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Studies on the Composition and *In-Vitro* Antioxidant Activities of Concentrates from Coconut Testa and Tender Coconut Water

Geetha V, Bhavana KP, Chetana R, Gopala Krishna AG and Suresh Kumar G*

Department of Traditional Foods and Sensory Science, CSIR-Central Food Technological Research Institute, Mysore, India

Abstract

Tender coconut water is known to have health beneficial effects. Antioxidants are the bioactives which helps in the amelioration of wide range of diseases like diabetes, obesity, cancer etc. In the current study two concentrates were prepared from coconut testa, as phenolic concentrate (PHE) and tender coconut water concentrate (TCW).These concentrates were evaluated for proximate composition, phenolic acids and antioxidant activities. Protein (3.7% and 5.2%), carbohydrates (56.6% and 53.5%), phenolics (3.4% and 2.6%) and flavonoids (1.9% and 1.4%) were observed in PHE and TCW respectively. Phenolic acids composition was estimated by HPLC and major phenolic acids were found to be gallic/tannic, protocateuchic and ferulic acid. Both concentrates had good reducing power ability with IC50 values of 68.4 µg (PHE) and 73.5 µg (TCW). Further, DNA protection assay evidenced the dose dependence protection for free radical induced oxidation by PHE and TCW. Hence, concentrates of PHE and TCW are useful in preventing stress induced ailments. Since the concentrates are stable it can be used in different food preparations.

Keywords: Coconut; Tender coconut water; Phenolic concentrate; Free radicals; DNA protection

Introduction

Coconut palm (*Cocos nucifera* L.) is one of the most useful tropical trees having religious and also traditional values in Asian countries. Jean et al. [1] described tender coconut water as a natural sterile liquid found in the young immature coconuts also known as liquid endosperm and is slightly translucent, it contains good amount of minerals, sugars, amino acids, proteins, vitamins and antioxidants, it is used to treat dehydration especially in diarrhea; vitamin C and riboflavin present in tender coconut water plays a major role. It reduces blood pressure; hepatoprotective in nature [2], it eliminates mineral poisoning [3]. Based on the compositional and functional properties it is also considered as sport beverage [4] therefore has drawn the attention of manufacturers and industries. However, perishability of coconut water is very high when exposed to air; it develops sour, off flavor and taste. Its natural freshness is lost within 24 h to 36 h even under cold, unless treated scientifically. Therefore an attempt was made to convert the TCW into stable concentrate.

Thin brown outer skin of coconut kernel known as coconut testa, coconut testa is removed during the processing of virgin coconut oil, preparation of desiccated coconut powder [5], therefore it is byproduct of virgin coconut oil processing industry, it contains plenty of bioactives such as phenolic acids, flavonoids that are known to have health beneficial effects and are under-utilized [5]. We made an attempt to prepare phenolic rich concentrate (PHE) from testa material.

Free radicals are reactive molecules and are short lived species having unpaired electrons. Bansal and Bilaspuri [6] described free radicals induce cell damage by attacking them resulting in oxidation of cell components and molecules. Oxygen is the element of life; however in pathological condition it generates reactive oxygen species (ROS) through various processes within the biological system that ultimately induces cell death *via* necrosis. Oxidative stress causes loss of structure and functionality of healthy cells. Pathogenesis of about more than 50 diseases has been implicated by free radicals [7]. Interventions of antioxidants limit the damage caused to DNA, proteins, and other macromolecules. Oxidation by free radicals to the tissues results in wide variety of diseases, most notably cancer and heart disease [8]. The most effective way is to prevent the damaging activity of free radicals in the system is by consuming diets rich in polyphenols, polyphenols are found mostly in plant materials [9] these antioxidants offer some protection against development of cancers, cardiovascular diseases, obesity and diabetes [10,11]. In the present study, tender coconut water concentrate (TCW) and phenolic concentrate from coconut testa (PHE) were prepared at CFTRI, Mysore. They were analysed for proximate composition, phenolic acids and antioxidant assays. The main objective of the present work is the analysis of the developed concentrated and their nutraceutical value, which can be further used for the preparation of health beneficial foods.

