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Evaluation of Phenolic Content and Antioxidant Property of Hydrolysed Extracts of *Terminalia catappa* L. Leaf

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Abstract: The influence of acid and alkaline hydrolysis on the phenolic content and antioxidant activity of *Terminalia catappa* L. leaves were evaluated in this study. Polyphenolic content was determined using total phenolic, total flavonoid and total tannin assays. Four *in vitro* antioxidant assays such as DPPH, ABTS, Ferric Reducing Antioxidant Potency (FRAP) and total antioxidant capacity assays were followed to determine the antioxidant potency of the extracts and the values were expressed as mg vitamin C equivalent antioxidant capacity (VCEAC)/g extract. The results of this study indicated that acid hydrolysed and alkaline hydrolysed extracts possess fewer amounts of polyphenolic constituents in comparison with non hydrolysed extract. Even the VCEAC values of non hydrolysed extract in antioxidant assays were significantly higher ($p<0.05$) than acid and alkaline hydrolysed extracts. Moreover, the UV-visible spectral study also showed the absence of some of the absorption peaks of polyphenolic constituents in acid and alkaline hydrolysed extracts and hence, it supported our *in vitro* results. A strong correlation was observed for polyphenolic content and antioxidant activities of these extracts ensuring the involvement of polyphenolic content for the antioxidant activity. However, the results of this study may not be generalised for all plants as different plants possess different phytoconstituents in varying quantities.

Key words: *Terminalia catappa*, DPPH, ABTS, Vitamin C, Hydrolysis

INTRODUCTION

Polyphenols present in plants, fruits and vegetables are an important source of natural antioxidants as they act as reducing agents, hydrogen donors, singlet oxygen quenchers and potential metal chelators (Kahkonen *et al*., 1999; Rice-Evans *et al*., 1995). Consumption of fruits, vegetables and plants rich in polyphenols is associated with the reduced risk of certain cancers, cardiovascular diseases, atherosclerosis, diabetes and Alzheimer's disease (Halliwell and Gutteridge, 1989; Arts and Hollman, 2005; Kurosawa *et al*., 2005; Youdim *et al*., 2004). The number of natural polyphenols has been estimated to be more than 5000, including 2000 flavonoids and the number is still increasing (Harborne, 1993).

Terminalia catappa L. leaf belongs to the family *Combretaceae*, rich in polyphenolic components such as punicalagin, punicalin, chebulagic acid, corialgin (Tanaka *et al*., 1986), gallic acid, ellagic acid, isovitexin, vitexin, rutin (Lin *et al*., 2000). It also contains triterpenoids such ursolic acid and 2α, 3β, 23-trihydroxyurs-12-en-28 oic acid (Fan *et al*., 2004). Different parts of this plant have long been used as folk medicine in India, Philippines, Malaysia and Indonesia for antidiarrheic, antipyretic and haemostatic purposes (Lin, 1992). The leaves of *T. catappa* reported to possess several pharmacological activities including antioxidative, hepatoprotective, analgesic anti-inflammatory, antidiabetic and anti-HIV reverse transcriptase activity (Chyau *et al*., 2002; Tang *et al*., 2004; Annegowda *et al*., 2010; Fan *et al*., 2004; Nagappa *et al*., 2003; Tan *et al*., 1991).

Phenolic compounds are always present in the form of glycosides in plants and are rarely present in the free form. Hence, several hydrolytic procedures like acid hydrolysis using hydrochloric acid (HCl) and alkaline hydrolysis using sodium hydroxide (NaOH) have been used to hydrolyse glycosides to aglycones (Krygier *et al*., 1982; Hertog *et al*., 1992).

It has been reported by earlier researchers that *T. catappa* leaves was found to possess good antioxidant activity, reducing power and inhibitors of peroxidation (Chyau *et al*., 2002; Mau *et al*., 2003). Till date, the classical solvent extraction, soxhlet extraction and supercritical fluid extraction have been used for the extraction of *T. catappa* (Chyau *et al*., 2002; Mau *et al*., 2003). To our knowledge, so far no investigation has been reported regarding the hydrolysis of *T. catappa* leaf extract which will offer a method for the quantitative

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determination of phenolic compounds. Hence, the objective of this study is to evaluate the influence of acid and alkaline hydrolysis on the phenolic content and also to provide the antioxidant information of *T. catappa* leaf extract for development and application of the resource.

