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Antioxidant Properties and Toxicity Assessment of the *Crescentia cujete* Extracts in Brine Shrimp (*Artemia salina*)

(Sifat Antioksidan dan Ketoksikan Penilaian ekstrak *Crescentia cujete* pada Udang Air Garam (*Artemia salina*)

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ABSTRACT

Crescentia cujete has traditionally been used to treat various ailments. The present study attempted to determine the antioxidant activities of 100% ethanol, 50% ethanol and aqueous extracts of the leaves, bark and fruit of the herb. Additionally, the toxicity of the extracts was investigated in brine shrimp. The results showed that 100% ethanol leaf extract had the highest antioxidant activity with an IC_{50} value of $261.97 \pm 0.57 \mu\text{g/mL}$ according to the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging assay. Estimation of the total phenolic content (TPC) using the Folin-Ciocalteu reagent showed that the leaves extracted with 100% ethanol had the highest concentration of TPC compared to the extracts prepared with other solvents of all parts of the plant. Analysis of the minerals using inductively coupled plasma atomic emission spectroscopy (ICP-OES) showed that the parts of the plant primarily consist of high levels of phosphorus, magnesium and calcium. Titration analysis of vitamin C showed that the highest concentration of the vitamin is present in the bark. High performance liquid chromatography (HPLC) analysis of vitamin E indicated that the content is the highest in the fruit, and the content of vitamin A was the highest in the leaves. Brine shrimp lethality assay (BSLA) results showed that 50% ethanol extract of the leaves is the most toxic during a 24 h treatment. Thus, the leaves and bark exhibit excellent antioxidant effects and can be potentially developed as functional food ingredients. The findings of the present study suggest further research in cell lines and in vivo.

Keywords: Calabash; minerals; polyphenol; toxicity; vitamins

ABSTRAK

Crescentia cujete telah digunakan secara tradisi untuk merawat pelbagai penyakit. Kajian ini bertujuan untuk menentukan aktiviti antioksidan terhadap 100% etanol, 50% etanol dan akues ekstrak daun, kulit kayu dan buah. Di samping itu, penilaian ketoksikan ekstrak ke atas udang brin juga dikaji. Keputusan menunjukkan daun yang diekstrak dengan 100% etanol mempunyai aktiviti antioksidan yang paling tinggi dengan nilai IC_{50} $261.97 \pm 0.57 \mu\text{g/mL}$, diuji menggunakan ujian esei radikal 2,2-diphenil-1-picrylhidrazil (DPPH). Penilaian jumlah kandungan fenolik (TPC) menggunakan reagen Folin-Ciocalteu menunjukkan bahawa daun yang diekstrak menggunakan 100% etanol mempunyai kepekatan tertinggi berbanding dengan ekstrak bahagian tumbuhan lain yang disediakan dengan pelarut lain. Analisis mineral menggunakan spektrometer pancaran optik-plasma gandingan aruhan (ICP-OES) menunjukkan bahawa semua bahagian tumbuhan yang telah diuji mengandungi fosforus, magnesium dan kalsium yang tinggi. Titrasi vitamin C telah menunjukkan kandungan tertinggi dalam kulit. Selain itu, analisa kromatografi cecair berprestasi tinggi (HPLC) vitamin E menunjukkan kandungan tertinggi dalam buah sementara vitamin A adalah paling tinggi dalam daun. Keputusan analisis kemautan udang brin (BSLA) menunjukkan bahawa ekstrak daun dengan menggunakan etanol 50% adalah yang paling toksik semasa pengujian selama 24 jam. Kajian ini dengan jelas menunjukkan bahawa daun, kulit dan buah *Crescentia cujete* adalah toksik dan bioaktif. Oleh itu, daun dan kulit mengandungi antioksidan dan berpotensi untuk dibangunkan sebagai bahan makanan fungsian. Keputusan kajian ini mencadangkan kajian lanjut dalam lapisan sel dan in vivo.

Kata kunci: Ketoksikan; labu kayu; mineral; polifenol; vitamin

INTRODUCTION

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen that are mainly produced by the mitochondrial electron transport chain (ETC) as by-products of the common cellular metabolism (He et al. 2017). ROS are small but highly reactive. ROS play important roles in such cellular functions as communication and homeostasis; however, at elevated levels, ROS may harm human health (Gorlach et al. 2015). The balance of

ROS in the body is commonly regulated by the antioxidant capacity of a cell. Interference that alters this balanced state results in a pro-oxidant state known as oxidative stress. Oxidative stress can cause a number of negative effects in the human body especially due to chemical reactions of ROS with the lipids and proteins due to the highly reactive nature of ROS (Gorlach et al. 2015). Some of the negative effects include cardiovascular diseases, cancer, neurodegeneration, diabetes, and ageing, cataract

(Morell 2008; Ohta 2012; Poljsak et al. 2013). Therefore, maintenance of the normal cellular ROS concentration is critical to ensure the regulation of normal physiological functions throughout the body. However, the formation of these reactive species can be counteracted by consuming sufficient amounts of exogenous antioxidants (Poljsak et al. 2013).