Materials and Methods

Materials

Coconut testa was obtained from the local industry in Mysore, tender coconut water from local market. Standards like bradford reagent, 1,1-Diphenyl-2-picryl hydrazyl (DPPH), calf thymus DNA, gallic acid, p-coumaric, ferulic, caffeic, gentisic, protocatechuic, vanillic acids, syringic etc., from Sigma chemical co. (St. Louis Missouri, USA). HPLC column was Shimadzu C_{18} column (250 mm × 4.6 mm, 5µm). The other chemicals such as ferric chloride, hydrogen peroxide, trichloro acetic acid and solvents used were of the analytical grade purchased locally.

Preparation of concentrates

The concentrates; phenolic concentrate from mature coconut testa (PHE) and tender coconut water concentrate(TCW) from tender coconut water, based on process developed from CSIR-CFTRI, Mysore.

***Corresponding author:** Suresh kumar G, Department of Traditional Foods and Sensory Science, CSIR- Central Food Technological Research Institute, Mysore 570 020, India, Tel: +918212514153; E-mail: sureshg@cftri.res.in

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Estimation of moisture, reducing sugar, total carbohydrates and protein contents

Moisture was estimated by oven methods, a method that dries samples at 135°C for 2 h [12], reducing sugar [13] total carbohydrate [14] and proteins [15]

Estimation of total polyphenols

Total phenolics in the sample were measured calorimetrically according to Singleton and Rossi [16] an aliquot of appropriately diluted samples and standard gallic acid (0-100 µg) in methanol were taken in clean dry test tubes in triplicates. Standard solution was prepared using gallic acid (0-100 µg/mL). 2.5ml of (1:10) diluted Folin- Ciocalteu's reagent was mixed, after 5 minutes 2.0 ml of Na_2CO_3 (7.5%) was added and mixed, the samples were incubated in the dark at room temperature for 2 hours. The absorbance was taken at 765 nm. The concentration of polyphenols in the sample expressed as g/100 g of gallic acid equivalent (GAE).

Estimation of total flavonoids

Total flavonoids in the sample were measured calorimetrically according to Zhishen et al. [17], an aliquot of suitably diluted sample were taken in clean dry test tubes in triplicates. Distilled water (4 ml) was added, at different interval of time *viz.*, at 0^{th} min. 0.3 ml $NaNO_2$ (5%), after 5 min. 0.3 ml $AlCl_3$ (10%), at 6^{th} min. 2 ml 1M NaOH were added to the mixture. Immediately, the reaction flask was diluted to volume of 10 ml using distilled water and mixed thoroughly. Absorbance read at 510nm using water as blank. Total flavonoids were determined using standard graph of catechin (0-100 µg/mL) and expressed as g/100g of Catechin equivalents (CE).

Antioxidant activities

Free radical scavenging effect was studied according to Lai et al. [18] using 1,1-diphenyl-2picrylhydrazyl (DPPH), the samples (5-80 µg , GAE) were mixed with 750 µM DPPH in methanol and the final volume made to 500 µl. The mixture was incubated for 15 min. at room temperature and the absorbance was read at 517 nm against sample blank. Gallic acid was used as standard. The scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{absorbance of sample} \times 100}{\text{Absorbance of control}}$$

Reducing power assay

Different concentrations of the samples were mixed with 2.5 ml of phosphate buffer (0.2M) and 2.5 ml of potassium ferricyanide (1% w/v). This mixture was heated at 50°C in water bath for 20 minutes. On cooling 2.5 ml of trichloro acetic acid (10%) was added and centrifuged for 10 minutes at 6000 rpm. The upper layer (2.5 ml) of solution was mixed with equal volume of distilled water, mixed well and 0.5 ml of freshly prepared ferric chloride solution was added, absorbance was measured at 700 nm and graph prepared and compared with standard GA.

DNA damage study

Protection of calf thymus DNA from oxidation by free radicals was analyzed according to the method by Rodriguez and Akman [19] DNA from calf thymus (sigma) was oxidized using Fenton's reagent (30 µM H_2O_2, 50 µM ascorbic acid and 80 µM $FeCl_3$) in presence and absence of test sample and relative difference in the migration of oxidized DNA was observed on 1% agarose gel by electrophoresis after staining with ethidium bromide. Gels were documented and protection were analysed based on native and oxidized means.

