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## Evaluation of Chemical Constituents and Antioxidant Activity of Coconut Water (*Cocos nucifera* L.) and Caffeic Acid in Cell Culture

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### ABSTRACT

Coconut water contains several uncharacterized substances and is widely used in the human consumption. In this paper we detected and quantified ascorbic acid and caffeic acid and total phenolics in several varieties of coconut using HPLS/MS/MS ( $25.8 \pm 0.6 \mu\text{g/mL}$  and  $1.078 \pm 0.013 \mu\text{g/mL}$  and  $99.7 \mu\text{g/mL}$ , respectively, in the green dwarf coconut water, or 10 mg and 539  $\mu\text{g}$  and 39.8 mg for units of coconut consumed, 500  $\pm$  50 mL). The antioxidant potential of four coconut varieties (green dwarf, yellow dwarf, red dwarf and yellow Malaysian) was compared with two industrialized coconut waters and the lyophilized water of the green dwarf variety. All varieties were effective in scavenging the DPPH radical ( $\text{IC}_{50}=73 \mu\text{L}$ ) and oxide nitric (0.1 mL with an IP of 29.9%) as well as in inhibiting the *in vitro* production of thiobarbituric acid reactive substances (1 mL with an IP of 34.4%), highlighting the antioxidant properties of the green dwarf which it is the most common used. In cell culture, the green dwarf water was efficient in protecting against oxidative damages induced by hydrogen peroxide.

**Key words:** Antioxidant activity, Ascorbic acid, Caffeic acid, *Cocos nucifera*, Polyphenols.

### INTRODUCTION

Coconut palm, botanically known as *Cocos nucifera*, belongs to the family of Arecaceae (Palmae), an important member of monocotyledons. Brazil is the fourth largest coconut producer, accounting for 5% of world production. In addition, considering the consumption of coconut water, Brazil is the largest world producer, highlighting the Northeast region, with 85.6% of the national

culture production and a planted area, in 2002, of approximately 280,835 ha (Neto et al. 2007).

Around the world, coconut fruit products have been used in popular medicine for the treatment of various diseases, such as arthritis and diarrhea (Esquenazi et al. 2002). Studies carried out with the coconut husk fiber have proven its antiproliferative activity against lymphocytes (Kirsztberg et al. 2003), and also determined its analgesic and antioxidant activities (Alviano et al. 2004).

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Coconut water (CW), the liquid endosperm obtained from immature coconuts, in its natural form is a refreshing and nutritious beverage, widely consumed around the world due to its beneficial health properties (Pummer et al. 2001). Moreover, coconut water plays an important alternative role for oral rehydration and even for intravenous hydration of patients in remote regions (Campbell-Falck et al. 2000) in addition to protect against induction of myocardial infarction (Anurag and Rajamohan 2003). Antioxidant activities of polyphenolics derived from plants have claimed beneficial health functions for retarding aging and preventing cancer and cardiovascular diseases (Scalbert et al. 2005). Moreover, the presence of ascorbic acid in the natural CW was correlated with antioxidant properties (Mantena et al. 2003). A previous study confirmed the presence of caffeic acid in the coconut oil from the copra (Seneviratne and Dissanayake 2008). Chakraborty and Mitra (2008) proved the existence of chlorogenic, caffeoylshikimic and dicaffeoylquinic acids, three caffeic acid derivatives, in the methanolic extract of young, mature and old coconut mesocarp. Until the present moment, any study showed the identification and quantification of caffeic acid in coconut water (*Cocos nucifera* L). These findings highlight the need for studies to identify and quantify coconut water compounds with nutraceutical proprieties, specially the caffeic acid, since it is found in other parts of the coconut, such as the mesocarp which is in direct contact with CW indicating that this compound can be present in the coconut water.