An antioxidant is any substance that delays, prevents and inhibits oxidative damage to the target molecules. Antioxidants can repair the injuries caused by oxidative stress by acting as free radical scavengers, inhibiting the initiation or propagation of the oxidative chain reactions and quenching the singlet oxygen (Xu et al. 2017). The main sources of exogenous antioxidants include food and medicinal plants, such as spices, fruits and vegetables (Xu et al. 2017). The natural compounds from these sources are rich in vitamins (A, C and E), minerals (selenium) and carotenoids, such as β -carotene, lycopene, lutein and polyphenols (lignans, phenolic acids, anthocyanins, flavonoids and stilbenes). These compounds have proven antioxidant activities that help to strengthen the mechanisms of the defence against damage caused by 'oxidative stress' (Wasek et al. 2014; Xu et al. 2017). Natural antioxidants have been the main choice for consumers due to toxicological reasons because synthetic antioxidants may lead to a number of health hazards (Taghvaei & Jafari 2015). Therefore, numerous studies are focusing on the search, characterization and application of natural antioxidants.

Crescentia cujete is called 'labu kayu' by local population in Malaysia. This herb is one of the thousands of plants that were traditionally used to heal numerous ailments. The parts of the plant, especially fruit, leaves and bark, are usually boiled in water or alcohol before being used to treat diseases. Several studies had supported the traditional application of the parts of this plant and proved that various parts of the *Crescentia cujete* tree possess numerous health benefits, such as anti-inflammatory, antibacterial, DPPH radical scavenging, antioxidant, cytotoxic, antivenom, central nervous system depressant and wound healing activities (Dawodu et al. 2016). Moreover, Das et al. (2014) has reported that the leaves of this plant have high potential for development as a natural antioxidant. However, there are lack of studies using ethanol extracts which are excellent for the extraction of antioxidant polyphenols. Therefore, a comprehensive study of the efficacy of the water and ethanolic extracts in scavenging free radicals and of the toxicity level of the extracts should be performed. The results will deliver ample information related to the benefits and safety of the extracts in domestic consumption. Development of this plant as one of the potent natural antioxidant sources can be considered an expansion of the medical, nutraceutical and functional food industry. Additionally, determining the lethal concentration of the extracts may provide a proper guidance for safe consumption of the parts of this plant.

MATERIALS AND METHODS

SAMPLES

Fresh leaves, bark and fruit of *Crescentia cujete* were harvested from the garden trees at Sitiawan, Perak, Malaysia. The plant materials were ensured to be free of physical and microbial damage. The identification of the samples was conducted by a botanist from Institute of Bioscience, Universiti Putra Malaysia. The specimen voucher number is SK 3136/17.

MATERIALS

Ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu phenol reagent, gallic acid, anhydrous sodium carbonate and 2,6-indophenol standard solution were purchased from Merck (Darmstadt, Germany). Potassium dichromate, ascorbic acid, metaphosphoric acid, anhydrous sodium sulphate, α -tocopherol standard solution, β -carotene standard solution, acetic acid, acetone, ethyl acetate, hexane, hydrochloric acid, methanol, nitric acid, petroleum ether, phenolphthalein, potassium hydroxide, sodium chloride and sodium hydroxide were of analytical and HPLC grades and were obtained from Sigma-Aldrich (New York, USA).

SAMPLE PREPARATION

The method was adapted from Vuthijumnok et al. (2013) with some modifications; the deep freezing temperature was decreased from -20°C to -80°C . All the plant materials were initially cleaned under running tap water and the pulps were cut into cubes ($2 \times 2 \times 2$) cm^3 . The cut fruits were immediately packed into a plastic zip lock bag to prevent oxidation and were deep frozen at $-80^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for at least 24 h. The deep-frozen samples were then freeze-dried at -50°C for at least 3 days until they were completely dry using a freeze-dryer (LABANCO, Labanco Corporation, Kansas City, Missouri, USA) at 0.120 mBar. The freeze-dried samples were then blended using a blender (HR 2001, Philips, Selangor, Malaysia) and sieved to obtain a fine powder. The samples were kept at 4°C in an air tight container until further analysis.

SAMPLE EXTRACTION

The extraction method was adapted from Huda-Faujan et al. (2015) with some modifications; the ratio of the samples to the solvents was increased from 1:7 (w/v) to 1:10 (w/v). The solvents (50% ethanol, 100% ethanol and 100% distilled water) were added into the samples at the ratio of 1:10 (w/v) and the mixtures were stored in the dark condition for 72 h. Then, the extracts were filtered using a Buchner funnel through a Whatman No. 1 filter paper and the residual solvent was evaporated using a rotary evaporator (N-100, Eyela, Tokyo, Japan) with an aspirator (A-1000S, Eyela, Tokyo, Japan) at 75°C and 50 rpm rotation. The crude extracts obtained were then

weighed. The yield percentage was calculated using the following equation:

$$\% \text{ Yield} = \frac{W_2 - W_1}{W_0} \times 100,$$

where W_2 is the weight of the extract and the container; W_1 is the weight of the container alone; and W_0 is the weight of the freeze-dried sample.

