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Pressurized hot water extraction of phenolic and antioxidant activity of *Clinacanthus nutan* leaves using accelerated solvent extractor

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ABSTRACT

Accelerated solvent extractor (*ASE*) is classified as the Pressurized Hot Water Extraction (*PHWE*) which is known as green extraction process mainly used for bioactive extraction from plant matrices. In the present work, '*belalai gajah'* leaves or *Clinacanthus nutan* was used for extraction of phenolic compound and antioxidant activity using *ASE*. In this study, the effect of temperature and time on total phenolic content (*TPC*), total flavonoid content (*TFC*) and antioxidant activity (*AA*) was investigated. Operating variable consists of temperature ranging from 100 to 180°C and time 5 to 30 minutes; at 1500psi. The results indicate the highest TPC was obtained at 160°C, 5 minutes with value of 20.97 ± 0.03 mg gallic acid g^{-1} dry sample and *TFC* was found highest, 20.99 ± 0.01 mg quercetin g^{-1} dry sample at 180°C, 30 minutes. Meanwhile, greatest *AA* achieved till 68% at 180°C, 5 minutes. Thus, the *TFC* in *C. nutans* contribute more to antioxidant activity than *TPC* with R^2 more than 80%. *TFC* can be used as a guidance in assessing the antioxidant activity of herb plants. From this study, the present investigation reveals that *TFC* is mainly responsible for *DPPH* free radical scavenging capacity.

INTRODUCTION

Currently, the herb plants are extensively used for remedy of numerous acute diseases which encouraged more attention as they are healthier and aware of the side effects caused by synthetic products or drugs (Ambrose *et al*., 2016; Tee *et al*., 2015). Their effectiveness, economical and less toxicity has made it popular and approved as a mode of treating diseases even in this modern times (Kumar *et al*., 2014). Some herbal medicinal plants have shown to possess significant phytochemicals and bioactive especially phenolic compounds which form a major bioactive compound in herbs plant (Venugopal and Liu, 2012). Phenolic compounds have an important roles in food industry and health benefits as it hold various biological effects, including antiseptic, anti-cancer, antiviral, anti-inflammatory, and antioxidant activity (Kanmaz *et al*., 2014; Quideau *et al*., 2011).

Clinacanthus nutan (*C. nutan*) is a vital herb species that widely cultivated in Southeast Asia region. *C. nutan* is belonging to the Acanthaceae family known as a snake grass or recognized as *'belalai gajah'* (Aslam *et al*., 2016; P'ng *et al*., 2014). Different part such as leaves and stems of *C. nutan* are used traditionally as medicine for treating various disease including skin infections, animal bites, skin damage caused by herpes simplex virus, metabolic diseases, fever, gout in diuretics and swelling due to fall or boils Malaysia, Indonesia, Thailand and China (Alam *et al*., 2016; Chiwapreecha *et al*., 2011). The leaves can be consumed raw or as refreshing beverages (Shim *et al*., 2013). Many studies has been discovered on phytochemical and

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pharmacological activity from C. nutan leaves (Shim *et al*., 2013). Numerous bioactive compounds such as phenolics, flavonoids, C-glycosyl flavones, β-sitosterol, stigmasterol and chlorophyll derivatives being extracted. The investigation is to demonstrate of pharmaceutical approach like antioxidant activity, anti-inflammatory and others (Aslam *et al*., 2015; Mustapa *et al*., 2015). Furthermore, the bioactive compounds from this plants especially phenolics and flavonoids are found greatest in the leaves compared in stems (Raya *et al*., 2015).

The existences of conventional techniques such as soxhlet, soaking and maceration presented with an organic solvent used for extraction of phenolic compounds from C. nutan leaves (Sulaiman *et al*., 2015). In Karim and Muhamad, (2015) also Mustapa *et al*. (2015) studies, the phenolic and flavonoid contents of C. nutans being quantified using soxhlet method ranging from 8 to 25 mg gallic acid g-1 dry extract and 3 to 6 mg quercertin g-1 dry extract, respectively. Meanwhile, about 18 mg gallic acid g-1 dry extract of phenolic and 6 mg quercertin g-1 dry extract of flavonoid content through soaking method resulted from Ghasemzadeh *et al*. (2014). However, least of phenolic and flavonoid contents about 0.78 mg gallic acid g-1 dry extract and 0.2 mg quercertin g-1 dry extract obtained using maceration method (P'ng *et al*., 2014). Soxhlet extraction are often performed at high temperatures for longer time which may cause thermal degradation of bioactive compounds due to prolonged heat exposure (Mustapa *et al*., 2015). Moreover, these traditional methods employed large amount of toxic solvents (Herrero *et al*., 2006) such as methanol, ethanol and hexane which may give hazardous effect to human health and environment (Sulaiman *et al*., 2015; Kanmaz, 2014). To date, the number of interest has expanded that focus on green technology application that precludes toxicity associated to the solvents. Pressurized hot water extraction meets the requirement to be considered as green technology.