The aims of this study were to: evaluate the CW antioxidant properties of four different varieties (green dwarf, yellow dwarf, red dwarf and yellow Malaysian), test the CW capacity to reduce the production of intracellular reactive oxygen species (ROS) in lung fibroblast (IMR-90) and establish a possible correlation between the observed antioxidant properties and the phenolic compounds present in CW, ascorbic and caffeic acids, as others which were not identified as three caffeic and chlorogenic acids.

## MATERIALS AND METHODS

### COCONUT WATER

Ten young coconuts (3-4 months) of each different variety (green dwarf, yellow dwarf, red dwarf and yellow Malaysian) were collected from the Brazilian Agricultural Research Corporation (EMBRAPA) - Coastal Tablelands - Aracaju-SE/ Brazil. They were stored at 24°C for 24 hours, cleaned and opened only at the moment of the analysis. Boxes containing UHT (ultra high temperature) processed coconut water and bottles containing coconut water of the green dwarf variety were purchased from a local market.

### CHEMICALS

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2',7'-dichloro-fluorescein-diacetate (DCFH-DA) and 3,4-dihydroxycinnamic acid (caffeic acid) were bought from Sigma-Aldrich (St. Louis, MO, USA), 4,6-dihydroxy-2-mercaptopyrimidine (thiobarbituric acid - TBA) was bought from Acros Organics (Geel, Belgium), trichloroacetic acid (TCA) was bought from Fluka (St. Louis, MO, USA), sodium nitroprusside and 2,4-dinitrophenylhydrazine from Merck (Darmstadt, Germany), L-threoascorbic acid (vitamin C) from Quimex (Chicago, USA), Folin solution from Deel (São Paulo, Brazil), sulphonilamide from Cromoline (São Paulo, Brazil), N-(1-naphthyl) ethylenediamine dihydrochloride from Acros Organics (Geel, Belgium) and quercetin (3,3',4,5',7-pentahydroxyflavone dehydrate) from Aldrich (St. Louis, MO, USA). All the solvents for chromatographic purpose were HPLC grade, purchased from Merck (Darmstadt, Germany). All the other chemicals were purchased from Merck (Darmstadt, Germany).

### SPECTROSCOPY

UV spectra were obtained with a Shimadzu UV-1650PC (Shimadzu, Kyoto, Japan) scanning array dual beam spectrophotometer. One pair of 4 mL quartz cuvettes were used in the analysis.

#### EXTRACTION AND QUANTIFICATION OF PHENOLIC COMPOUNDS

Five green dwarf coconut water ( $500 \pm 50$  mL) were lyophilized resulting in a  $21.2 \pm 2.1$  g of a highly hygroscopic mass, with a yield of  $4.2\% \pm 0.4\%$ . The lyophilized (75 mg/mL) was solubilized in methanol 50%. The methanolic extract was centrifuged at 6,000 rpm for 10 min for precipitating the debris. The supernatant was filtered at a  $0.22 \mu\text{m}$  Durapore membrane and 150  $\mu\text{L}$  was analysed by high pressure liquid chromatography coupled to mass spectrometer (HPLC/MS/MS).

#### HPLC-UV/VIS AND HPLC/MS/MS ANALYSIS

The analysis was carried out using a Shimadzu HPLC apparatus coupled to UV-Vis detector SPD-10AVP (Shimadzu, Kyoto, Japan) coupled in mass spectrometer Quattro II (Micromass, Manchester, UK) with source ionization electrospray in positive mode (ESP<sup>+</sup>). Phenomenex<sup>TM</sup> 5  $\mu\text{m}$  Luna C18 100A column (150 X 3.00 mm 5  $\mu\text{m}$  particular size) was used under 20°C, flow 0.6 mL/min and as mobile phase acetic acid 0.1% (A) and acetonitrile with acetic acid 0.1% (B) at gradient: 0 min - 10% of B; 40 min - 90% of B; 45 min - 90% of B; 47 min - 10% of B; 60 min - Stop.

