Antioxidant Capacities of Fruit Extracts of *Garcinia indica* with Different Assays and Maturity Stages

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This study provides a basis and principle for developing an integrated antioxidant assay of *Garcinia indica* (kokum). Three maturity stages (unripe, medium ripe and fully ripe) of kokum fruit were analyzed for their total phenolic contents (TPC), 1, 1-diphenyl-2-picrylhydrazyl-scavenging ability (DPPH-SC), ferric reducing antioxidant power (FRAP), and oxygen radical antioxidant capacity (ORAC). Significant correlations were obtained for the four assays used (*r* ranging from 0.380 to 0.767, all p < 0.01). Two-way ANOVA revealed that there were significant effects of maturity stage, and the interaction maturity stages for TPC, FRAP and ORAC, whereas only maturity stage and the interaction term were significant for DPPH-SC activity. Overall, the present results provide basic data for choosing kokum fruits with higher antioxidant activity for direct consumption.

Keywords: *Garcinia indica* (Kokum), Antioxidant, *in vitro*, TPC, DPPH-SC, FRAP, oxygen radical antioxidant capacity (ORAC).

INTRODUCTION

Growing evidence suggests that there is a positive correlation between the consumption of diet rich in fruits and vegetables and the reduction of risks for chronic diseases, such as cardiovascular diseases, arthritis, chronic inflammation, and cancers (Aruoma 2003). It is also believed that the physiological functions of fruits to be partly attributed to their antioxidant activity (Wang *et al.* 1996). Various berry fruits have been shown to exhibit a high antioxidant potential (Aaby *et al.* 2005, Huang *et al.* 2005). *In vitro* studies indicate that berries possess a remarkably high scavenging activity towards chemically generated reactive oxygen species (ROS) (Ozgen *et al.* 2009, Zafra-Stone *et al.* 2007)

Kokum (*Garcinia indica* Choisy**)** is a slow growing, ornamental, slender tree, with a dense canopy of green and red-tinged tender emerging leaves with drooping branches growing to a height of 10-18 meters. The kokum crop requires six to seven years to bear fruits. The trees flower from November to February and yields ripened fruits from April to May in every year. The fruits are harvested manually in spring. The ripe *Kokum* fruit is dark purple or red in color with a yellow tinge. It contains 3 to 8 large seeds embedded in a regular pattern like orange segments in the white pulpy material as shown in Figure 1. The fruit has a pleasant flavor and sour taste. It is traditionally used as an acidulant in many Indian dishes. The major organic acid component that imparts the savory taste to the fruit is hydroxyl citric acid (HCA), which is an important ingredient in many fat reducing supplements and it is claimed to increase fat burning (Jena *et al.* 2002)

The chemical and spectral investigations revealed the kokum rind contains garcinol, a fat-soluble yellow pigment and two water soluble anthocyanin pigments as cyanidin-3-glucoside and cyanidin-3-sambubioside. The constituents of *kokum* are as shown in Table 1 (Krishnamurthy and Sampathu, 1988). Several methods are commonly used to determine "antioxidant activity," including oxygen radical ab

sorbance capacity (ORAC), (Wang and Lin 2000) and Zheng and Wang 2003) ferric ion reducing antioxidant parameter (FRAP), (Heinonen *et al.* 1998, Lin and Tang 2007) and 1,1-diphenyl-2-picrylhydrazyl-scavenging activity (DPPH-SC) (Huang 2007 and Naderi *et al.* 2004). Different methods for determina-

Table 1. Chemical constituents of *kokum* fruit

Constituents	%
Moisture (g/100g)	80.0
Protein (N x6.25%)	1.0
Total Ash (%)	2.6
Tannin (%)	1.7
Pectin (%)	0.9
Total sugars	4.1
Crude fat (%)	1.4
Organic acid (as HCA) (%)	5.9
Pigment (%)	2.4

Source: Krishnamurthy and Sampathu (1988).