DETERMINATION OF THE TOTAL PHENOLIC CONTENT

This method was adapted from Singleton and Rossi (1965) with some modifications; the extracts were dissolved using the corresponding extraction solvents instead of using methanol. Standard gallic acid solutions (3.125 ppm-100 ppm) were prepared to construct a standard calibration curve. Folin-Ciocalteu reagent (2.5 mL of 10%) and 2 mL of 7.5% sodium were added into 0.5 mL of plant extract. The mixtures were vortexed using a vortex (LMS-1003, Genius 3, Gyeonggi-do, KOREA) for 10 s and incubated in an incubation oven (100-800, Memmert, Schwabach, German) at 40°C for 1 h in the dark. The absorbance was measured at 756 nm using a spectrophotometer (UV-1650PC, Shimadzu, Kyoto, Japan). The results were expressed as $\mu\text{g GAE/g}$ extract.

DPPH RADICAL SCAVENGING ACTIVITY

The method was adapted from Jing et al. (2015) with some modifications; the concentration of DPPH solution was increased from 100 μM to 500 μM and the absorbance was measured at 515 nm instead of 517 nm. The samples (31.25 $\mu\text{g/mL}$ -500 $\mu\text{g/mL}$) or positive control (ascorbic acid) methanol solution (100 μL) were added to 100 μL of 500 μM DPPH methanol solutions (5.9 mg/100 mL methanol) in a 96-well microplate. The remaining DPPH radicals were determined by the absorbance at 515 nm using a microplate reader (EL800, BioTek, Vermont, USA) after incubation at room temperature for 30 min. Scavenging activities were determined by the following equation:

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

The IC_{50} value obtained by extrapolation using linear regression analysis corresponds to the concentration of the sample required to scavenge 50% of the DPPH radicals.

MINERAL ANALYSIS

Fresh leaves, fruit and bark were used for mineral analysis. The sample (0.1 g) was weighed into a vessel. Then, 10 mL of HNO_3 and 5 mL of HCl were added into a vessel. The vessel was placed into a microwave digester for 1 h. After 1 h, the sample was cooled, and the volume was adjusted to 45 mL with 1% HNO_3 . The samples were analysed using an ICP-OES system (5300 DV, Perkin Elmer, Massachusetts,

USA). Calculations were performed according to the following equation:

$$\% \text{ Element} = \frac{\text{Reading from ICP-OES} \times \text{Dilution} \times \text{Volume (mL)}}{\text{Weight of test material in g} \times 10\,000} \times 100,$$

DETERMINATION OF ANTIOXIDANT VITAMINS

VITAMIN A

The method was adapted from AOAC Official Method (AOAC 2005). Eighty millilitres of 95% ethanol was added to a flask containing 20 g samples followed by 20 mL of 20% potassium hydroxide and a few boiling chips. Then, the flask was attached to a water-cooled refluxing apparatus, and the samples were heated with refluxing for 30 min. After that step, the unit was cooled down to room temperature. Then, the hydrolysates were extracted three times with 50 mL of hexane. The hexane extracts were combined and washed with distilled water until the solution was neutral according to phenolphthalein. The hexane extracts were filter-washed using anhydrous sodium sulphate. The volumes of the extracts were reduced using a rotary evaporator. The residues were immediately re-dissolved in 5 mL mobile phase (methanol) and transferred to a 10 mL volumetric flask. The volume was adjusted to the mark with the mobile phase. The extracts were filtered using a 0.45 μm membrane filter (PTFE). Twenty microliters of the extract was injected into an HPLC system (2695 & 996, Waters Alliance, California, USA) for analysis. Beta-carotene was used as the standard solution. Vitamin A content was calculated according to the following equation:

$$\beta\text{-carotene, ppm} = \frac{C \times 10}{W_s}$$

where C is the concentration according to the standard curve (ppm); and W_s is the sample weight (g).

VITAMIN C

The method was adapted from AOAC Official Method (AOAC 1990). Fresh samples were used for this analysis. Freshly prepared ascorbic acid solution was used as the standard. Approximately 10 g of the sample and metaphosphoric acid-acetic acid solution were added into a conical flask, and the volume was adjusted to 200 mL. Then, the filtrates were titrated with standardized indophenol reagent until pink colour remained for at least 5 s, and the results are expressed as mg ascorbic acid per 100 g sample according to the following equation:

$$\text{mg ascorbic acid per 100 g sample} = X \times A \times \frac{V}{Y} \times \frac{100}{W}$$

where X is the volume of indophenol used to titrate the sample (mL); A is the volume of indophenol used to titrate the ascorbic acid standard (equivalent to mg of ascorbic acid present in the sample) (mL); V is the total volume

(mL) of the test sample; Y is the total volume (mL) of the sample used in titration to pink colour (10 mL); and W is the weight of the sample (g).