#### ANTIOXIDANT ACTIVITY BY DPPH ASSAY

The radical scavenging activity was determined by the spectrophotometric method based on the reduction of MeOH solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Soler-Rivas et al. 2000). First, 100, 500 and 1,000  $\mu\text{L}$  of each coconut water variety (green dwarf, yellow dwarf, red dwarf, yellow Malaysian), of the industrialized products and the green dwarf lyophilized CW, solubilized in distilled water, were added to 2 mL of MeOH solution of DPPH (90  $\mu\text{M}$ ). After an incubation period (30 min at 24°C), the absorbance was read against a blank of MeOH at 515 nm. The remaining DPPH, measured after a certain time, inversely

corresponded to the radical scavenging activity of the antioxidant. Finally, the inhibition percent (IP) and  $\text{IC}_{50}$  for each sample were calculated using an incubation time of 30 minutes. Quercetin, ascorbic and caffeic acids were used as positive controls.

#### SCAVENGING OF NITRIC OXIDE *in vitro*

In order to evaluate NO scavenging *in vitro*, it was used a nitroprusside solution (NPS) as generator of nitric oxide, as described by Green et al. (1982), in which this volatile radical spontaneously oxidizes to nitrite and then, reacts with sulfanilamide. The diazo compound formed reacts with the hydrochloride of N-(1-naphthyl)-ethylenediamine, generating a pink chromophore. The NPS solution 5 mM was diluted in phosphate buffered saline (0.1 M, pH 7.0) and mixed at the proportion of 0.1, 0.5, and 1.0 mL of the analite: 4 mL of NPS with the analite of each coconut water variety, the industrialized water and lyophilized CW solubilized in distilled water and then incubated at 24°C for 150 min. In parallel, a negative control experiment was conducted with water. In 30 min intervals, 0.5 mL of the mixture was mixed with 1 mL of water and 0.5 mL of Griess reagent (sulphanilamide 1% in  $\text{H}_3\text{PO}_4$  5% and N-(1-naphthyl)-ethylenediamine dihydrochloride 0.1%). The chromophore formed was measured at 546 nm. Inhibition percent (IP) and  $\text{IC}_{50}$  for each sample were then calculated using the incubation time of 150 min. Quercetin, ascorbic and caffeic acids were used as positive controls.

#### DETERMINATION OF ANTIOXIDANT ACTIVITY WITH THIOBARBITURIC ACID (TBARS) *in vitro*

A modified TBARS assay was performed to measure the antioxidant potential capacity using egg yolk homogenate as a lipid rich medium (Hartwig et al. 1993). Briefly, 0.5 mL of 10% (w/v) egg yolk homogenate and 0.1, 0.5 and 1 mL of the sample solutions to be tested - each coconut water variety, the industrialized water (UHT processed and bottle) and the green dwarf lyophilized water solubilized in distilled water,

prepared immediately before use - and 0.9 mL of 10 mM of hydrogen peroxide in water were added to induce lipid peroxidation. After that, 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL 1% (w/v) thiobarbituric acid were subsequently added to the resulting mixture, vortexed and then heated at 90°C for 40 min. After cooling, 3.0 mL of 1-butanol were added to each tube and then extensively vortexed and centrifuged at 1,800 g for 17 min. The absorbance of the organic upper layer was measured using a spectrophotometer (UNICO – UV - 2100 spectrophotometer), set at 532 nm. All the calculated values were based on the antioxidant inhibition percentage index (IP %):  $IP\% = (1 - At / Ac) \times 100$ , where Ac is the absorbance value of the fully oxidized control and At is the absorbance of the test sample. The concentration of the reactive substances with tiobarbituric acid (TBARS) was calculated using the molar absorption coefficient of 157,000/M/cm. Quercetin, caffeic and ascorbic acids were used as positive controls.

#### DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

For this purpose, it was used the Folin-Ciocalteu method, with modifications (Singleton et al. 1999). The concentrations of total phenolics were calculated using the standard curve of gallic acid ( $r=0.9987$ ) and the results were expressed in milligrams of gallic acid equivalents (GAE) per liter.

