Antioxidant Activity of *Caryota urens* L. (*Kithul*) Sap

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ABSTRACT. *In Sri Lanka young inflorescence of Caryota urens L (Family: Arecaceae) is tapped for sweet sap that is then used for preparation of fermented beverage and sweeteners. In folkloric, these products are claimed to have health benefits. However, as yet biological activities of C. urens sap have not been scientifically investigated. Recent scientific investigations reported the nutritional value of C. urens sap and its potential as a healthy natural beverage. In this study, antioxidant properties of C. urens sap were studied as a major biological function that may contribute to the claimed health benefits. Antioxidant activity of C. urens sap collected from three major tapping locations of the country (Kotmale, Gampola and Matara) was estimated using1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{1}), Ferric Reducing Antioxidant Power (FRAP) and metal ion chelating assays. From each location 15 samples were used. Radical scavenging activity and FRAP value were calculated as Trolox Equivalent Antioxidant Concentration (TEAC) per 100 g of sap and expressed as mmol Trolox Equivalent (TE). DPPH radical scavenging activities were 0.74±0.05, 0.58±0.07 and 1.15±0.05 mmol TE/100 g and ABTS^{1} radical scavenging activities were 1.44±0.10, 1.22±0.14 and 1.64±0.11 mmol TE/100 g for Kotmale, Gampola and Matara, respectively. FRAP values were 2.54±0.25, 2.22±0.34 and 2.92±0.28 mmol TE/100 g and metal ion chelating activity as Inhibitory Concentration 50% (IC_{50}) was 6.02±0.31, 3.37±0.40 and 3.73±0.33 mg/ml for Kotmale, Gampola and Matara, respectively. This study shows, for the first time, antioxidant activity of C. urens sap, as determined by free radical scavenging activity, electron donating reducing power and metal ion chelating capacity. The results provide some scientific information for traditionally claimed health benefits of C. urens.*

Keywords: *Antioxidant activities, Caryota urens, natural beverage, palm sap*

INTRODUCTION

Caryota urens L. Family: Arecaceae, is a underutilized palm species mainly distributed in several countries in south Asia: India, Sri Lanka, Malaysia and Indonesia to Philippines (Dalibard, 1999). This palm has several synonyms such as fish tail palm, toddy palm, jaggery palm and popular as Kithul in Sri Lanka. In Sri Lanka, traditionally, young inflorescence of *C. urens* palm is tapped for its sweet phloem sap which is then used to produce sweeteners (treacle & jaggery) and fermented beverage (toddy). According to folkloric knowledge, *C.*

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urens sap and sap based products posses health promoting properties. However, as yet these functional properties of *C. urens* sap have not been scientifically investigated.

A strong possibility exists that traditionally claimed health benefits of the *C. urens* sap such as anti-ageing, anti-hyperglycemic *etc.*, are mediated largely *via* antioxidant effects (free radical scavenging, metal ion chelating, *etc.*). Also, to the best of our knowledge, there are no reports available on antioxidant activity of any palm sap. Therefore, the aim of the study was to determine the antioxidant potential of *C. urens* sap.

Recent scientific investigations report that *C. urens* sap is nutritionally rich and contains mixture of simple sugars such as sucrose, glucose and fructose (Somasiri *et al.*, 2008) and further fresh sap has shown potential for beverages. Therefore, scientific information of functional properties of fresh *C. urens* sap would help to improve the value and importance of these products.

MATERIALS AND METHODS

Sample collection and preparation

C. urens palm sap samples were collected from three main *C. urens* palm tapping localtions in Sri Lanka namely, Kotmale, Gampola and Matara during December 2007 to February 2008. Samples were directly collected into sterilized sample collection vials and immediatly stored in ice. Then samples were centrifuged at 10,000 rpm for 5 min. and supernatant stored at-40 °C. From each location 15 different samples were used in the study.