VITAMIN E

The method was adapted from AOAC Official Method (AOAC 1990). Ten grams of the sample, 50 mL of 95% ethanol, 50% potassium hydroxide and 0.25 g of ascorbic acid were added into a 250 mL boiling flask. The flask was then attached to a water-cooled reflux apparatus. After 30 min heating, the unit was cooled to room temperature. Then, the solutions were transferred into a separating funnel, and the flask was rinsed with 50 mL distilled water. The extraction was performed using 25 mL petroleum ether with vigorous shaking. The upper layer contained the petroleum ether extract and was collected by removing the lower layer into a beaker. The extraction step was repeated twice by adding 25 mL of ether. Finally, the ether extracts were combined and washed with distilled water until the solution was neutral according to phenolphthalein. Then, the extracts were filter-washed using anhydrous sodium sulphate and evaporated using nitrogen gas. The extracts were diluted with methanol and filtered through a 0.45 µm membrane filter (PTFE). Ten microliters of the extract were injected into an HPLC system (385-LC, Varian, Shropshire, UK) for analysis. Vitamin E (α -tocopherol) was used as the standard solution, and the samples were spiked with 0.5 µL of the stock standard solution. The column was reversed-phase C18 (5 µm; 4.6 × 150 mm ID) attached to a fluorescence detector with excitation wavelength of 296 nm and emission wavelength of 330 nm. Flow rate was 1.0 mL/min using the gradient pump mode. Vitamin E content was calculated according to the following equation:

$$\text{Vitamin E, mg/kg} = \frac{C \times 10}{W_s}$$

where, C is the concentration according to the standard curve (ppm); and W_s is the sample weight (g).

BRINE SHRIMP LETHALITY TEST

The method was adapted from Syahmi et al. (2010) with some modifications; the extracts were dissolved using the corresponding extraction solvents instead of methanol and petri dishes were used for 24 h treatment instead of bijoux bottles. Brine shrimp eggs, *Artemia salina* was hatched and used to test the potential toxicity of the samples' crude extracts. By referring to the Meyer's toxicity index, the crude extracts with $LC_{50} < 1000$ µg/mL were considered toxic, and the crude extracts with $LC_{50} > 1000$ µg/mL were considered non-toxic (Hamidi et al. 2014).

ARTEMIA SALINA LETHALITY ASSAY

The sample extracts were dissolved using the corresponding extraction solvents and diluted with artificial seawater (3.8% NaCl solution). The sample extracts were serially

diluted with artificial seawater (1.953 µg/mL - 1000 µg/mL). Twenty millilitres of the solution of each concentration was transferred into a corresponding Petri dish and incubated for 24 h. After that step, potassium dichromate ($K_2Cr_2O_7$) was dissolved in artificial seawater, and the solution was used as a positive control with concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.907, and 1.953 µg/mL. Negative control was prepared using ethanol and distilled water. The Petri dishes were observed, and the dead larvae from each Petri dish were counted after 24 h. Based on the mortality percentage, the concentration that caused 50% lethality (LC_{50}) of the nauplii was determined by using the plot of the mean mortality percentage versus the log of concentration.

$$\text{Percentage of death (\%)} : \frac{(\% \text{ Death in the test plate}) - (\% \text{ Death in the control plate})}{100 - \% \text{ Death in the test plate}} \times 100\%$$

STATISTICAL ANALYSIS

The results are expressed as the mean of the triplicates ± standard deviation. Significant difference was set at the 95% confidence level. All analyses were conducted by Tukey's test using the Minitab 16 software.

RESULTS AND DISCUSSION

CONTENTS OF MINERALS AND ANTIOXIDANT VITAMINS IN *C. cujete*

The mineral and vitamin contents in different parts of *C. cujete* are presented in Table 1. The bark contains the highest level of sodium, phosphorus, zinc and copper followed by the leaves and fruit. Conversely, the leaves had the highest amount of calcium, magnesium, manganese, iron and selenium. This finding is consistent with the data of Ejelonu et al. (2001), who reported the very low levels of calcium and magnesium in the fruit (4 mg/100 g and 0.01 µg/mL, respectively). Similarly, Olusi et al. (2015) found exceptionally high levels of calcium, phosphorus, magnesium and iron in the bark (2267.24 mg/100 g, 56.64 mg/100 g, 216.65 mg/100 g and 89.84 mg/100 g, respectively). However, in contrast to the earlier findings, low levels of manganese and sodium were found in the bark of *C. cujete* at 4.36 mg/100 g and 0.07 mg/100 g, respectively (Olushi et al. 2015). The recommended nutrient intake (RNI) for healthy adult Malaysian men and women 19-50 year of age is 1000 mg per day (MOH 2017). Therefore, *C. cujete* leaves may provide for the daily calcium needs by consumption of less than 300 g leaves. Certain nutrients serve as antioxidants or are the components of the antioxidant enzymes, such as selenium, copper, zinc, manganese and iron. Deficiency of these elements causes oxidative stress and damage to the biological molecules and membranes which will occur when these elements are not consumed in sufficient amount (McDowell et al. 2007). In general, the leaves, bark and fruit of *C. cujete* had different composition of the nutrients,

and the nutrient contents are significantly ($p < 0.05$) higher in the leaves. The distribution and allocation of the nutrients in the leaves, bark and fruit indicate the capacity of the plants to obtain, transport and store the nutrients (Zhang et al. 2015). However, the mineral distribution in the parts of a plant may vary because of climate, soil composition or nutrient level, harvest time, genotypes, preparation method and maturity level (San et al. 2009).