#### DETERMINATION OF GLUCOSE

The aldo sacharids react with o-toluidine in glacial acetic acid medium producing a greenish coloration, as described by Hyvarinen and Nikkila (1962).

#### DETERMINATION OF TOTAL LIPIDS

The method used for determination of total lipids was described by Postma and Stroes (1968), which consists in the addition of concentrated sulfuric acid and vanillin solution (5 mM) in phosphoric acid (14.3 M), which in the presence of lipids resulting in a pink chromophore quantified in 537 nm.

#### DETERMINATION OF ASCORBIC ACID

The method is based on the oxidation of ascorbic acid to dehydroascorbic acid and subsequent reaction with dinitrophenylhydrazine as described by Natelson (1957), with modifications. Briefly, 500 µL of CW were added to tubes containing 200 µL of dinitrophenylhydrazine reagent (2 g of 2-4 dinitrophenylhydrazine, 250 mg of thiourea and 30 g of  $CuSO_4 \cdot 5H_2O$  dissolved in 100 mL of ice cold  $H_2SO_4$  9N) and placed in a water bath at 37°C for three hours, in order to test if this solution is stable for one week when kept under refrigeration. The solution was cooled in an ice bath, and 800 µL of cold  $H_2SO_4$  65% were added and rested for half an hour at room temperature. Finally, 500 µL of  $H_2SO_4$  65% were added to a final volume of 2 mL to develop the red color which was measured at 520 nm in a spectrophotometer.

#### CELL CULTURE

Lung is one of the organs suffered from high concentration of oxygen and exposed high level of oxidative stress. So lung has good defense systems against oxidative stresses (Pryor et al. 1998, Kelly et al. 2003). To study the effect of caffeic acid on oxidative stress induced by hydrogen peroxide, we used lung fibroblasts (IMR-90). The cells IMR-90 from the American Type Culture Collection were maintained at 37°C in an incubator with a humidified atmosphere of 5%  $CO_2$  and cultured in RPMI containing 1% non-essential amino acid, 10% heat-inactivated fetal calf serum, streptomycin (100m g/mL) and penicillin (100 units/mL).

#### DCFH-DA (2',7'-DICHLORFLUORESCIN-DIACETATE) ASSAY

The accumulation of intracellular ROS was measured using the fluorescent probe DCFH-DA (Cho et al. 2009) in lung fibroblast cells IMR-90. The DCFH-DA can be deacetylated in cells, where it can quantitatively react with intracellular oxidants ( $H_2O_2$ ) to be converted in its fluorescent product,



the DCF, which is retained within cells and thus provides an index of oxidation in cell cytosol. The cells were cultured in a 24-well plate (2 mL of DMEM), washed during 24 h and 20 µg/mL of DCFH-DA were loaded for 20 min. After that, the cells were washed again and then, pre treated for 30 min with the green dwarf lyophilized water (0.1% and 1% in 2 mL DMEM) or ascorbic acid (25 µg/mL) solubilized in water, washed and then exposed to 100 µM of H<sub>2</sub>O<sub>2</sub> for 5 min. The cells were examined at 535 nm with excitation at 485 nm using a fluorescence spectrophotometer (Tecan Infinite M200 with XFluor 4 software, Austria).

#### STATISTICAL ANALYSIS

Values were expressed as the mean ± SD. For multiple comparisons, ANOVA, followed by Tukey's test, were applied. The IC<sub>50</sub> values were obtained by non-linear regressions of concentration-response curves.