Extraction plays a crucial function for natural products extraction of herbal plants and foodstuffs and it is defined as a separation process to separate a solute from one phase into another (Herrero *et al*., 2006). In extraction of bioactive compounds from plant sources are widely used approach of pressurized hot water extraction that provides a vast benefits compare to the conventional method. Commonly, this technology used water as a main solvent at temperature of 100 to 374°C (Plaza *et al*., 2013). Capability in selection of different types of compound vastly be influenced by a temperature, whereby the high polar compound obtained at a low temperature and at high temperature is favorable for less polar compound. Pressurizes liquid extractor or known as accelerated solvent extractor has gained attention since this technique provides a fast extraction and consumes less solvent than other extraction technique (Kanmaz, 2014; Kanmaz and Ova, 2013). The process of accelerated solvent extraction usually consume less than 30 minutes under elevated pressure up to 1500psi and temperature around 100 to 200°C (Santos *et al*., 2012; Camel, 2001). Various plant matrices has been successfully extracted by this method to extract antioxidant, phenolic compounds and functional compounds such as mango leaves (Fernandez *et al*., 2012), sea buckthorn (Gong *et al*., 2015), thyme (Vergara-Salinas *et al*., 2012) and others.

C. nutan leaves has been widely extracted using various extraction techniques, however the pressurized hot water extraction of phenolic using accelerated solvent extractor has never been reported. Thus, the objective of this study is to evaluate the consequences of temperature and time on phenolic, flavonoid content and antioxidant activity.

Experimental:

Materials:

The *C. nutan* leaves was collected from local herbs farm which is located in Temerloh, Pahang, Malaysia. All chemicals and reagents used were gallic acid, sodium carbonate, Folin-Ciocalteu reagent, (Friendemann Schmidt Chemicals, UK), sodium nitrite, sodium hydroxide, aluminium chloride, quercetin and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) standard (Sigma Chemical Co, USA).

Sample preparation:

The *C. nutan* leaves was first separated from the stems and washed thoroughly to remove impurities. Then, dried the leaves at 40°C for overnight. Further grounded of dried leaves using a grinder and sieved through 1.18mm mesh size. Kept stored the powdered leaves in air-tight vacuum pack at room temperature prior to extraction process (Chelyn *et al*., 2014)

Accelerated Solvent Extraction:

The extraction process performed using accelerated solvent extraction (ASE 350 model) in batch wise at temperature (100 to 180ºC) and extraction time (5 to 30 min) functioned by a solvent controller. About 5 g of powdered leaves was mixed with 3 g of diatomaceous earth in 100 mL extraction that inserted with cellulose filter. Firstly, the sample was auto-filled with water solvent and loaded to extraction cell at 1500psi. Then, heating process started and the static extraction was performed at respective set temperature and time. Solvent purging from extraction cell using nitrogen gas and finally, the pressure released from the system. Kept stored the collected extracts at -20ºC for further analysis.

Analysis of phenolic content:

The phenolic analysis was described by Susanti *et al*. (2015) with slightly changes. Concisely, 0.5 mL of leaves extract (10%) was added into test tube containing 2.5 mL Folin-Ciocalteu reagent. Then the mixture was left for 5 minutes before adding 2 mL of sodium carbonate (75g/L). Afterwards, kept allowed the reaction for two hours. The total phenolic content was measured spectrophotometrically at 765 nm and phenolic amount quantified based on mg gallic acid equivalent per gram of dry sample. All the analyses were conducted in triplicates.