MATERIALS AND METHODS

Plant material: Fresh leaves of *Terminalia catappa* were collected from Universiti Sains Malaysia (USM) campus Penang, Malaysia during November 2008. This plant was then authentified by a botanist and the voucher specimen (No. 11048) was deposited in Herbarium, School of Biological Sciences, USM. Collected and authentified leaves were then washed with running water to remove the dirt, dried and ground to obtain powder of 40 mesh size.

Chemicals: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Folin–Ciocalteu reagent, potassium persulphate, vanillin, sodium carbonate, sodium hydroxide, aluminium chloride hexahydrate, ferric chloride, sodium acetate, ascorbic acid, gallic acid, catechin were purchased from Sigma Chemicals, Germany. All the other solvents used in this study were of analytical grade and purchased from Fisher Scientific Sdn. Bhd, Malaysia.

Extraction: The method described (Hertog *et al*., 1992) with slight modification was followed for the hydrolysis of *T. catappa* leaf powder at Centre for Drug Research, USM, Penang, Malaysia. Acid hydrolysis was performed by refluxing 20 g of leaf powder in round bottom flask with 200 mL of 0.5 N HCl for 2 h at 90°C. After cooling, the solution was filtered, centrifuged and the supernatant liquid was collected which was then extracted twice with ethyl acetate (100 mL). The ethyl acetate layer collected was then completely removed from extract using rotary evaporator under vacuum at 45°C yielding an acid hydrolysed extract (sample 1). Similar procedure of acid hydrolysis was followed for the alkaline hydrolysis and control (without hydrolysis) in which we used 0.5 N NaOH and distilled water to obtain alkaline hydrolysed (sample 2) and non hydrolysed extract or control (sample 3) respectively. All the extracts kept at 4°C in air tight container until further analysis.

Determination of total phenolic, total flavonoids and total tannins: The colorimetric method described by Singleton and Rossi (1965) was used to determine the total phenolic content of the *T. catappa* hydrolysed as well as control extracts and the values were expressed in terms of mg gallic acid equivalents (mg GAE) per gram extract. Aluminium chloride colorimetric method described by Sakanaka *et al*. (2005) was followed to find out the total flavonoid content in the extracts and the values were expressed in terms of mg catechin equivalents (mg CAE) per gram extract. Vanillin-HCl method with slight modifications was used to measure the total tannins concentrations in the extracts and the values were expressed in terms of mg catechin equivalents (mg CAE) per gram extract (Burns, 1971).

ABTS radical scavenging assay: ABTS radical scavenging activity of *T. catappa* hydrolysed as well as control extracts were determined according to the previously described procedure of Re *et al*. (1999). ABTS solution was freshly prepared by adding 5 mL of a 4.9 mM potassium persulphate solution to 5 mL of a 14 mM ABTS solution and the resulting solution was kept for 16 h in dark at room temperature (25±1°C). This solution was diluted with methanol to yield an absorbance of 0.700±0.02 at 734 nm and the same solution was used for the antioxidant assay. One milliliter of reaction mixture of standard and extracts comprised 950 μL of ABTS solution and 50 μL of the samples. This solution was vortexed for 10 sec and the absorbance was recorded at 734 nm after 6 min using a UV-visible spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) which was compared with the control ABTS solution. The calibration curve of vitamin C was prepared by plotting the percentage inhibition of vitamin C at various concentrations (5-100 μg mL^{-1}). The percentage inhibition was calculated using the following formula:

$$\text{Percentage inhibition of vitamin C and extracts} = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of samples.

The results were expressed as mg Vitamin C Equivalent Antioxidant Capacity (VCEAC) per gram extract.