Analysis of phenolic acids using HPLC

The phenolic acid compositions were determined by HPLC (model LC-10 AVP Shimadzu Corp., Tokyo, Japan) coupled with UV detector connected to spherisorb C-18 reverse-phase column (250 mm × 4.6 mm, 5 µm). Following conditions were used λ 280nm, mobile phase; water: methanol: acetic acid (80:18:2), isocratic with flow rate of 1 mL/min. Standards of various phenolic acids; gallic acid (GA), protocateuchic acid (PC), caffeic acid (CA), ferulic acid (FA), syringic acid(SA), vanillic acid (VA), *p*-coumaric acid(pCA) and cinnamic acid (CiA) were used for the identification of phenolic acids in the samples and expressed in relative percentage.

Identification of peaks by LC- Mass spectrometry (LC-MS)

The unknown peak fraction was collected from the HPLC were subjected to LC-MS for phenolic acid identifications. The mass of the peak was identified using the instrument Q-Tof Ultima, Waters corp. UK, Alliance HPLC system was equipped with PDA detector, Waters 2996. The source used was ESI –ve, Capillary (kV): 3.5, Cone: 100 V, Source temperature was 120°C, Desolventation temperature: 350°C, Cone gas: 50 L/Hr, Desolventation gas: 500 L/Hr

Statistical analysis

All the analyses were carried out in triplicate and the average values were expressed mean ± SD. The significance of difference was calculated by Student's *t*-test and $p<0.05$ were considered to be significant. The correlations between antioxidant activity and phenolic contents were calculated using trial version graph pad prism software.

Results and Discussion

Proximate composition of phenolic and tender coconut water concentrates of coconut

Prepared concentrates of phenolic (PHE) and tender coconut water (TCW) subjected to proximate composition, and the results indicated as in (Table.1) showed that the moisture (22.7% and 36.4%), protein (3.7% and 5.2%), total sugar (56.6% and 53.5%), reducing sugar (31.0% and 34.3%), total polyphenols (3.4% and 2.6%) and total flavonoids (1.9% and 1.4%), PHE and TCW respectively The moisture and protein content of TCW was slightly higher than PHE. However total sugar and total phenolics were slightly less in TCW when compared with PHE concentrate.

Proximate composition of TCW showed higher protein content due to the process of concentrating,which increased the soluble protein. Phenolics/flavonoids and other free sugars are generally extracted using 70% ethanol [20] and hence phenolic extracts contain more free sugars and phenolics (small molecule antioxidants). Presence of high amount of sugar in the concentrate acts as preservative, with effective amount of phenolics, it can be used to treat stress induced ailments

Sl.no	Parameters	PHE	TCW
1	Protein (%)	03.7 ± 0.5	05.2 ± 0.8
2	Reducing sugars (%)	31.0 ± 1.2	34.3 ± 1.4
3	Total sugars (%)	56.6 ± 2.1	53.5 ± 2.1
4	Moisture (%)	22.7 ± 1.1	36.4 ± 1.8
5	Total Polyphenols (%)	03.4 ± 0.4	02.6 ± 0.2
6	Total Flavonoids (%)	01.9 ± 0.1	01.4 ± 0.1

Table 1: Proximate composition of PHE and TCW concentrates.

[21]. Thereby the concentrates may be useful in preparation of foods for the stress induced diseases.

In-vitro antioxidant assays of phenolic (PHE) and tender coconut water (TCW) concentrate

Free radical scavenging assay was performed using a colored free radicals DPPH and will be decolorized by neutralizing it. IC_{50} value is a concentration at which 50% of the free radicals will be scavenged by the test sample. The IC_{50} values was found in the order GA < PHE < TCW (Figure 1A) indicates PHE has the scavenging ability at 68.4 µg/mL which is lesser than that of TCW 73.5 µg/mL and hence PHE concentrate has a better antioxidant activity.

Reducing power assay shows the reduction of free radicals by antioxidants, in this method, antioxidant compound forms a coloured complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700 nm. Increase in absorbance of the reaction mixture indicates the reducing power of the samples [22]. PHE concentrate showed dose dependent reducing ability which is better than the TCW concentrate (Figure 1B). Gallic acid (GA) is well known phenolics having good antioxidants ability and has highest reducing power ability when compared to the test samples.