Analysis of flavonoid content:

The flavonoid analysis was established using aluminium chloride method adapted from Tan *et al*. (2014). Initially, about 0.5 mL of aliquot leaves extract was assemble with 2 mL of distilled water and 0.15 mL of sodium nitrite (5%). After 5 minutes, 0.15 mL of aluminium chloride (10%) added, mixed thoroughly and left for 6 minutes. Additional of aluminium chloride in the mixture is to develop a deep yellow-colored complexes of flavonoids. Subsequently, 2 mL of sodium hydroxide (4%) was added and top up the volume of mixture to 5 mL with distilled water. Vortex the mixture and left in dark for 15 minutes. Finally, flavonoid content was measured at absorbance of 510 nm via blank based on mg quercetin equivalent per gram of dry sample. All determination performs in triplicates.

Analysis of free radical scavenging activity:

The radical scavenging activity is estimated as mentioned by Sharifi *et al*. (2013) with some modification. About 3.5 mL of (0.004%) ethanolic 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) was mixed with 0.5 mL ethanolic leaves extract (25%). Next, left the mixture for 30 minutes before spectrophotometrically measured at 517 nm of absorbance. The radical scavenging activity was evaluated by comparing the sample absorbance with the control. Each measurement was done in triplicate. The estimation of scavenging activity is based on following equation:

$$\text{DPPH \%} = [\frac{Ac - As}{Ac} \times 100]$$

Where A_c = absorbance of control and A_s = absorbance of tested sample.

Statistical analysis:

The data was computed and statistically analyzed using the Microsoft Excel 2013 software. The data presented were the mean values of two or more repeated experiments and the average absolute relative deviation (AAD) was calculated using the following equation:

$$AAD = \frac{1}{N}\frac{[x_i - x_{avg}]}{x_i}$$

Where, N is the number of the experimental data, x_i is the experimental point and x_{avg} is the average value at every condition. Two way analysis of variance (ANOVA) was established using the (R software version 3.1.2) for estimation the outcome of time and temperature instantaneously. All parameters were measured to provide statistically significant different to the process. Whereby if the *p* value less than 0.01 was achieved regarding to 99% confidence level. Meanwhile, the higher *f* value would responded as high significant effect to the process (Sarip *et al.,* 2016).

RESULTS AND DISCUSSION

Analysis of phenolic content:

The figure 1 demonstrates the total phenolic content (*TPC*) of *C. nutan* leaves as performed of temperature and extraction time. The extraction condition at temperature of 100 to 180°C and time 5 to 30 minutes correspond to the *TPC* amounted from 13.71 ± 0.01 to 20.97 ± 0.03 mg GAE/g of dry sample. At 5 minutes, 160°C, the *TPC* value achieved highest to 20.97 ± 0.03 mg GAE/g of dry sample. The *TPC* value of *C. nutan* leaves is slightly lower than that in the study of Karim and Muhamad (2015), which differs about 8% using decoction method. However, the presence *TPC* value was 30% higher than Mustapa *et al*. (2015) using microwave irradiation extraction (MAE).

As extended the extraction time, our *TPC* value showed decreased within temperature of 140 to 160°C. According to Zullaikah *et al*. (2015), at higher temperature over 150°C typically enhance the extraction rate thus increase of yield compound. Besides that, at this extreme temperature certain compound being extensively exposed become degraded and eventually loss of desirable compounds because of thermal instability. Moreover, at 5 min, 180°C of extraction condition the *TPC* of *C. nutan* leaves gradually decreased. Yet,

prolong the extraction time the *TPC* derived increased. This increment is because of derivatives compound produced from the caramelization and maillard reaction (Howard and Pandjaitan, 2008; Montilla *et al*., 2006).