DPPH radical scavenging assay: The DPPH radical scavenging activity of *T. catappa* hydrolysed as well as control extracts were measured according to the method described by Brand-Williams *et al*. (1995). Briefly, 2.9 mL of DPPH solution (100 μM in methanol) was added to 0.1 mL of the crude extract (100 μg mL^{-1}). Further, this solution was incubated in dark for 30 min at room temperature. The decrease in the absorbance (due to the proton donating activity) was measured at 517 nm against a blank consist of 0.1 mL of ethanol and 2.9 mL of DPPH solution. Vitamin C calibration curve was prepared by

plotting the percentage inhibition of vitamin C at various concentrations (25-250 μg mL^{-1}). The percentage inhibition was calculated using the formula mentioned in DPPH assay and the results were expressed as mg VCEAC ability per gram extract. All the measurements were done in triplicate.

Ferric Reducing Antioxidant Potency (FRAP) assay: The ability of the hydrolysed and non hydrolysed *T. catappa* leaf extracts to reduce ferric ions was measured according to the modified method described by Benzie and Strain (1996). Briefly, a known aliquot of the sample (200 μL) was added to 3 mL of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3 \cdot 6H_2O$ solution) and the reaction mixture was incubated in a water bath (at 37°C). The increase in absorbance at 593 nm was measured after 30 min of time interval. Vitamin C various concentrations (5-100 μg mL^{-1}) was used for the preparation of standard calibration curve. The FRAP values were expressed as mg VCEAC per gram of plant material.

Total Antioxidant capacity assay: The method described by Prieto *et al.* (1999) was adapted to measure the antioxidant capacity of the hydrolysed and non hydrolysed *T. catappa* leaf extracts. In brief, to a known aliquot of the sample solution (0.4 mL) taken in a vial, 4 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added and incubated in a water bath at 95°C for 90 min. The blank solution contained 4 mL of reagent solution and 0.4 mL of methanol. After cooling the samples to room temperature, absorbance was measured at 695 nm against a blank. Calibration curve was prepared by using standard solution of vitamin C (5-100 μg mL^{-1}) and the antioxidant activity was expressed as mg VCEAC per gram extract.

UV-visible spectroscopy: The UV-Visible absorption pattern of phytoconstituents can be measured in very dilute solution against a solvent blank using UV-Visible spectrophotometer. Hence, in order to find out the difference among hydrolysed and non hydrolysed extracts, we qualitatively analysed these samples using UV-Visible spectrophotometer. Sample solutions (0.1 mg mL^{-1}) prepared in methanol were used for this analysis and the spectra were recorded against control (methanol). The wave length maxima (λ_{max}) and the intensity of the vitamin C of the absorption spectrum were recorded for each extracts.

Statistical analyses: All assays were carried out in triplicates and results are presented as Mean±SD (n = 3). One way analysis of variance was performed (one way ANOVA) and the significant differences between mean values were determined by Tukey's pair wise test at a level of significance of $p<0.05$. Correlation between polyphenolic content and different antioxidant activities were calculated using Pearson correlation coefficient. The statistical analyses were carried out using SPSS 17 (SPSS Inc USA).

RESULTS AND DISCUSSION

Extraction is an important step in the recovery of antioxidants from the fruits, vegetables and medicinal plant material. On the basis of literature available about hydrolysis, it is clear that no single method is suitable for the hydrolysis of plant material hence we used acid and alkaline hydrolysis methods. Results of different methods of hydrolysis of *T. catappa* leaves showed that highest yield was obtained by acid hydrolysis (2.70%), followed by aqueous hydrolysis (1.10%) and lowest yield (0.50%) with alkaline hydrolysis.