Table 2. Two-way ANOVA for TPC, DPPH-SC, FRAP, and ORAC of three maturity stages of kokum fruit.[a]

Stage	TPC[b]	DPPH-SC[c]	FRAP[d]	ORAC[e]
Unripe (U)	425±45	442±62	5.4±0.2	11.5±2.2
	1013±24	540±19	6.5±0.3	14.8±4.4
Medium ripe (M)	1078±54	927±55	8.5±0.1	16.7±3.1
	1162±14	1012±115	9.5±0.1	16.4±2.4
Fully ripe (F)	2192±93	1669±409	15.3±0.1	22.0±0.3
	2207±101	2018±223	15.6±0.3	22.9±2.0

a: TPC: Total phenolic contents; DPPH-SC: DPPH- scavenging activity; FRAP: Ferric reducing antioxidant power; ORAC: Oxygen radical absorbance capacity. Data are represented as the means ± SD (n = 4).
b: Data are expressed as mg gallic acid equivalent (GAE)/100 g FW.
c: Data are expressed as µg BHT equivalent/g FW.
d: Data are expressed as mM ascorbic acid equivalent/100g FW.

tion of antioxidant activities are based on different reaction mechanisms, so they often give inconsistent results (Prior *et al.* 2005). No single method can give a comprehensive prediction of antioxidant activity, and it is not surprising that different antioxidant assay methods could produce inconsistent results (Prior *et al.* 1998). For example, the FRAP assay determines the reducing capability based on ferric ion, and ORAC only determines antioxidant activity against peroxyl radicals (Ogawa *et al.* 2008). Therefore, the use of more than one method has been recommended (Aruoma 2003).

Kokum possesses a highly complex mixture of phytochemicals, but there is no systematic method to integrate different antioxidant characteristics. Therefore, the aim of this research was to set up a reliable and simple procedure to estimate antioxidant activities by integrating multiple assays in kokum fruits of different maturity stages. A combination of different antioxidant assays was used, which included total phenolic contents (TPC), FRAP, DPPH-SC, and ORAC. A multivariate statistical technique was performed in order to integrate the patterns of data, to reduce the dimension of data set, and to highlight the similarities and differences in the data (Bhat 2005)

MATERIALS AND METHODS
Chemicals
AAPH (2,2-azo,bis (2-amidinopropane) dihydrochloride) was purchased from HiMedia Laboratory Ltd. Mumbai. B-PE (B-phycoerythrin from *Porphyridium cruentum*), Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-2 -carboxylic acid), DPPH (1,1-diphenyl-2-

picrylhydrazyl), TPTZ (2,4,6-tripyridyl s-triazine), Folin-Ciocalteau reagent, $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, and butylated hydroxytoluene (BHT) were obtained from Sigma (Sigma-Aldrich, Bombay). All other chemicals and organic solvents of analytical grade were obtained from Merck (Merck Specialities, Mumbai).

Sample Preparation
Kokum fruits start developing from February and were harvested between late March and April of 2011. The unripe stage referred to green fruit, were harvested when the fresh weight reached approximately 15 g. The medium ripe stage referred to red fruits, were harvested when the fresh weight reached approximately 23 g. The fully ripe stage referred to black-purple fruit, were harvested when the fresh weight reached approximately 47g.

To prepare the fruit extracts, samples weighing at least five grams from four replicates of each kokum fruit ripeness stage were extracted twice with 10 mL of 80% ethanol containing 0.2% formic acid using a Soxhlet extraction unit for 1hr 45 min and then centrifuged at 13000g for 10 min at 4°C. The supernatants were combined and transferred to vials, stored at -80°C, and then used for analyses of FRAP, ORAC, DPPH-SC and TPC.