DNA protection assay wherein DNA was oxidized using fenton's reagent containing different source of free radicals along with and without test samples as presented in the gel images of PHE (Figure 2a) and TCW (Figure 2b).

Free radicals are continuously produced in our body and play a major role in the pathogenesis and tissue damage in many clinical disorders [23-25] they are generated by various processes in biological system which are also the by-products of normal metabolism. Free radicals like Reactive oxygen species (ROS), Superoxide(O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) are highly reactive and attack the essential biomolecules, destabilizes them, which further results in the collapse of the cell [26]. Ability of TCW and PHE concentrate in bringing down the free radicals to some extent is very important which was shown by our prepared concentrates, there are no studies showing the radical scavenging activity of coconut concentrates. Mantena et al. [27]; Leong and Shui [28] studied antioxidant activities in tender coconut water. There are many methods to test antioxidant properties, DPPH is the most commonly used assay for the determination of IC_{50} values (50% of free radicals scavenging concentration) recently, DNA protection assay is gaining importance. The use of more than one method is recommended for quantifying the antioxidant activity of any samples [1] such *in-vitro* studies would be cost effective and can be screened quickly for the potency of the samples.

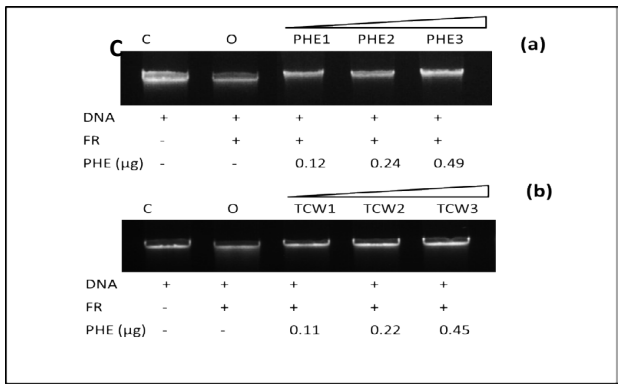

Figure 1: HPLC chromatogram of phenolic concentrate and tender water concentrate a) Standard phenolics, b) Testa phenolic concentrate, c) Tender coconut water concentrate.

Reducing properties of the active components present in the samples is important, even at lower concentration the extracts should be effective. Though the biological system has the mechanisms to eliminate free radicals by either natural enzymatic and non-enzymatic antioxidant defense system, external antioxidants are useful in minimizing the stress induced abnormalities. Vishakh et al. [29] have shown the beneficial effects of phytochemicals present in tender coconut water and also studied their antioxidants activities *invitro*. Tender coconut water has been tested in CCL_4 induced liver damage in rats, which showed that antioxidant enzymes activities were ameliorated [2]. It also reverses the blood pressure, improves insulin sensitivity by ameliorating antioxidant activity [30,31]. TCW reduces the nicotine and its metabolite cotinine cytotoxic effect on spermatozoa DNA damage [32]. Anurag and Rajamohan [33] reported the beneficial effects of TCW with several factors viz. potassium, calcium, magnesium, L-arginine.

Phenolic compositions of concentrates of PHE and TCW by HPLC and LC-MS

HPLC analysis for the PHE and TCW were carried out and the chromatogram presented as in Figure 3. Standard phenolic acids mixture was resolved as shown in the figure followed by the samples PHE and TCW. The phenolics acids such as GA, PC, CA, and FA, SA, VA, pCA and CiN were identified in the fractions. The PC, GA, SA and FA are the major in PHE whereas GA, FA and PC in TCW (Table 2). The peak unidentified (U) in PHE was fractionated and subjected to Mass spectroscopy as shown in Figure 3 fragmentation was at 198.32 whose mass pattern was similar to standard synergic acid (197.4).

Prepared TCW and PHE concentrate from coconut have GA/ TA, pCA and FA. Phenolics and flavonoids are present in the kernel part of the nuts [34]. Since our concentrates were kernel origin therefore showed good antioxidant activity by scavenging free radicals. FA

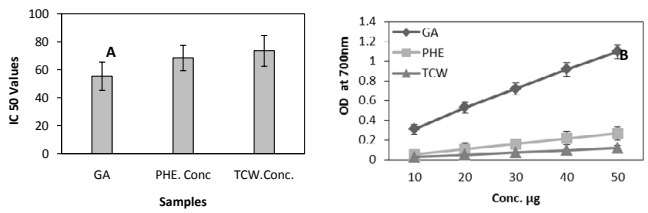

Figure 2: Antioxidant activities of TCW and PHE by Free radical scavenging activity. (A) DPPH (B) Reducing power assay.