Vitamin analysis showed that the fruit contains the highest amount of vitamin E; the bark had the highest level of vitamin C; and the leaves contain the highest amount of vitamin A. Vitamin E is a fat-soluble vitamin well known for its high antioxidant potency (Ye et al. 2013). The vitamin E contents in *C. kujete* were relatively low in all parts of the plant ($p < 0.05$). Additionally, the amount of vitamin C is significantly ($p < 0.05$) different between the parts of the plant. However, when the vitamin C levels were compared in a study of Ogunlesi et al. (2010), it was demonstrated that the vitamin C contents in *C. kujete* were lower than that in the tropical vegetables. In their study, the samples identified as rich in vitamin C included red pepper (123.73 mg/100 g) and curry plant (140.50 mg/100 g). The recommended nutrient intake (RNI) of vitamin C for healthy Malaysian men and women are 70 mg per day (MOH 2017). *C. kujete* contains relatively low amount of vitamin C considering the daily human needs because a larger serving is required. Vitamin A is best known for its excellent antioxidant properties and is the best quencher

of the singlet oxygen (Fiedor & Burda 2014). *C. kujete* contains a reasonable amount of vitamin A in the leaves (22.94 ± 0.32 mg/100 g), bark (14.47 ± 0.08 mg/100 g) and fruit (0.15 ± 0.00 mg/100 g) ($p < 0.05$). In this study, the amount of vitamin A was higher than that in other studies. Igwenyi and Elekwa (2014) reported the vitamin A content in a traditional herb, *Geranium robertianum*, of 1.44 ± 0.02 mg/100 g; therefore, the herb can be a good source of vitamin A. Hence, the leaves and bark of *C. kujete* can serve as an excellent source of vitamin A.

QUANTIFICATION OF TOTAL PHENOLIC AND ANTIOXIDANT ACTIVITY

The fruit, leaves and bark of *C. kujete* were freeze-dried and extracted using 100% ethanol, 50% ethanol and water (aqueous). Ethanol and water are less toxic and safer than other solvents, such as hexane, acetone and methanol (Chew et al. 2011). Table 2 presents the extraction yield, total phenolic content and the IC₅₀ values of the fruit, leaf and bark extracts of *C. kujete*. The results indicate that the extraction yield is the highest in the fruit followed by the leaves and bark ($p < 0.05$). Additionally, the results showed that water is the most effective solvent for extraction of the compounds from the *C. kujete* fruit, whereas 50% ethanol is the most effective solvent for extraction of the compounds from the *C. kujete* leaves and bark. A possible explanation for this may be that the fruit is

TABLE 1. Content of minerals and vitamins in fruit, leaves and bark of *Crescentia kujete*

	Content			Adult RNI (man, woman)*	Adult DRIs (man, woman)#
	Fruit	Leaves	Bark		
Mineral					
Sodium (mg/100 g)	9.03 ± 2.75 ^b	8.03 ± 0.89 ^b	18.20 ± 0.62 ^a	(1500, 1500) mg/d	(2300, 2300) mg/d
Phosphorus (mg/100 g)	40.36 ± 5.32 ^b	38.64 ± 2.90 ^b	60.53 ± 0.12 ^a	(700, 700) mg/d	(580, 580) mg/d
Calcium (mg/100 g)	4.50 ± 1.13 ^c	474.24 ± 32.19 ^a	268.84 ± 12.42 ^b	(1000, 1000 for 19-50; 1200 for 51-59 y) mg/d	(1000, 1000) mg/d
Magnesium (mg/100 g)	19.79 ± 2.68 ^c	75.10 ± 6.10 ^a	45.39 ± 0.55 ^b	(420, 310 for 19-29 y; 320 for 30-50 y; 420 for 51-59 y) mg/d	(420, 320) mg/d
Manganese (mg/100 g)	0.09 ± 0.02 ^c	0.61 ± 0.04 ^a	0.25 ± 0.03 ^b	(2.3, 1.8) mg/d	(2.3, 1.8) mg/d
Iron (mg/100 g)	0.29 ± 0.11 ^c	1.56 ± 0.12 ^a	0.78 ± 0.11 ^b	(14, 29 for 19-50 y; 11 for 51-59) mg/d	(45, 45) mg/d
Copper (µg/100 g)	200.00 ± 0.04 ^b	310.00 ± 0.04 ^b	560.00 ± 0.10 ^a	(900, 900) µg/d	(10,000, 10,000) µg/d
Zinc (mg/100 g)	0.37 ± 0.09 ^b	0.57 ± 0.01 ^a	0.61 ± 0.10 ^a	(6.5, 4.7 for 19-29 y; 4.6 for 30-59) mg/d	(9.4, 6.8) mg/d
Selenium (µg/100 g)	1.13 ± 0.00 ^c	13.72 ± 0.01 ^a	5.87 ± 0.00 ^b	(32, 24) µg/d	(45, 45) µg/d
Vitamin					
Vit. A (mg/100g)	0.15 ± 0.00 ^c	22.94 ± 0.32 ^a	14.47 ± 0.08 ^b	(600,600) µg/d	(500,550) µg/d
Vit. C (mg/100g)	5.76 ± 0.78 ^c	7.95 ± 0.05 ^b	9.32 ± 0.00 ^a	(70,70) mg/d	(60,70) mg/d
Vit. E (mg/100g)	0.02 ± 0.00 ^a	0.003 ± 0.00 ^b	0.002 ± 0.00 ^c	(10, 7.5) mg/d	(12, 12) mg/d