The polyphenolic content of hydrolysed as well as control extracts of *T. catappa* leaves are shown in Table 1, organic layer of non hydrolysed extract (sample 3) has significantly high ($p<0.05$) total phenolic content followed by acid hydrolysed (sample 1) and alkaline hydrolysed extract (sample 2) with mg GAE g^{-1} extract of 432.90±1.43, 338.09±4.26 and 33.64±0.84, respectively. Moreover, the phenolic content of sample 1 was ten times less than sample 2 and thirteen times less than sample 3. Similar trend of total phenolic was observed for total flavonoid and total tannin assay where in the values were ranged from 9.34±1.77 to 70.36±2.64 and 3.54±0.56 to 13.87±0.96 mg CAE g^{-1} extract for total flavonoid and total tannin respectively. As evident from the Table 1, sample 3 possessed significantly high ($p<0.05$) flavonoid and tannin content followed by sample 1 and 2.

The decrease in the polyphenolic content by acid hydrolysis in comparison with control might be due to the degradation of polyphenolic content by acid treatment. Similar findings by Nuutila *et al.* (2002)

Table 1: Total phenolic, flavonoid and tannins content of *T. catappa* extracts

Samples	Total phenolic[1]	Total flavonoid[2]	Total tannins[2]
1	338.09±4.26b	50.77±0.93b	10.57±0.58b
2	33.64±0.84a	9.34±1.77a	3.54±0.56a
3	432.90±1.43c	70.36±2.64c	13.87±0.96c[1]

[1]Total phenolic expressed as mg gallic acid equivalent (GAE)/g extract. [2]Total flavonoid expressed as mg catechin equivalent (CAE)/g extract. Values were expressed as the Mean±SD. Values with different alphabets in the same column were significantly different ($p<0.05$) from each other

and Biesaga *et al.* (2007) reported the process of acid hydrolysis, leading to decreased flavonoid content. Moreover, significant decrease in the phenolic content by alkaline hydrolysis in comparison with control might also be due to the loss of phenolic glycosides by alkaline treatment. Similar findings were also corroborated by Krygier *et al.* (1982) and Nardini *et al.* (2002) in which they mentioned alkaline hydrolysis lead to the loss of important phenolic acids such as caffeic acid, dihydrocaffeic acid and homogentisic acid and they suggested the addition of ascorbic acid and ethylenediaminetetraacetic acid (EDTA) during alkaline hydrolysis to prevent degradation of phenolic acids. In addition, Hertog *et al.* (1992) reported that hydrolysis was responsible for the degradation of flavonols present in plant material but not the standard flavonols. Hence, the presence of some unknown phytoconstituents in the plant material accelerating the degradation of flavonols might not be ruled out.

Four *in vitro* antioxidant methods including, DPPH, ABTS assay, FRAP and total antioxidant capacity assays were used to evaluate the antioxidant activity of *T. catappa* leaf hydrolysed as well as non hydrolysed extracts. Antioxidant activity of these extracts were expressed as mg VCEAC g^{-1} extract as this method found to be simple and reliable to measure antioxidant capacity of foods and herbs (Kim *et al.*, 2002). Moreover, antioxidant activity measured in terms of mg VCEAC g^{-1} extract is more meaningful and descriptive as the results provide a direct comparison of antioxidant activity of the sample with vitamin C.

DPPH and ABTS radicals are the two stable radicals with high level of sensitivity used for the evaluation of antioxidant potency of numerous pure compounds, fruit juices and plant extracts (Arnao, 2000; Re *et al.*, 1999; Rapisarda *et al.*, 1999). Hence these two radicals were used to investigate the antiradical activity of hydrolysed extracts of *T. catappa* leaves. Result of DPPH radical scavenging assay showed that significant ($p<0.05$) radical scavenging activity was found in sample 3 followed by sample 1 and sample 2 with the value of 906.67±20.56, 482.43±13.69 and 102.86±5.43 mg VCEAC g^{-1} extract respectively (Table 2). As it is evident from the Table 2, even the ABTS radical scavenging assay shown the similar trend of DPPH assay. Sample 3 possessed significantly high ($p<0.05$) antioxidant valued followed by sample 1 and sample 2 with the value of 816.39±7.61, 690.62±8.33 and 109.54±7.83 mg VCEAC g^{-1} extract. This shows that more antioxidant components are present in sample 1 and 2 which could promptly react with DPPH radicals either by donating hydrogen or electron to form stable radical.