Chemicals and equipment

L-(+)-ascorbic acid, gallic acid, quercetin and 6-hydroxy-2-5-7-8-tetramethylchroman-2-carboxylic acid (Trolox), which were used as standards for calibration curves and 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(-2-pyridyl)-5-6-diphenyl-1-2-4-triazineferrozine-4,4'-disulfonic acid sodium salt (ferrozine) and potassium persulphate were purchased from Sigma-Aldrich (USA). All the other chemicals used for the preparation of buffers and solvents were of analytical grade. All glass ware and plastic-ware used in sample handing in every experiment were sterilized. All the analyses were carried out using 96-well micro-plates (Greiner Bio-One, Germany). SPECTRAmaxPLUS^{384} (Molecular Devices, California, USA) micro-plate reader was used for plate reading.

Free radical scavenging assays

Radical scavenging activity of *C. urens* sap was determined using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2-azinobis-3-ethylbenzothiozoline-6-sulfonic acid ($ABTS^{+}$) radicals. Five different *C. urens* sap concentrations (0.23, 0.47, 0.94, 1.88 and 3.75 mg/ml) of each sample were prepared in methanol and in phosphate buffer separately for DPPH and $ABTS^{+}$ radical scavenging assays, respectively.

DPPH free radical scavenging assay was performed using 96 well micro-plates based on the methods described by Blois (1958). Briefly, 50 µl from each concentration of samples was mixed with 70 µl of methanol in 96 well micro plate and pre-plate reading was recorded at 517 nm. Then 80 µl from 0.5 mM DPPH solution (in methanol) was added to each well and

incubated for 15 minutes at 25±2 °C in dark before reading the plate at 517 nm using SPECTRAmaxPLUS^{384} micro-plate reader. Methanol was used as blank while Trolox (0.62, 1.25, 2.50, 5.00 and 10.0 µg/ml concentrations) served as the standard antioxidant.

2,2-azinobis-3-ethylbenzothiozoline-6-sulfonic ($ABTS^{+}$) radicals were generated as described by Re *et al.* (1999), using 7 mM ABTS and 4.5 mM potassium persulpahte solution. Then the $ABTS^{+}$ solution was diluted 7 times with 50 mM phosphate buffered saline (PBS pH 7.4) immediately before use. 50 µl from sample was mixed with 110 µl of 0.2 M PBS in 96 well micro-plate and pre-plate reading was recorded at 734 nm. Then 40 µl from diluted $ABTS^{+}$ solution was added and incubated at 25±2 °C for 10 min. and absorbance was recorded at 734 nm. Phosphate buffer served as the blank and five different concentrations of Trolox (0.78, 1.56, 3.12, 6.25 and 12.5 µg/ml) were used to construct the standard curve.

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay was performed according to the method described by Kucuk *et al.* (2007), using 96 well micro-plates. In brief, 25 µl from *C. urens* sap samples (3.75 mg/ml) was mixed with 250 µl of 0.2 M phosphate buffer (pH 6.6) and 250 µl of 1% potassium ferric-cyanide and incubated at 50 °C in a water bath for 20 min. This was followed by addition of 250 µl of 10% tricholoro-acetic acid and centrifuged at 10,000 rpm for 10 min. Then, 125 µl from the supernatant was mixed with 55 µl of de-ionized water and 20 µl from 0.1% ferric chloride solution in 96 well micro-plate and the plate reading was recorded at 700 nm. Trolox in five different concentrations (0.03, 0.06, 0.13, 0.25 and 0.50 mg/ml) was used to construct the standard curve.

Ferrous iron chelating assay

The ferrous iron chelating capacity of *C. urens* palm sap was determined using 3-(-2-pyridyl)-5-6-diphenyl-1-2-4-triazineferrozine-4,4'-disulfonic acid (ferrozine) reagent according to the method by Carter (1971). In brief, 65 µl from 325 µM ferrous sulphate and 70 µl from test sample were mixed in micro-plate and plate was pre-read at 562 nm. Then 65 µl from 800 µM ferrozine was added and absorbance was recorded at 562 nm after 10 min. of incubation at room temperature (25±2 °C). EDTA in five different concentrations (1.88, 3.75, 7.50, 15.0 and 30.0 µg/ml) was used as the positive control.

