the leaves extract of *Eucommia ulmoides* from different province showed that growing locations had significant impacts.

The standardised *T. crispa* aqueos extract obtained from the ~~optimised~~optimized extraction procedures was further evaluated for its antioxidant properties. The DPPH scavenging activity has been widely used to evaluate the antiradical activity of various samples (Tirzitis & Bartosz,

2010; Piao et al., 2004). Antioxidants react with DPPH reducing a number of DPPH molecules

which equal to the number of their available hydroxyl groups (Sanchez et *al.,* 1998). DPPH is

a stable radical with a maximum absorption at 517nm that can readily undergo scavenging by an antioxidants (Lu & Yeap, 2001). In the present study, the free radical scavenging ability of STCAE was assessed by the discoloration of a 1, 1 –diphenyl-2-picrylhydrazyl radical (DPPH) solution and was compared to vitamin C and BHT.

Results from present study show that STCAE able to scavenge DPPH radical with the percentage of inhibition of 87.06±0.23%. The results are in accordance with the previous reports by Zulkhairi et al., (2008) who revealed 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. The reason for the slight differences might be due to the extraction periods used in preparing the extract. According to Albu *et al.,* 2004, temperature and incubation periods are important factors involved in the extraction process ~~in order to~~to produce a high antioxidant reading. This was supported by Aziz et al., (2003) who stated, optimization of extraction protocols are essentially required in order to enhance the concentration of biologically active constituents and to maintain their activities.

Zulkifli et al., (2013) suggested, the high antioxidant activity of the stem extract of *Tinospora crispa* is most probably due to the presence of apigenin and magnoflorine as its possess hydroxyl group that donates the electron to reduce the DPPH radicals. This was supported by Rackova et al., (2004) who reported that magnoflorine isolated from *Mahonia aquifolium* showed high antiradical activity toward DPPH radical. Magnoflorine has also been shown to have high antioxidant activity in the root of *Tinospora cordifolia* (Rekha & Veena 2011) and in the seed of *Xanthoxylum piperitum* (Hisatomi et al. 2000). Besides that, Kim, et al., (1999) 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 on the other hand has demonstrated the peroxyl radical scavenging capacity comparable to that of glutathione as reported by Kim, et al., (2010). Therefore, it is suggested in present study that the free radical scavenging effect of STCAE might possibly attributed by syringin as well.

Thiobarbituric acid (TBA) method is a colorimetric technique which is widely used for measuring the extent of lipid oxidation in the samples (Rhee, 1978). The peroxidation of polyunsaturated fatty acid (PUFA) will yield the lower molecular compounds such as malondialdehyde (MDA) (Ledwozyw *et al*., 1986). The result 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 done by Zulkhairi et al., (2008) 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. Naczk and Shahidi, (2004) reported that, prolonged extraction time increases the chance of decomposition and oxidation of phenolics due to their long exposure to ~~unfavourable~~unfavorable environmental factors like temperature, light and oxygen.

In the FRAP assay, antioxidant properties in plant extracts can be measured by looking at the reduction of ferric 2, 4, 6-tripyridyl-s-triazin complex (Fe3+-TPTZ) to the ferrous form (Fe2+-TPTZ). A blue colored FeII-tripyridyltriazine compound was formed from ~~colourless~~colorless oxidised FeIII by the action of electron donating antioxidants in plant sample. FRAP assay can provide a quick information on total antioxidant potentials in cells, without having to run ~~more lengthy~~lengthier test. The FRAP is versatile and can be readily applied to both aqueous and organic extracts of different plants. This method is a simple, ~~reproducible~~reproducible, and inexpensive. From the result showed in Figure 16, the difference of FRAP value between STCAE with vitamin C and BHT were about 0.09 mmol/L and 0.35 mmol/L respectively. This result clearly showed that STCAE has high antioxidant property due to the small difference of FRAP value between STCAE and controls. According to David et al., (2007), antioxidant compounds in some herbs are likely to be heat labile. The processes of steaming, ~~flaking~~flaking, and boiling of plants have been reported to decrease their biological compounds (Bryngelsson et al., 2002). 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.

Standardization of herbal formulation is important ~~in order to~~to maintain high quality products in the market based on the concentration of their active principles as well as In-vitro and In-vivo evaluation of their biological activities (Rasheed & Roja, 2012). Standardization minimizes batch to batch variation; assure safety, efficacy, ~~quality~~quality, and acceptability of the poly herbal formulations (Ahmad et al., 2006). The bioactive extract should be standardized ~~on the basis of~~based on active principles or major compounds along with the chromatographic fingerprints by using Thin Layer chromatography (TLC), High Performance Thin Layer Chromatography (HPLC), very sensitive High Performance Thin Layer Chromatography (HPTLC) and Gas Chromatography (GC) (Rasheed & Roja, 2012). HPLC analysis was performed in this present study in which HPLC fingerprint of TCAE showed the present of syringin.

Syringin has been reported to possess a significant cholesterol-lowering effect in hyperlipidemia animal model. It has also been applied in the development of drug combination for treating cardiovascular and cerebrovascular diseases. Instead, syringin was reported to be safe and nontoxic and has strong pharmacological action and good drug effect. Thus, STCAE was produced with at least 0.40 wt. % of syringin from total extract. The results from this study can be used to formulate a new nutraceutical product utilizing STCAE in the form of capsule and tablet.

6.0 CONCLUSION

Based on the findings of this study, it was concluded that ~~in order to~~to obtain high extraction yield and high syringin concentration of *T. crispa* extract, 60°C was selected as suitable temperature. The best extraction time was at 1 hour whereas the optimum ratio of solvent to solid was at 1:15 g/ml. The standardised *T. crispa* aqueous extract (STCAE) was produced containing at least 0.4 wt% of syringin. STCAE obtained was found to possess high antioxidant activities through DPPH, FRAP and TBA bioassays. The results obtained in this study suggested that *T. crispa* could be used as an easily accessible source of natural

antioxidants and can be utilized further as possible health supplement in pharmaceutical industry.

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