#### RESULTS AND DISCUSSION

Coconut water is widely used in tropical countries due to its flavor, nutritional properties and low cost. The results (Table I) demonstrate that, among all the varieties analyzed, glucose and total lipids showed a significant difference in all samples ( $p < 0.05$ ), indicating that CW is a good source of caloric

replacement of glucose and lipids, besides its other nutrients as amino acids and protein (DebMandal and Mandal 2011). Coconut has multifarious utility. The coconut water, the liquid endosperm, is an excellent natural drink with a caloric value of 17.4/100 g. The wide applications of coconut water can be justified by its unique chemical composition of sugars, vitamins, minerals, amino acids and phytohormones (Young et al. 2009, DebMandal and Mandal 2011). The green dwarf variety exhibited the highest amount of total phenols and ascorbic acid (Table I). In addition, the green dwarf variety is the most consumed by the population; therefore it was selected for lyophilization. We made the choice of the concentration (75 mg/mL) to make a correlation between the green dwarf and the process of reconstitution of the lyophilized. The analysis of the lyophilized demonstrated that this process reduced by half the phenolic concentration, possibly due to the drag of low molecular weight phenols associated with the removal of water, however, the ascorbic acid concentration demonstrated no significative change in this process (Table I). In the UHT commercial samples, the phenolic content was very low possibly due to the use of high temperatures at sterilization which degrades compounds and ascorbic acid. Moreover, we consider the possibility that the acid ascorbic was added after the process.

**TABLE I**  
Comparison of the constituents of four coconut varieties, industrialized products and lyophilized coconut water.

Samples	Glucose (mg/dL)	Total Lipids (mg/dL)	Ascorbic Acid µg/mL)	Total Phenolics (µg/mL)
Green dwarf	509.6 ± 1.0 <sup>b</sup>	428.7 ± 99.9 <sup>b</sup>	25.8 ± 0.6	99.4 ± 12.4
Yellow dwarf	192.0 ± 2.9	222.0 ± 46.8	20.1 ± 3.3	23.8 ± 7.5 <sup>a</sup>
Red dwarf	610.1 ± 60.2 <sup>b</sup>	233.1 ± 17.4	22.1 ± 2.1	18.4 ± 3.6 <sup>a</sup>
Yellow Malaysian	807.2 ± 69.1 <sup>b</sup>	392.7 ± 62.3 <sup>b</sup>	11.3 ± 2.5	22.3 ± 6.3 <sup>a</sup>
UHT processed	546.5 ± 1.1 <sup>b</sup>	305.0 ± 22.4	13.9 ± 3.9	10.9 ± 1.1 <sup>a</sup>
Bottle	548.8 ± 1.0 <sup>b</sup>	300.4 ± 24.7	29.1 ± 1.6	13.0 ± 3.1 <sup>a</sup>
Lyophilized (75 mg/mL)	670.1 ± 48.6 <sup>b</sup>	307.4 ± 13.7	21.8 ± 0.8	48.8 ± 10.7 <sup>a</sup>

Data are means ± SD (n=10). (a) These data are significantly different when compared with the Green dwarf group ( $p < 0.05$ ). (b) These data are significantly different when compared with the Yellow dwarf group ( $p < 0.05$ ). For multiple comparisons, ANOVA, followed by Tukey's test, were applied.

The results (Table II) showed that all varieties were able to scavenge the free radical DPPH and the green dwarf variety showed the best antioxidant activity with an  $IC_{50}$  of 150  $\mu\text{g/mL}$  DPPH. In the inhibition analysis of nitrite formation from NPS 5 mM solution, the red dwarf variety was highlighted for its relatively high IP value of 57.7%, followed by the yellow dwarf (IP=38.4%) and the yellow Malaysian (IP=36.5%). Therefore, this activity provide the basis of the popular anti-inflammatory utilization of the red dwarf, since the action of endogenous nitric oxide (NO) is the modulation of the inflammatory state, acting on the platelet aggregation and vasodilatation (Saha and Pahan 2006). As shown by a previous study (Maia et al. 2010), natural substances such as gallic acid have an ability to scavenge nitric oxide. In high concentrations NO can react with superoxide anion and produce potentially harmful peroxynitrite (Peluffo and Radi 2007), which is involved in the genesis of several disorders including induced lipid peroxidation, membrane destabilization (Virág et al. 2003), oxidation, protein nitration, and DNA damage (Ippoushi et al. 2009). Thus, substances able to scavenge NO may inhibit the development of these disorders. In previous studies, we proved that natural substances such as lycopene can protect cultured mammalian cells against biomolecular damage induced by Fe-NTA/ascorbate treatment (Matos et al. 2000), showing the *in vivo* protective effect of lycopene against iron-induced DNA, membrane damage and histopathologic changes in liver of rats (Matos et al. 2001) as well as that both lycopene and  $\beta$ -carotene protected *in vivo* damage of iron-induced oxidative stress in rat prostate (Matos et al. 2006).