Estimation of total phenol content in *Caryota urens* sap

The Folin-Ciocalteu method (Singleton *et al.*, 1999) was adopted for 96 well micro-plate assay to determine total phenolic contents in *C. urens* palm sap. Each sap sample was diluted with de-ionized water (100 mg/ml) and 20 µl from this solution was mixed with 110 µl of 10 time diluted Folin-Ciocalteu reagent and 70 µl from 10% sodium carbonate solution in 96 well micro-plate. The plate was incubated at room temperature (25±2 °C) for 30 min. and absorbance was recorded at 670 nm. Six different concentrations of gallic acid (0.006, 0.012, 0.025, 0.05, 0.01 and 0.02 mg/ml) were used to construct the standard curve for each plate.

Estimation of ascorbic acid content in *Caryota urens* sap

Ascorbic acid content of the sap was determined by 2,4-dinitrophenylhydrazine method (US Department of Health and Human Services, 1979). In brief, 100 µl from each sample (10 mg/ml) was mixed with 100 µl of metaphospheric acid (60 mg/ml) and 150 µl of

dinitrophenylhydrazine-thiourea-copper sulphate (DTCS) reagent followed by incubation at 37 °C for 3 h in a water bath. Then 500 µl of 12 M H_2SO_4 was added to each sample on ice. Then 200 µl from the mixture was loaded into 96 well micro-plate and absorbance was recorded at 520 nm. Ascorbic acid in metaphospheric acid (4, 8, 16, 31 and 63 µg/ml) was used to construct the calibration curve.

Estimation of total flavonoid content in *Caryota urens* sap

Total flavonoid content of the sap was determined by aluminium chloride method using 96 well micro-plates. Sap samples were diluted in methanol (7.5 mg/ml) and 100 µl from this solution was mixed with 100 µl of 2% aluminium chloride in methanol in 96 well micro-plate and absorbance was recorded at 367 nm after 10 min. of incubation at room temperature (25±2 °C). Pre-plate reading was recorded before adding the aluminium chloride solution. Six different concentrations of quercetin were used to construct the calibration curve.

Data analysis

Softmax Pro5.2v software of the micro plate reader (SPECTRAmaxPLUS^{384} Molecular Devices, California, USA) was used to calculate total phenol content, total flavonoid content, ascorbic acid content, FARP value, and percentage of radical scavenging activity, percentage ferrous iron chelating activity and IC_{50} values of all *C. urens* sap samples. For each sample of each test, 3 replicates (wells) were used. Data of each experiment were statistically analysed using GLM procedure of SAS software release 6.12 followed by comparison of means using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Radical scavenging activity of *Caryota urens* sap

DPPH radical scavenging activity of sap, estimated as inhibitory concentration 50% (IC_{50}) and Trolox equivalent antioxidant concentration (TEAC) are given in Table 1. The sap collected from Matara showed significantly higher ($p<0.05$) DPPH radical scavenging activity compared to other two locations (Table 1). Similarly, $ABTS^+$ radical scavenging activities of *C. urens* sap, estimated as IC_{50}. and TEAC are also summarized in the Table 1. The sap collected from Matara showed significantly higher ($P<0.05$) $ABTS^+$ radical scavenging activity than that of Kotmale samples (Table 1).

Ferric reducing antioxidant power (FRAP) of *Caryota urens* sap

The FRAP values of *C. urens* sap collected from different location are summarized in Table 2. FRAP values of *C. urens* sap collected from three different localities were not significantly different (Table 2).

Ferrous ion chelating activity of *Caryota urens* sap

Ferrous ion chelating activity of *C. urens* sap collected from different locations is presented in Table 2 as IC_{50} values. Ferrous ion chelating activity of sap from Matara and Gampola locations were significantly ($P<0.05$) higher than Kotmale (Table 2).