FRAP and total antioxidant capacity assays were based on the reduction of ferric ion to ferrous and Mo(VI) to Mo(V) by the antioxidants in the samples respectively. Stronger absorbance value indicates the higher antioxidant activity. As evident from Table 2, sample 3 showed significantly high ($p<0.05$) VCEAC value followed by sample 1 and 2 with the antioxidant value of 219.04±0.28, 120.12±0.09, 50.69±0.18 and 366.23±1.86, 291.81±4.72 and 80.90±3.46 mg VCEAC g^{-1} extract for FRAP and total antioxidant capacity assays respectively. The variation in the polyphenolic constituents might be influencing the antioxidant values of these extracts.

The difference in the results of FRAP, total antioxidant capacity, DPPH and ABTS radical scavenging assay might be because these assays acts by different mechanism. FRAP and total antioxidant capacity assays are based on electron transfer reaction where as, DPPH and ABTS radical scavenging assays are based on electron transfer as well as hydrogen atom transfer reactions (Prior *et al.*, 2005). Hence, similar antioxidants present in plant extracts might react differently in different antioxidant assays. It is evident from the results that extracts obtained from different hydrolysis techniques exhibited varying degrees of polyphenolic content and antioxidant activities.

Table 3 summarise the correlation between the polyphenolic contents present in the hydrolysed and non hydrolysed extracts with the VCEAC values of four different antioxidant activities. The polyphenolic components present in the extracts obtained by different methods of hydrolysis exhibited good correlation with ABTS radical scavenging assay, total antioxidant capacity assay, DPPH assay and FRAP assay. In addition, flavonoid content presented a significant correlation with all the antioxidant activities. This confirms that

Table 2: DPPH and ABTS radical scavenging activity, FRAP and total antioxidant capacity of *T. catappa* extracts

Samples	DPPH assay[1]	ABTS assay[1]	FRAP assay[1]	Total antioxidant capacity assay[1]
1	482.43±13.69b	690.62±8.33b	120.12±0.09b	291.81±4.72a
2	102.86±5.43a	109.54±7.83a	50.69±0.18a	80.90±3.46a
3	906.67±20.56c	816.39±7.61c	219.04±0.28b	366.23±1.86b

[1]mg vitamin C equivalent antioxidant capacity (VCEAC)/g extract. Values were expressed as the Mean±SD. Values with different alphabets in the same column were significantly different ($p<0.05$) from each other

Table 3: Correlation between VCEAC values of antioxidant activities and total phenolic, flavonoid and tannin contents

Antioxidant assays	Correlation R^2		
	Total phenolic	Total flavonoid	Total tannin
VCEAC of DPPH assay	0.8959	0.9431	0.8895
VCEAC of ABTS assay	0.9956	0.9735	0.9298
VCEAC of FRAP assay	0.8517	0.9069	0.8697
VCEAC of total antioxidant capacity assay	0.9984	0.9911	0.9556