Folin-Ciocalteau Assay for Total Phenolic Content (TPC)

Fig 1. *Gardcina indica*
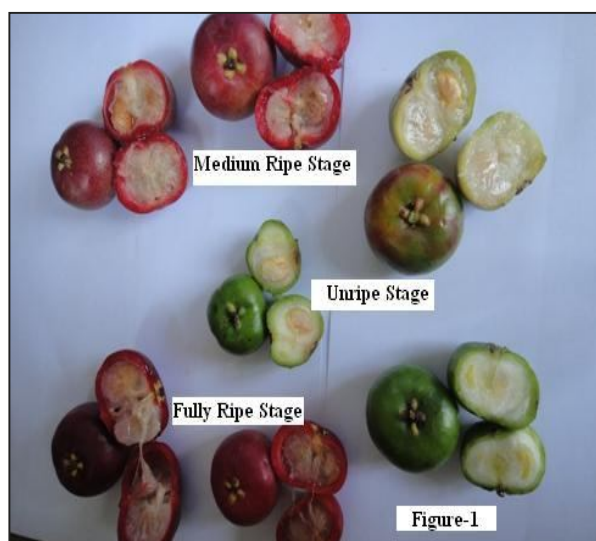
TPC in kokum fruit extract was determined according to a modified Folin-Ciocalteau method (Mahattanatawee *et al.,* 2006) using gallic acid as the standard. Samples and standards were dissolved in 5 mL of 0.3% HCl in methanol/water (60:40, v/v). The solution (100 µL) was added to 2% Na_2CO_3 (2 mL). After 2 min, 50% Folin-Ciocalteau reagent (100 µL) was added to the mixture, and set aside for 30min. Absorbance was measured at 750nm by a UV spectrophotometer (Elico Inc.) and results were expressed as mg of gallic acid equivalent/100g of fresh weight.

DPPH-Scavenging Activity Assay

DPPH, a stable radical, has been widely used for the determination of primary antioxidant activity, that is, the free radical scavenging activities of antioxidants, which produced a decrease in absorbance. DPPH-SC in kokum fruit extract was determined according to a modified DPPH-SC assay (Naderi *et al.* 2004). Briefly, aliquots (160µL) of samples, controls, or the standard, BHT, were mixed with 40µL of 10mM DPPH in ethanol, respectively. The mixture was shaken vigorously and left to stand for 30min at room temperature in the dark. Absorbance of the testing solution was measured at 525nm by UV spectrophotometer and results were expressed as µM BHT equivalent/g FW.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay method was based on the reduction of Fe (III) $(TPTZ)_2 Cl_3$ complex (as an oxidant) by antioxidants to form colored $Fe(II)(TPTZ)_2$. FRAP in kokum fruit extract was determined according to a modified FRAP assay (Ogawa *et al.,* 2008). Briefly, 200µL of the freshly prepared FRAP reagent, containing $Fe(III)(TPTZ)_2Cl_3$ and acetate buffer, was mixed with 30µL of distilled water and 10µL of the test sample or the blank (solvent control), pH 3.6 and was prepared freshly and warmed to 37°C. Absorbance at 595 nm was measured after incubation at 37°C for 5min by a UV spectrophotometer. $FeSO_4$ was used as the standard. Results were expressed as mM ascorbic acid equivalent/g FW.

Oxygen Radical Absorbance Capacity (ORAC) Assay

All samples were measured using the ORAC assay, which uses B-phycoerythrin (B-PE) as an oxidizable protein substrate and AAPH as a peroxyl radical generator (Prior *et al.,* 1998). 50µL of samples, controls, or standard (Trolox at five different concentrations for construction of a standard curve) were mixed with 50 µL of 2.5 mg/L B-PE solution and 50 µL of PBS (pH 7.4, 75 mM). They were incubated at constant temperature (37°C, 15 min) and then 50 µL of 16 mM AAPH solution added to initiate the reaction. The fluorescence intensity [545 nm (Ex)/575 nm (Em)] was measured every five minutes for 70min at 37°C by UV absorption. The ORAC value of a sample was calculated on the basis of the Trolox standard curve, with ORAC value assigned 1 µM Trolox.