Table 1. DPPH and $ABTS^+$ radical scavenging activities of *Caryota urens* sap collected from different locations

Location	DPPH radical scavenging activity		$ABTS^+$ radical scavenging activity	
	IC_{50} (mg/ml)	TEAC (mmol TE /100g)	IC_{50} (mg/ml)	TEAC (mmol TE /100g)
Kotmale	3.40 ± 0.26^a	0.74 ± 0.05^a	1.82 ± 0.09^a	1.44 ± 0.10^{ab}
Gampola	4.62 ± 0.35^b	0.58 ± 0.07^b	2.08 ± 0.13^{ab}	1.22 ± 0.14^a
Matara	2.07 ± 0.29^c	1.15 ± 0.05^c	1.69 ± 0.10^b	1.64 ± 0.11^b
Pooled value	3.36±1.27	0.82±0.29	1.86±0.19	1.43±0.21
Standard (Trolox µg/ml)	5.84±0.21	-	6.50±0.20	-

Values are presented as mean ± SEM of 15 independent replicates (for standards 3 replicates)
Values in a column with same superscript letters are not significantly different (P<0.05)

Table 2. Ferric reducing antioxidant power and ferrous ion chelating activity of *Caryota urens* sap collected from different locations

Location	FRAP value (mmol TE/100g)	Ferrous ion chelating activity IC_{50} (mg/ml)
Kotmale	2.54 ± 0.25^a	6.02 ± 0.31^a
Gampola	2.22 ± 0.34^a	3.37 ± 0.40^b
Matara	2.92 ± 0.28^a	3.73 ± 0.33^b
Pooled value	2.56±0.35	4.37±1.44
Standard (EDTA µg/ml)		17.01±0.61

Values are presented as mean ± SEM of 15 independent replicates (for standards 3 replicates)
Values in a column with same superscript letters are not significantly different (P<0.05)

Ascorbic acid, total phenol and flavonoid content of *Caryota urens* sap

Ascorbic Acid Content (AAC), total phenolic content and total flavonoid content of *C. urens* sap collected from different locations are presented in Table 3.

Table 3. Ascorbic acid, total phenolic, total flavonoid, soluable solid content and pH of *Caryota urens* sap

Localities	*Ascorbic acid* content (mg AAE /100g of sap)	*Total phenol* content (mg GAE /100g of sap)	*Total flavonoid* content (mg QE /100g of sap)	*Soluble solid* content (g/100ml)	*pH value*
Kotmale	30 ± 2^a	143 ± 11^{ab}	5.2 ± 0.4^a	12.5 ± 0.8^a	5.8 ± 0.1^a
Gampola	24 ± 3^a	120 ± 15^a	1.2 ± 0.7^b	11.6 ± 0.4^a	5.8 ± 0.1^a
Matara	30 ± 2^a	161 ± 12^b	3.6 ± 0.5^c	10.3 ± 0.8^a	5.7 ± 0.1^a
Pooled value	28±40	141±21	3.4±2.0	11.4±1.1	5.8±0.1

Values are presented as mean ± SEM of 15 independent replicates (for standards 3 replicates)
Values in a column with same superscript letters are not significantly different (P<0.05)
AAE= Ascorbic Acid Equivalent, GAE= Gallic Acid Equivalent, QE= Quercertin Equivalent

Ascorbic acid content of sap collected from different locations was not significantly ($P>0.05$) different. Total phenolic content (TPC) of sap collected from Matara showed significantly ($P<0.05$) higher value than Gampola. Total flavonoid contents (TFC) of Kotmale sap showed a significantly ($P<0.05$) higher value than two other locations (Table 3).

Further, there was no significant difference ($P>0.05$) in the total soluble solid content and pH of sap samples collected from Kotmale, Gampola and Matara (Table 3).

Results of this study showed that *C. urens* sap is rich in antioxidant activity in terms of free radical scavenging, electron donating reducing and metal ion chelating activities. These are important and different actions of dietary antioxidants which help to control cellular damages that occur under oxidative stress conditions. Oxidative stress is a condition where there is an imbalance between generation of oxidants and their neutralization by antioxidant defence system of the body (Abdollahi *et al.*, 2004). Antioxidant defence system is mainly composed of enzymatic and non-enzymatic defence systems and the antioxidants of dietary origin play an important role in strenghtening non-enzymatic defence (Valko *et al.*, 2005).