Small letters indicate significant ($p < 0.05$) differences of means between the different plant parts. They are expressed in mean ± SD ($n = 3$). RNI = Recommended Nutrient Intake for Malaysia. DRI = Daily Reference Intake. *Values are based on people aged 19-59 years from Ministry of Health Malaysia (2017). #Values are based on people aged 19-70 years from National Institute of Health (2015)

composed of highly water-soluble molecules, including proteins and carbohydrates. Therefore, high polarity of water compared to other solvents explains greater ability of water to extract the polar compounds from the samples (Delfanian et al. 2015). However, water cannot extract the compounds with high number of the carbon bonds. Hence, mixing ethanol and water at a suitable extraction coefficient may assist with the extraction of the compounds with high number of the carbon bonds (Delfanian et al. 2015). The results obtained in the leaves and bark support this hypothesis. These two parts of the plant may have compounds with higher number of the carbon bonds.

Total phenolic content of *C. kujete* was quantified using the Folin-Ciocalteu assay. The concentration of TPC was expressed as gallic acid equivalent (GAE)/g extract. The results showed that the highest concentration of TPC was observed in the leaves extracted using 100% ethanol ($1281.10 \pm 0.33 \mu\text{g GAE/g extract}$). Comparison of the parts of the plant indicates that 100% ethanol extracted the highest amount of TPC compared with that in the case of water and 50% ethanol. Overall, the contents decrease in the following order: leaves (100% ethanol) > leaves (aqueous) > leaves (50% ethanol) > bark (100% ethanol) > fruit (100% ethanol) > bark (50% ethanol) > bark (aqueous) > fruit (50% ethanol) > fruit (aqueous). Das et al. (2014) evaluated the total phenolic content of the extracted *C. kujete* bark and leaves in crude ethanol extract, chloroform fraction, ethyl acetate fraction and water fraction. The ethanolic extract had the highest concentration of TPC compared to that in the aqueous extract of the leaves and bark in agreement with the results

obtained in this study. However, the concentration of TPC was significantly higher compared to that obtained in this study. Differences in results obtained in the same species can be attributed to differences in plant origin, procedures and standards used to assess the TPC content (Huda-Faujan et al. 2015). The results of our study indicate that the leaves contain the highest TPC levels followed by the bark and fruit ($p < 0.05$) (Table 2). This finding is most likely due to relatively higher abundance of unbound low molecular weight polar phenolic compounds in the leaves compared to that in the bark and fruit (Ghazi et al. 2012; Russo et al. 2013). Moreover, bark and fruit usually contain high levels of structural polysaccharides that bind high proportion of polyphenols thus masking their detection in the assay of phenolic compounds (Perez-Jimenez & Saura-Calixto 2005).

The radical scavenging properties of *C. kujete* fruit, bark and leaves were evaluated using the DPPH assay. This assay has high reproducibility and sensitivity (Buenger et al. 2006). DPPH radical scavenging activities of 100% ethanol, 50% ethanol and aqueous extracts of the fruit, leaves and bark of *C. kujete* were tabulated and compared. Figure 1 shows that each part of the plant has variable scavenging activities depending on the solvent used for extraction. The results indicate that different solvents have different polarity, dispersibility and penetration thus resulting in selective extraction of various phytochemicals (Zhang 2015). Thus, the solvents used for extraction will affect the extraction efficiency of the target compounds and influence the scavenging activities of the extracts. The IC_{50} values of the extracts were calculated and the results are shown in Table 2. Lower IC_{50} values indicate

TABLE 2. Extraction yield, total phenolic content and IC_{50} values of fruit, leaves and bark extracts of *Crescentia kujete*

Sample	Extraction yield (%)	Total Phenolic (mg GAE/ 100 g extract)	IC_{50} ($\mu\text{g/mL}$)
Fruit			
Aqueous	86.05 ± 4.24^a	8.56 ± 1.47^c	ND
50% Ethanol	75.87 ± 2.18^a	16.34 ± 0.53^b	ND
100% Ethanol	16.69 ± 0.43^b	166.12 ± 3.81^a	ND
Leaves			
Aqueous	10.4 ± 0.06^b	1215.86 ± 0.32^b	296.88 ± 1.77^b
50% Ethanol	28.29 ± 0.54^a	672.94 ± 0.32^c	272.80 ± 1.48^c
100% Ethanol	8.12 ± 0.30^c	1281.10 ± 0.33^a	261.97 ± 0.57^d
Bark			
Aqueous	8.17 ± 0.05^b	91.94 ± 5.79^c	655.83 ± 16.04
50% Ethanol	12.54 ± 0.46^a	146.96 ± 3.66^b	766.53 ± 14.37
100% Ethanol	2.75 ± 0.12^c	278.68 ± 1.53^a	474.88 ± 1.35^a
Ascorbic acid ^b			27.74 ± 0.03^c