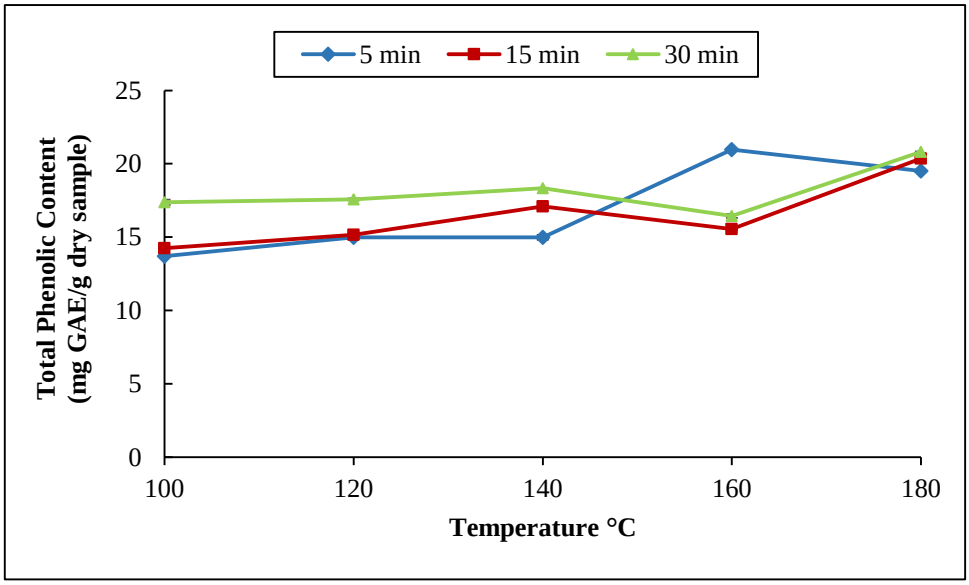


Fig. 1: The total phenolic content for *C. nutans* leaves extract

In addition, shorter extraction time is preferable as it will minimize the production of unwanted reaction and product during extraction. Therefore, the highest *TPC* could be obtained at 160°C and 5 minutes. According the two way ANOVA, the temperature showed a corresponding effect with higher *f* value of 14.96, while least *p* value of 0.002. However, the less significant effect could be observed on the extraction time with least *f* value of 1.74 and *p* value higher with 0.21. The least *f* value indicates that the extraction time has minimal effect on *TPC* from *C. nutans* extract through pressurized hot water extraction.

Analysis of flavonoid content:

Figure 2 shows the flavonoid content of *C. nutan* leaves ranging from 8.64 ± 0.06 to 20.99 ± 0.01 mg QE/g of dry sample which affected by increased of temperature and extraction time. As temperature increased within prolong extraction time, the disruption of solute-matrix interaction occurred, reducing the viscosity also contribute to optimum yield (Kanmaz, 2014; Plaza *et al*., 2013).

In this study, the highest total flavonoid content (*TFC*) (20.99 ± 0.08 mg QE/g of dry sample) was discovered at 180°C at extended time of 30 minutes that significantly higher about 54% than study by Karim and Muhamad, (2015) which using decoction extraction method. Likewise in Susanti *et al*. (2015), the flavonoid extraction greatest at 30 minutes. In addition the flavonoid extraction is completed within this interval, however the degradation of flavonoid occurred due to exposure to high temperature in prolong time. The less polar of apigenin glycoside mainly from flavonoid aglycone group consists of the quercetin, kaempferol, luteolin and C-glycosyl flavone that found to be major in *C. nutan* leaves (Ko *et al*., 2014; Ghasemzadeh *et al*., 2014).

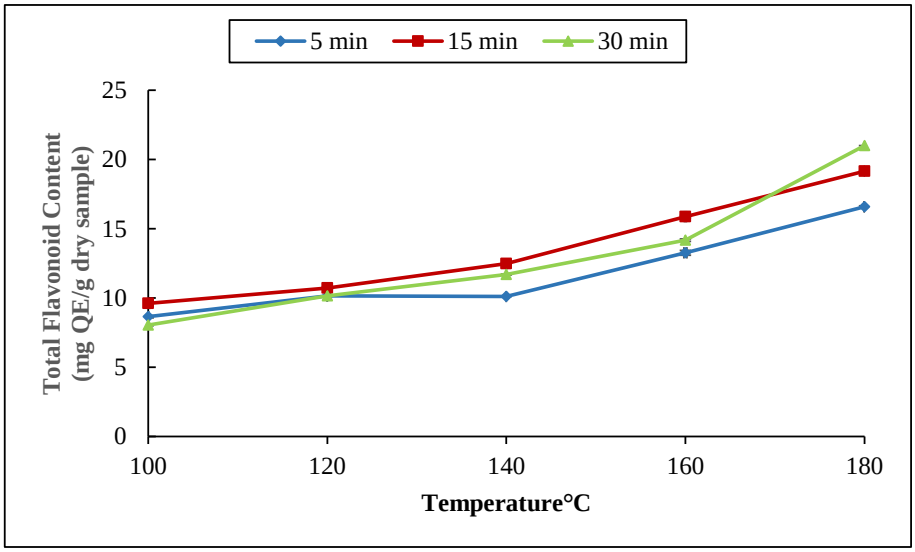


Fig. 2: The total flavonoid content *C. nutans* leaves extract

In pressurized hot water extraction with increase of temperature and time resulted decreasing of water polarity (Zullaikah *et al*., 2015) which encouraged higher *TFC* of *C. nutans* leaves. Statistically, the temperature showed more significant effect on *TFC* compared to extraction time with higher *f* value of 53.11, and least *p* value of 9.638 x 10^{-6}. Contrarily, the extraction time given minimal effect that showed less significant with least *f* value of 2.31 and *p* value higher with 0.15.