In this study, antioxdant activity of *C. urens* sap was determined using different methods which are widely used to estimate the antioxidant activities in similar samples (Payet *et al.*, 2005; Thaipong *et al.*, 2006; Kucuk *et al.*, 2007; Phillips *et al.*, 2009). Results of this study showed that *C. urens* sap exhibit free radical scavenging, electron donating and ferrous ion chelating activities in dose dependent manner (data not shown). There are no available literature on antioxidant activities of any plam sap for relevant comparision of our results and this is the first report on antioxidant activity of any palm sap. However, *C. urens* sap showed high antioxidant activity over other processed sweeteners such as granulated sugars, maple syrup, corn syrup and agave nectar (Payet *et al.*, 2005; Theriault *et al.*, 2006; Phillips *et al.*, 2009). Unlike all of these sweetners, *C. urens* sap showed metal ion chelating power that was estimated as ferrous ion chelation ability in the presence of ferrous ion binding ligen ferrozine. Metal ion chelating activity is known as an indirect antioxidant activity which is responsible to block fenton type reation which is implicated in many diseases (Halliwell & Gutteridge, 1990).

C. urens sap showed marked free radical scavenging activity, measured using DPPH (0.8 ± 0.29 mmol TE/100 g) and $ABTS^{+}$ (1.4 ± 0.21 mmol TE/100 g) radicals. Free radical scavenging activity of *C. urens* sap that was estimated using DPPH radicals showed lower activity compared to the activity estimated using $ABTS^{+}$ radicals (Table 2). There may be several reasons for this observed difference. In DPPH radical scavenging assay, the molecules that are soluble only in alcohols are responsible for the scavenging activity while in $ABTS^{+}$ the water soluble compounds are responsible. *C. urens* sap is an aqueous mixture of carbohydrates (sucrose, glucose, fructose, sugar acids and unidentified carbohydrates) (Somasiri *et al.*, 2008) and therefore, some compounds responsible for radical scavenging activity may not be available in methanolic solution for scavenging activity. In addition, the DPPH assay has a disadvantage of steric inaccessibility where the larger molecules cannot scavenge the DPPH radical (Prior *et al.*, 2005). Therefore, the larger molecules such as some carbohydrates which are having radical scavenging properties present in *C. urens* sap may be responsible for $ABTS^{+}$ radical scavenging activity but not scavenged the DPPH radical. Therefore, the radical scavenging activity of *C. urens* sap measured with $ABTS^{+}$ is higher than that was measured with DPPH. Payet *et al.* (2005) has reported a similar result from cane brown sugar supporting our notion.

Total phenolic content of *C. urens* sap showed significant correlation with FRAP values and free radical scavenging activities. However, ascorbic acid and flavonoid content did not show significant correlation. Generally, antioxidant activity of plant extarcts show a strong correlation with polyphenolic content (Shu-Jing & Lean-Teik, 2008) while antioxidant activity of fruit juices show a high correlation with the ascorbic acid content (Marinova *et al.*, 2005). In the present study, only total phenolic content of *C. urens* sap showed significant correlation with antioxidant activity of *C. urens* sap. Even though, the total phenolic content of *C. urens* sap was comparatively lower than some available sweetener alternatives such as maple (Phillips *et al.*, 2009; Theriault *et al.*, 2006) it showed higher antioxidant activities and metal ion chelating activity than maple (Phillips *et al.*, 2009; Theriault *et al.*, 2006). *C. urens* sap is rich in carbohydrates and organic acids (Somasiri *et al.*, 2008). Therefore, there might be bioactive carbohydrates in the sap contributing to higher antioxidant activity exhibited by *C. urens* sap. Previous studies (Kim *et al.*, 2002; Lim *et al.*, 2006) have reported antioxidant properties of carbohdydrate-rich fraction of *Duchesnea chrysantha* (False strawberry) and higher metal ion chelating activity from carbohydrate rich fruits.

In this study, antioxidant activity of *C. urens* sap collected from three main *C. urens* tapping locations was compared. Even though there were some differences in antioxidant activity, phenolic and flavonoid content of the sap collected from different locations were not markedly apart. Therefore, irrespective of origin of *C. urens* sap, it can be used as a raw material for natural beverage or sweetener with antioxidant properties.

CONCLUSIONS

The study showed antioxidants activity of *C. urens* sap, estimated as free radical scavenging activity, electron donating reducing power and metal ion chelating capacity, providing some scientific information for traditionally claimed health benefits. Further, it was found that there are no marked differences in antioxidant property of *C. urens* sap collected from three major Kithul palm tapping areas in the country.

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