Small letters indicate significant ($p < 0.05$) differences of means between the extracting solvent. They are expressed in mean \pm SD ($n = 3$). IC_{50} = The effective concentration providing 50% scavenging effect. ND = not detected. The IC_{50} value of the fruit extracts unable to be determined within the tested concentration (in the range of 0 - 3 mg/mL)

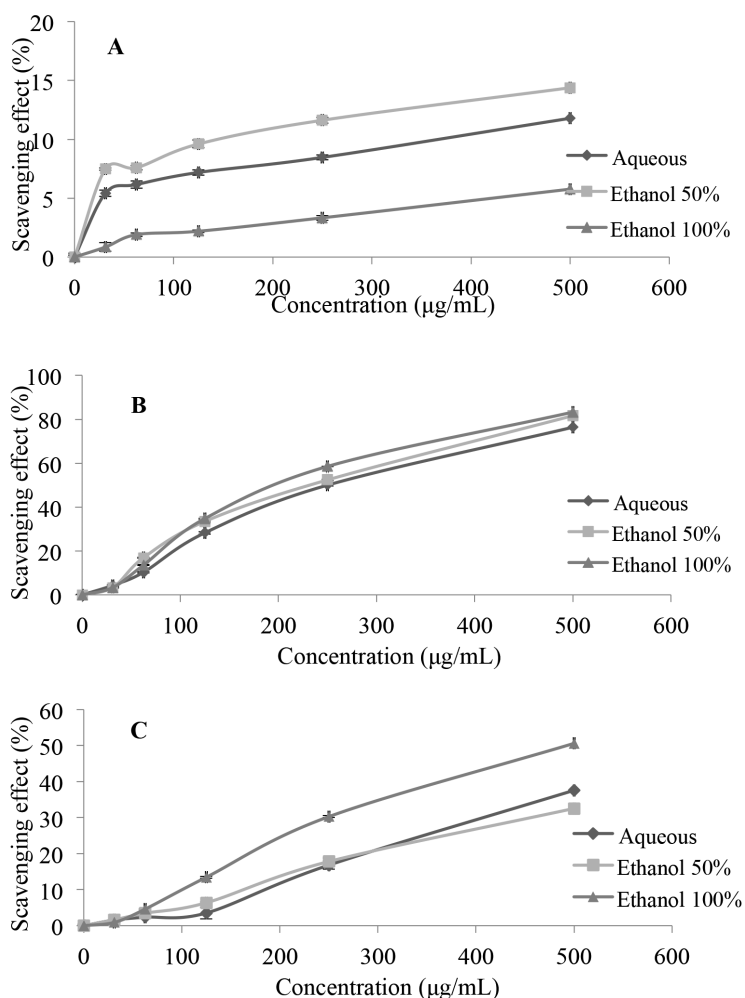
lower concentration needed to scavenge 50% of DPPH free radicals. As shown in Table 2, only leaf and bark extracts had the IC_{50} within the test concentrations. None of the fruit extracts have achieved the IC_{50} (Table 2). The strongest radical scavenging was shown by the *C. kujete* leaves extracted using 100% ethanol.

This finding contradicts the results of Das et al. (2014) who found that aqueous extract of the leaves had lower IC_{50} values indicating more potent radical scavenging compared to that of the ethanol extract. The contradictions between the results of these studies may be due to a number of factors including differences in the plant matrix and the processing conditions, such as temperature and time of extraction; moreover, all these factors may influence the antioxidant activities (Do et al. 2014). The scavenging activity of *C. kujete* leaf and bark extracts can be influenced by the presence of the compounds that are classified as free radical scavengers, such as flavonoid quercetin, apigenin, naphthoquinones, tannins and steroids (Das et al. 2014). Ethanol is effective in extracting the radical scavengers, such as phenolic compounds, with low and

medium molecular weight and medium polarity, including aglycones, flavonoids, anthocyanins, terpenoids, saponins, tannins, totarol, flavones, phenones and polyphenols (Widyawati et al. 2015). These results indicated that the leaves and barks contain the compounds with less polar properties and can be efficiently extracted by ethanol.