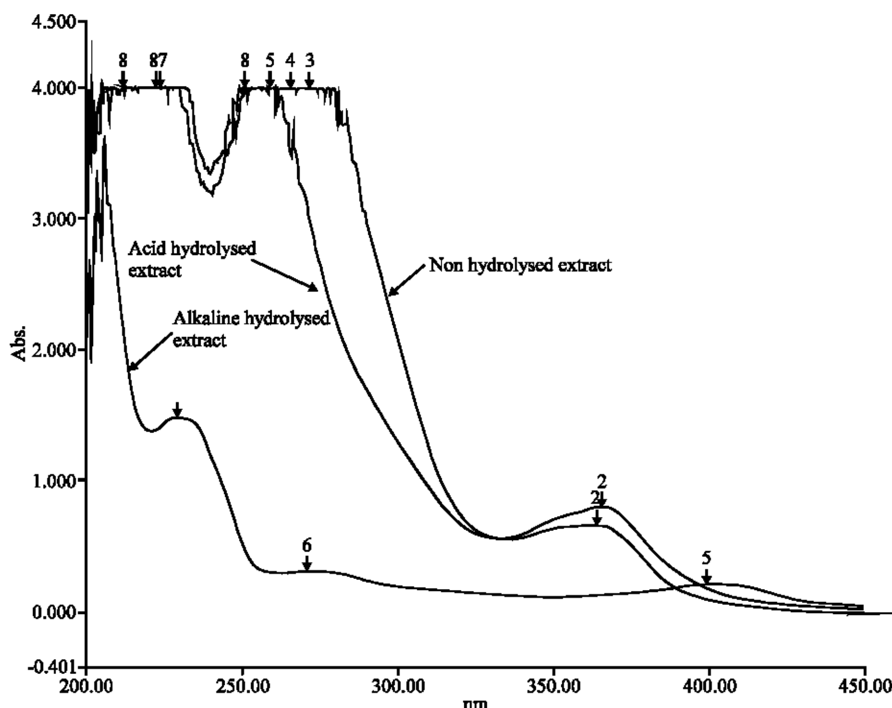

Fig. 1: UV spectra of nonhydrolysed and hydrolysed extracts of *T. catappa*

polyphenols are likely to contribute to the antioxidant activity of hydrolysed and non-hydrolysed extracts. Our results are in agreement with earlier reports that the polyphenolic components contribute significantly for the antioxidant activity of some medicinal plants (Kim *et al*., 2003; Bouayed *et al*., 2007).

UV-Visible spectrum obtained for the plant extracts helps in the identification of many phytoconstituents. The position of wave length maxima (λ_{max}) helps in the identification of unknown complex phytoconstituents present in the plant extracts (Harborne, 1998). Sample 3 shown λ_{max} at 364 nm, 272 nm, 265 nm, 259 nm 227 nm and sample 1 shown λ_{max} at 366 nm, 251 nm, 222 nm where as, sample 2 shown λ_{max} 664, 399, 270 and 229 nm (Fig. 1). Yellow flavonols shows intense peak between 350 to 390 nm in visible range and 250 to 270 nm in UV range even some of the phenolic acid such as gallic acid, p-hydroxyl benzoic acid, procatechuic acid and o- coumaric acid shows the maximum absorption at 272, 265, 260 and 227 nm respectively (Harborne, 1998). It is evident from UV-Visible spectrum (Fig. 1) that sample 3 might contain significant amount of flavonols and some of the phenolic acids as it shown the respective λ_{max} for them. But, number of absorption peaks for sample 1 was comparatively less than sample 3 where as, in sample 2 the absorption peaks responsible for these phytoconstituents are absent. Hence, spectral result has also supported the result of the phenolic and antioxidant assays where in sample 3 has highest phenolic content and antioxidant activity followed by sample 1 and 2.

CONCLUSION

Phenolic glycosides present in fruits, vegetables and medicinal plants are thought to have potential health benefits due to their antioxidant property. The bioactivity may be ascribed to aglycone moiety and not to the sugar moiety. Moreover, the antioxidant activity of phenolic glycosides is mainly due to catechol structure in the aglycones (Bors *et al*., 1990; Murota and Terao, 2003).

Acid and alkaline hydrolysis helps in obtaining extracts enriched with aglycone moieties from phenolic glycosides. We conclude from our study that the antioxidant activity of hydrolysed extract of *T. catappa* leaf is less than the non hydrolysed extract. Hence, this study confirms that there may not be any potential benefits of hydrolysis to obtain an extract enriched with antioxidant compounds. However, the strong correlation between polyphenolic content and antioxidant values confirmed the involvement of phenolic compounds in the antioxidant activity of *T. catappa* leaf extract. We even recommend to use a control while performing acid or alkaline hydrolysis in order to evaluate the advantage of

hydrolysis. However, this study can not be generalised to all plants as different plants contains different phytoconstituents in varying amount.

Currently studies are underway to optimise the acid and alkaline hydrolysis to obtain antioxidant rich extracts and HPLC studies to find out the presence of components in non hydrolysed and hydrolysed extracts.

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