Statistical Analyses

Data (mean ± SD) were analyzed using two-way ANOVA to determine the overall effect of maturity stage (S) of the antioxidant measurements. p value < 0.05 is considered statistically significant. Data pretreatment was conducted using the Z score transformation (mean = 0; standard deviation = 1) in order to eliminate the bias due to different units of the results from the four different antioxidant assays.

RESULTS AND DISCUSSION

Antioxidant Activity Analyses

The antioxidant values of the three stages of fruit obtained by the four assays (TPC, DPPH-SC, FRAP, and ORAC) varied widely; and the ranking of antioxidant values were inconsistent even maturity stage. For example, TPC ranged from 425 ± 45 mg GAE/100g FW in the medium ripe to 2207 ± 101 mg GAE/100g FW in the fully ripe, which is comparable to those (181 to 2570 mg GAE/100g FW) reported by others (Cam 2009, Lin and Tang 2007) in kokum fruits. The variation of TPC depends on many factors, such as the degree of maturity stage, and environmental conditions during fruit development (Lin and Tang 2007).

Two-way ANOVA revealed significant effects of im-

maturity stage (U) (p<0.001), maturity stage (F) (p<0.001), and interaction of immaturity and maturity stage (U ⊠ F) (p<0.001) for TPC, FRAP, OPRAC, whereas only F and U ⊠ F were significant for DPPH -SC (Table 2). In general, TPC, FRAP, OPRAC and DPPH-SC values increased with increasing maturity (i.e., from unripe to fully ripe), although TPC in some-what lower at the medium ripe stage than at the unripe stage. Interestingly, TPC in kokum fruits has also been shown to decrease to some extent from the unripe stage to the medium ripe stage, then to increase sub-stantially at the ripe stage (Wang and Lin 2000). For DPPH-SC, two-way ANOVA revealed significant effects of maturity stage (p< 0.001) and the interaction (U ⊠ F) (Table 1), indicating that pattern of changes in DPPH-SC value with fruit maturity were not the same for all cultivars.

Correlations of Antioxidant Assays

Significant correlations existed for the results from the four different antioxidant assays used in this study (r ranging from 0.380 to 0.767, all $p < 0.01$). The highest correlation coefficient was found between ORAC and FRAP ($r = 0.787$, $p < 0.001$, N = 60) and the lowest one was between DPPH-SC and TPC ($r = 0.380$, $p < 0.01$, N = 60). In addition, TPC gave sig-nificantly positive correlations with FRAP and ORAC ($r = 0.636$ and 0.545, both $p < 0.001$, N = 60), sug-gesting that phenolic compounds were the major con-tributor to of FRAP and ORAC in kokum. Significant correlations were also found between TPC and FRAP in other genotypes of kokum as well as between FRAP and ORAC in blackberry and strawberry. (Wong, 2006 and Aaby, 2005). The relatively low correlation between TPC and DPPH-SC may be due to the fact that the Folin-Ciocalteu's reagent can only roughly estimate the amount of phenolic compounds (Zheng and Wang 2003). A possible explanation for the observation that high TPC did not correspond to a high DPPH-SC could be due to the antagonistic and synergistic reactions between phenolics and other phy-tochemicals in kokum fruit. These results suggested that the antioxidant activity of mulberry determined by one method may only partially reflect its overall antioxidant potential.

CONCLUSIONS

It is important to provide an optimum method to assess kokum antioxidant activity so that more reli-able and useful information can be used by other re-searchers as well as the kokum food industry. In this study, we demonstrate significant correlations among the four antioxidant assays used. Two-way ANOVA revealed that there were significant effects of maturity stage, and the interaction of maturity stages for TPC, FRAP, and ORAC, whereas only maturity stage and the interaction were significant for DPPH-SC activity. Thus, the present results provided the basic data for choosing kokum fruit with higher antioxidant activity for direct consumption or for production of fruit juice.

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