As a benchmark for the analysis, we used pure substances such as quercetin and ascorbic acid in the concentrations of total phenols and ascorbic acid found in the green dwarf water. We also used caffeic acid, whose dose was established from the correlation with the concentration of chlorogenic acid, a

derivative of caffeic acid formed from association with quinic acid (Soares 2002) and found in the mesocarp of the same variety (Chakraborty and Mitra 2008). Of these, the ascorbic acid showed the highest antioxidant activity on DPPH radical scavenging and in inhibiting the formation of nitrite, while the caffeic acid, at the concentration used, recorded an IP of only 3.8% for DPPH, but it was not able to reach the  $IC_{50}$  for *in vitro* NO analysis (Table II).

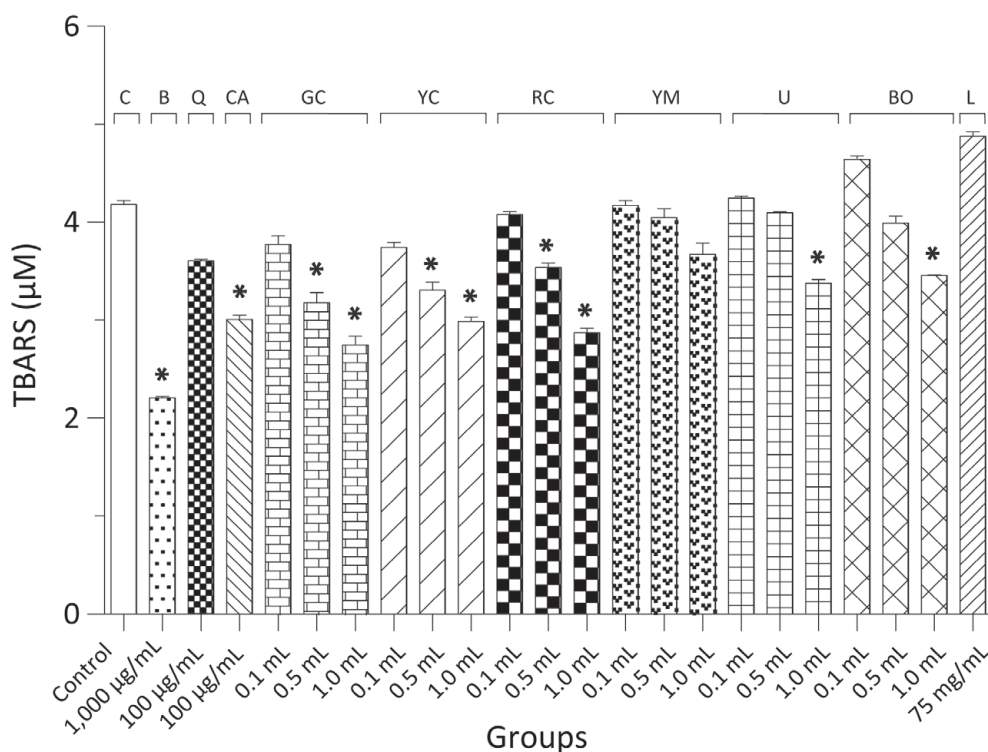
Regarding the TBARS determination, the green dwarf variety demonstrated the best antioxidant activity with 34.4% IP, followed by the red dwarf (IP=31.4%). The results obtained from the analysis of the lyophilized water demonstrated that even though it retained the concentration of ascorbic acid and phenolic content of half the original sample (green dwarf water), it was not able to inhibit the formation of TBARS, therefore causing a significant increase ( $p < 0.05$ ) in the same, when compared to the negative control. The yellow Malaysian and the lyophilized water showed low antioxidant activity. Among the pure compounds tested, the caffeic acid showed the best activity with 28.2% IP, close to the BHT result, a known standard antioxidant (Figure 1). The measured percentage of TBARS inhibition showed a high correlation with the total phenols levels (red dwarf:  $r = 1.000$ ; green dwarf:  $r = 0.988$ ; yellow dwarf:  $r = 0.988$ ; yellow Malaysian:  $r = 0.976$ ; UHT:  $r = 0.956$ ; Bottle:  $r = 0.993$ ; Lyophilized:  $r = 0.866$ ). Therefore, the total phenols levels may be responsible for the antioxidant activity against the inhibition of formation of TBARS.