Analysis of antioxidant activity:

Figure 3 displays the antioxidant activity as performed of temperature and extraction time by the changes of purple to yellowish color (Sharma and Bhat, 2009). The free radical scavenging is proportional with the temperature at 100 to 180°C ranging from 38.93 ± 0.16 to 68.05 ± 0.04% inhibition. At 5 minutes, 180°C the percent inhibition is 68.05 ± 0.04. This agreement with finding of Rodriguez *et al*. (2006) that indicate highest antioxidant acquired at highest temperature. However, at 160 to 180°C for all extraction times, the trend of antioxidant activity seems almost constant and expected to decrease at temperature more than 180°C. It is because of antioxidant compound degradation comprises of phenolic and flavonoid (Azmir *et al*., 2013; Ko *et al*., 2014). Additionally, at higher temperature possibly cause destruction of flavone except for the shorter extraction time about 5 minutes (Mustapa *et al*., 2015). Consequently, the *C. nutans* leaves extracted at shortest extraction time and higher temperature promising a good percent inhibition. Based on statistical analysis, antioxidant activity is more significant affected by a temperature with higher *f* value of 125.85 with least *p* values of 1.02x10^{-7}. Different with extraction time that is less significant effect by least *f* value of 9.48 and higher of *p* value.

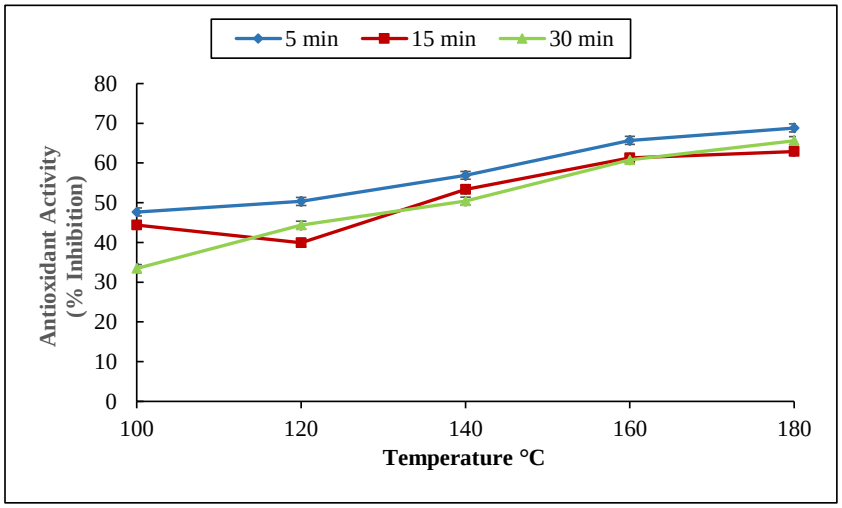


Fig. 3: The antioxidant activity of *C. nutans* leaves extract

Relationship of phenolic compound and antioxidant activity:

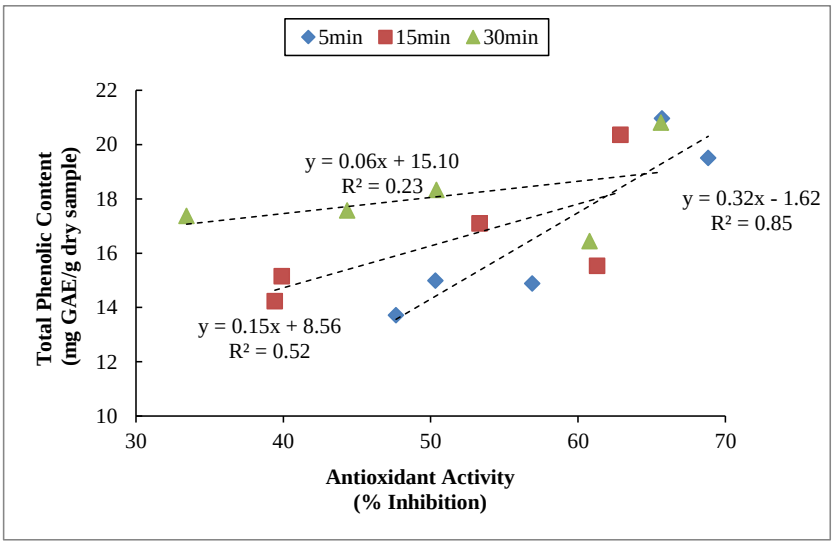
According to Zullaikah *et al*. (2015), the highest of polyphenolic compound contribute to most potent antioxidant activity (*AA*). A linear relationship between phenolic and flavonoid content with *AA* of *C. nutans* extracts are displayed in Figure 4 (a) and 4 (b), respectively. The slope and R^2 values are summarized in Table1.