TOXICITY ASSESSMENT

Brine shrimp lethality assay (BSLA) is one of the preliminary cytotoxicity methods based on the ability to kill laboratory-cultured *Artemia nauplii*. BSLA is an efficient, fast and less expensive assay for testing the bioactivity of the plant extracts. Moreover, a positive correlation between the brine shrimp lethality and oral lethality test in mice had been demonstrated in various studies of medicinal plants (Naidu et al. 2014). The median lethal concentration (LC_{50}) of the brine shrimp lethality assay was determined in this study. Median lethal concentration (LC_{50}) is the lethal concentration required to kill 50% of the population after 24 h of exposure. The LC_{50} values calculated for



DPPH = 2, 2-diphenyl-1-(2,4,6-trinitrophenyl-hydrazyl)

FIGURE 1. DPPH radical scavenging properties of fruit, leaves and bark extracts of *Crescentia kujete*: (A) fruit, (B) leaves and (C) bark

TABLE 3. LC₅₀ value of ethanolic and aqueous extracts of *Crescentia cujete*

Samples	LC ₅₀ (µg/mL)		
	Fruit	Leaves	Bark
Aqueous	38.74 ± 1.35 ^c	5.49 ± 0.32 ^b	25.74 ± 1.35 ^a
50% Ethanol	133.15 ± 4.50 ^b	4.84 ± 0.32 ^c	26.07 ± 4.50 ^a
100% Ethanol	292.17 ± 0.00 ^a	11.83 ± 0.33 ^a	14.04 ± 0.00 ^b
Potassium Dichromate (Positive control)		124.41 ± 2.08 ^c	

Small letters indicate significant differences of means between the extracting solvents. They are expressed in mean ± SD ($n = 3$). LC₅₀ = the concentration of the plant extracts and positive control sufficient to obtain 50% of nauplii mortality of *Artemia salina*

the ethanolic and aqueous extracts of *C. cujete* are listed in Table 3. Crude extracts with the LC₅₀ values of less than 1000 µg/mL are considered significantly active and toxic while the extracts with the LC₅₀ value of more than 1000 µg/mL are considered non-toxic according to the Meyer's toxicity index. The LC₅₀ values of all extracts including potassium dichromate were less than 1000 µg/mL. Therefore, all parts of the plant extracted with three types of solvents are bioactive and toxic. Leaves are the most toxic part of *C. cujete* followed by the bark and fruit (Table 3). The bark extracts exhibited the highest toxicity in the case of the 100% ethanol extract (14.04 ± 0.00 µg/mL), whereas there is no significant ($p < 0.05$) difference between the 50% ethanol and aqueous extracts (26.07 ± 4.50 µg/mL and 25.74 ± 1.35 µg/mL, respectively). Conversely, the aqueous fruit extracts were the least toxic compared to those of the other parts of the plant followed by 50% ethanol and 100% ethanol extracts (38.74 ± 1.35 µg/mL, 133.15 ± 4.50 µg/mL and 292.17 ± 0.00 µg/mL, respectively). The leaf extracts are more bioactive and toxic, apparently due to the high content of the bioactive compounds in the leaves compared to those in the fruit and bark.

The phytochemical screening of the crude ethanol extracts of the *C. cujete* leaves showed the presence of steroids, saponins, alkaloids, tannins, glycosides, terpenes and flavonoids (Das et al. 2014). Conversely, Billacura and Pangcoga (2017) reported that the crude ethanolic and aqueous extracts of *C. cujete* contains trace levels of tannins and alkaloids. These data indicate the reason for low toxicity of the fruit extracts. Hence, at higher levels, the compounds become toxic (Almadin & Jumawan 2015). Assuming that these leaves are boiled in water in the traditional way of consumption, they can be toxic to the consumers even at lower concentrations. In a study of volatile constituents of the *C. cujete* leaves by Dawodu et al. (2016), toxic agents were not detected within the volatile fraction of the compounds. Therefore, the compounds cannot be lost in the air during boiling and toxicity levels of the leaves should remain the same. However, the findings of the current study do not support the previous studies. Billacura and Laciapaq (2017) and Billacura and Pangcoga (2017) found that the median lethal concentrations of the aqueous and ethanolic extract of the *C. cujete* fruit and leaves are lower compared to

those detected in this study. However, the toxicity of the aqueous extracts of *C. cujete* is higher than that of the 100% ethanolic extracts in agreement with this study. The differences can be due to different preparation methods of the samples; the samples were air dried in the study of Billacura and Pangcoga (2017), whereas the samples were freeze-dried in this study. Furthermore, the differences in the extraction methods can be only one of the factors. The information on the antioxidant activities and the toxicity assessment obtained in the present work provide a rationale for the medicinal use of the plant extracts. A more thorough study of the toxicity of *C. cujete* is recommended to further assess the efficacy and safety of this medicinal species. Better extraction methods with alternative solvents should be explored because this plant can be very beneficial in a number of ways due to the presence of the valuable antioxidant compounds.

CONCLUSION

The results of the current study indicate that the leaves of the *Crescentia cujete* plant are rich in minerals and antioxidant vitamins. The extracts of the *C. cujete* plant were able to scavenge free radicals, especially the extracts of the leaves and bark prepared in water or ethanol. However, the beneficial effects may vary depending on the solvent used for the extraction. The leaves extracted with ethanol contain the highest TPC and the lowest TPC was detected in the fruit extracted with water. The parts of the *C. cujete* plant have high potential to be developed as natural antioxidants, especially the leaves. However, all extracts exhibited higher toxicity than potassium dichromate except the ethanolic extract of the fruit. Meyer's toxicity index indicates that 100% ethanol, 50% ethanol and aqueous extracts of the plant had high toxicity in brine shrimp *Artemia salina*. Thus, further studies of the toxicity must be conducted, and concentrations safe for consumption should be determined.

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