The green dwarf variety was selected for chemical characterization by HPLC and assessment of its antioxidant potential in cultured lung fibroblasts (IMR-90) as it is the most widely consumed and showed the best results in the antioxidant screening. Regarding the cell culture, fibroblasts were chosen taking in account that they usually exist in the lung, which is a highly vascularized organ with a large positive oxygen pressure, creating an environment adequate to oxidative reactions.

TABLE II

Samples	NO			DPPH	
	IP (%)	IC <sub>50</sub> (µg/mL NPS)	Nitrite concentration (µM)	IP (%)	IC <sub>50</sub> (µg/mL DPPH)
Nitroprusside 5 mM	----	----	26.8 ± 1.5 <sup>b</sup>	----	----
Caffeic acid (30 µg/mL)	18.6	ND	21.8 ± 0.7 <sup>b</sup>	3.8	3.5
Ascorbic acid (25 µg/mL)	57.0	20.3	11.5 ± 1.7 <sup>a</sup>	28.1	5.5
Quercetin (100 µg/mL)	40.5	697.5	15.4 ± 1.5 <sup>a</sup>	8.1	25.4
Green dwarf (0.1 mL/mL)	29.9	219.7	18.8 ± 2.0 <sup>ab</sup>	38.3	150.0
Yellow dwarf (0.1 mL/mL)	38.4	638.1	16.5 ± 2.4 <sup>a</sup>	38.1	186.0
Red dwarf (0.1 mL/mL)	57.7	25.5	10.2 ± 1.3 <sup>a</sup>	22.7	194.9
Yellow Malaysian (0.1 mL/mL)	36.5	81.7	17.0 ± 4.4 <sup>a</sup>	3.6	390.2
UHT processed (0.1 mL/mL)	52.3	14.5	12.8 ± 1.8 <sup>a</sup>	51.3	30.2
Bottle (0.1 mL/mL)	19.7	234.3	21.5 ± 2.0 <sup>b</sup>	14.5	156.0
Lyophilized (75 mg/mL)	57.3	175.9	11.4 ± 2.2 <sup>a</sup>	20.2	549.2

ND – non-detected. Data are means  $\pm$  SD (n=10). **(a)** These data are significantly different when compared with the nitroprusside group ( $p<0.05$ ). **(b)** These data are significantly different when compared with the ascorbic acid group ( $p<0.05$ ). For multiple comparisons, ANOVA, followed by Tukey's test, were applied.