Peak number	Phenolic acids	PHE Conc. (µg/mg)	TCW Conc. (µg/mg)
1	Gallic acid	33.05 ± 0.13	38.94 ± 1.34
2	Protocateuchic acid	41.43 ± 1.23	15.59 ± 0.45
3	Caffeic acid	06.34 ± 0.03	04.20 ± 0.05
4	Ferulic acid	19.83 ± 0.06	24.35 ± 1.57
6	Vanillic acid	nd	02.65 ± 0.03
7	p-coumaric acid	18.80 ± 0.48	05.80 ± 0.06
8	Syringic acid	28.25 ± 0.53(U)	nd
10	Cinnamic acid	10.80 ± 0.21	nd
*nd: not defined			

Table 2: Phenolic acid composition of phenolic concentrate and tender water concentrate by HPLC.

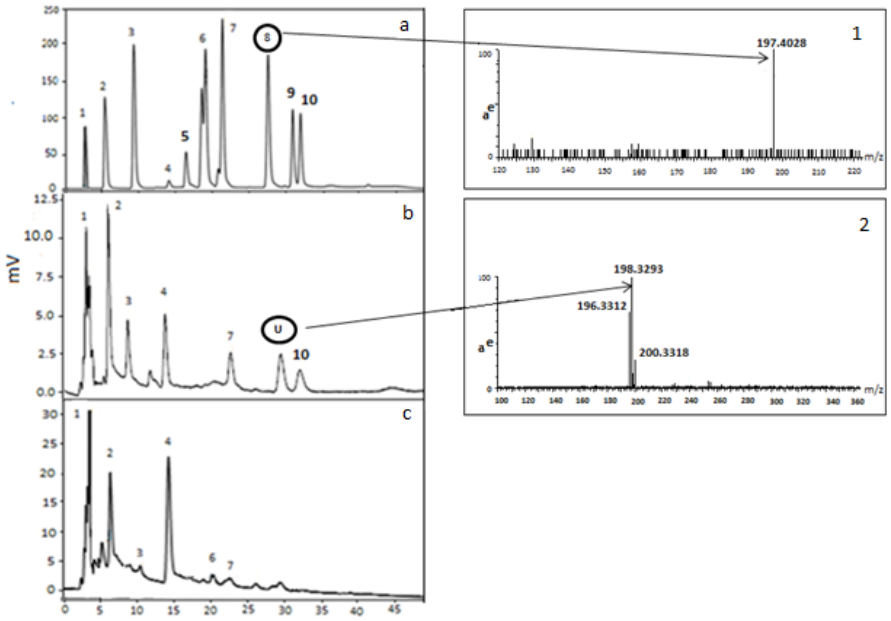

Figure 3: HPLC chromatograms (left) a) Standard phenolics, b)PHE ,c) TCW concentrate.
Mass spectra(right),Mass spectra of PHE concentrate- 1) Standard syringic acid,2) Peak 8-MS for the HPLC chromatogram of PHE concentrate.

(Ferulic acid), a well-known compound, reduces various metabolic disorders including obesity and diabetes and is available in both free and bound forms [35,36]. The availability of FA in TCW along with L-arginine amino acid is important [37], which has health beneficial effects apart from other bioactives. Busarakorn, et al. [38] have shown the presence and antioxidant activity in TCW *in- vitro*. The present study of the prepared concentrates (PHE and TCW) for the phenolic content and its potentiality as antioxidants is useful, which confirmed the scientific background for development of food product from the concentrates.

Conclusion

Prepared tender coconut water (TCW) and phenolic concentrates (PHE) retained most of bioactives, had free radical scavenging and DNA protecting properties, which confirms the antioxidant property. Phenolic acids such as gallic acid, ferulic acid, Protocatechuic acid etc., were identified in the concentrates and they are responsible for the antioxidant properties. Hence TCW can be used as ready to drink food product having natural health beneficial nutrients. PHE concentrate is rich in phenolics, can be blended with any food products for health beneficial effect.

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