As increased of extraction time, the total phenolic content (*TPC*) slope showed decreasing from 0.32 to 0.06 along with R^2 values 0.85 to 0.22. Figure 5 (a) shows at 5 minutes, 160°C gave a quite reasonable high correlation of *TPC* and antioxidant activity with highest R^2 value of 0.85. While, at 15 minutes, 180°C showed a less correlation with R^2 = 0.52 and there is no correlation at 30 minutes, 180°C with least R^2 value of 0.21.

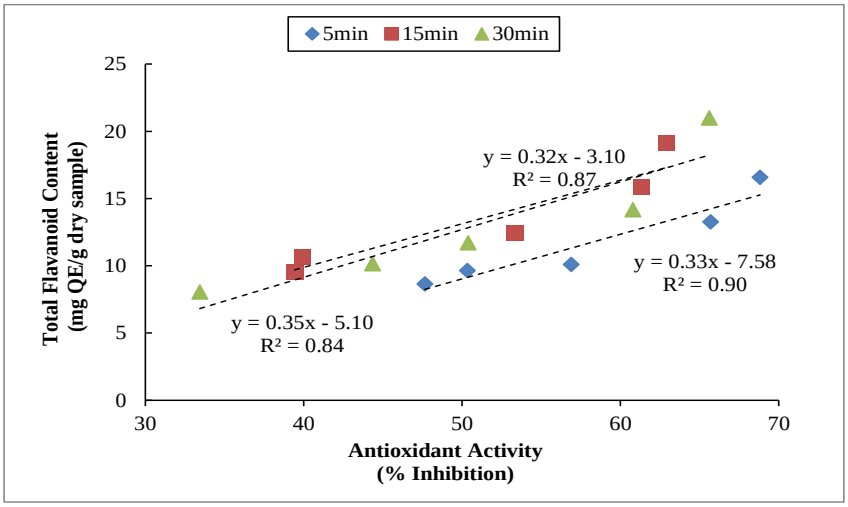
While, the slope and R^2 for total flavonoid content (*TFC*) is almost constant ranging from 0.33 to 0.35 and 0.83 to 0.89, respectively. In figure 5 (b) indicates the positive correlation between highest *TFC* and *AA* in extraction condition of 5 to 30 minutes at 180°C with R^2 value 0.84 to 0.90. In addition, the biological functions of flavonoid compound which affected by the substitution and increasing of hydroxyl group enhanced the *AA* (Ko *et al*., 2005). Thus, it shows that the *TFC* in *C. nutans* leaves are more contributes to *AA* than the *TPC*.

Table 1: The slope and R^2 values for correlation of phenolic, flavonoid content against antioxidant activity

Time (min)	Antioxidant Activity			
	Total Phenolic Content		Total Flavonoid Content	
	Slope	R^2	Slope	R^2
5	0.32	0.85	0.33	0.90
15	0.15	0.52	0.32	0.87
30	0.06	0.22	0.35	0.84



(a)



(b)

Fig. 4: Correlation between phenolic compound and antioxidant activity;
(a) Total phenolic content, and (b) Total flavonoid content

Conclusion:

The present investigation reveals that the phenolic, flavonoid content and antioxidant activity has been successfully extracted from *C. nutan* leaves at elevated temperature and time in pressurized hot water extraction process using accelerated solvent extractor. The temperature shows more significant effect against the phenolic, flavonoid and antioxidant activity. Yet, the flavonoid is more participate to antioxidant activity than phenolic with R^2 more than 80%. Hence, the pressurized hot water extraction system proposed in this study has promises of being used for the herbs utilization of beneficial food materials, nutraceuticals and pharmaceuticals at manufacturing industry.

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