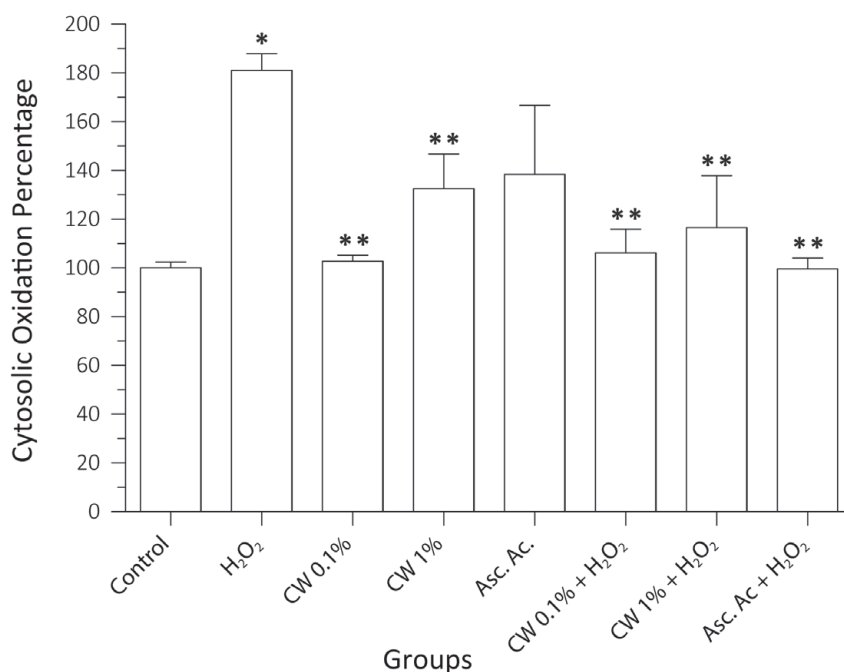


**Figure 1** - Effects of four different coconut water varieties, industrialized and the lyophilized water in preventing TBARS formation. (C) Negative control; (B) BHT; (Q) Quercetin; (CA) Caffeic acid; (GC) Green dwarf; (YC) Yellow dwarf; (RC) Red dwarf; (YM) Yellow Malaysian; (U) UHT processed; (BO) Bottle; (L) Lyophilized. Data are means  $\pm$  SD (n=10). (\*) These data are significantly different when compared with the negative control (p<0.05). For multiple comparisons, ANOVA, followed by Tukey's test, were applied.



The results obtained in evaluating the antioxidant potential in the cytosol of cultured fibroblasts ( $1 \times 10^6$  per plate) confirmed that there was a significant difference ( $p < 0.05$ ) among all groups when compared with the  $H_2O_2$  group, showing that the pretreatment with coconut water was able to reduce the concentration of intracellular ROS when compared to the ascorbic acid (Figure 2). In a previous study it was demonstrated that treatment of WI-38 lung fibroblast cells with caffeic acid (0.5, 5 and 5  $\mu M$ ) reduced the fluorescence intensity by  $H_2O_2$  treatment, reflecting a reduction of ROS

generation (Kang et al. 2006). Fonseca et al. (2009) confirmed the presence of long chain fatty acids such as the palmitic and oleic acids in green dwarf water, as well as the possibility that  $\beta$ -oxidation, occurring at the peroxisome level, generated a slight rise of peroxide, which is responsible by increasing intracellular ROS in CW 1% group. About the ascorbic acid treated group, because of its high concentration in the cytosol of cells, there is the possibility that ascorbic acid interfered in the method, since ascorbate may be a reducing agent, such as it occurs in yeast (Monteiro et al. 2007).



**Figure 2** - Accumulation of intracellular ROS expressed in cytosolic oxidation percentage. The samples were pre-incubated with Coconut Water (CW) at 0.1 and 1 % of final concentration or Ascorbic Acid (Asc. Ac.) 25  $\mu g/mL$  at the well plate, washed and exposed to the same equitoxic concentrations of  $H_2O_2$  (100  $\mu M$ ) for 5 minutes and measured after 20 minutes of DCFH-DA incubation. A negative control group using distilled water (Control) was performed. Data are means  $\pm$  SD ( $n=3$ ). (\*) These data are significantly different when compared with the Control group ( $p < 0.05$ ). (\*\*) These data are significantly different when compared with the  $H_2O_2$  group ( $p < 0.05$ ). For multiple comparisons, ANOVA, followed by Tukey's test, were applied.

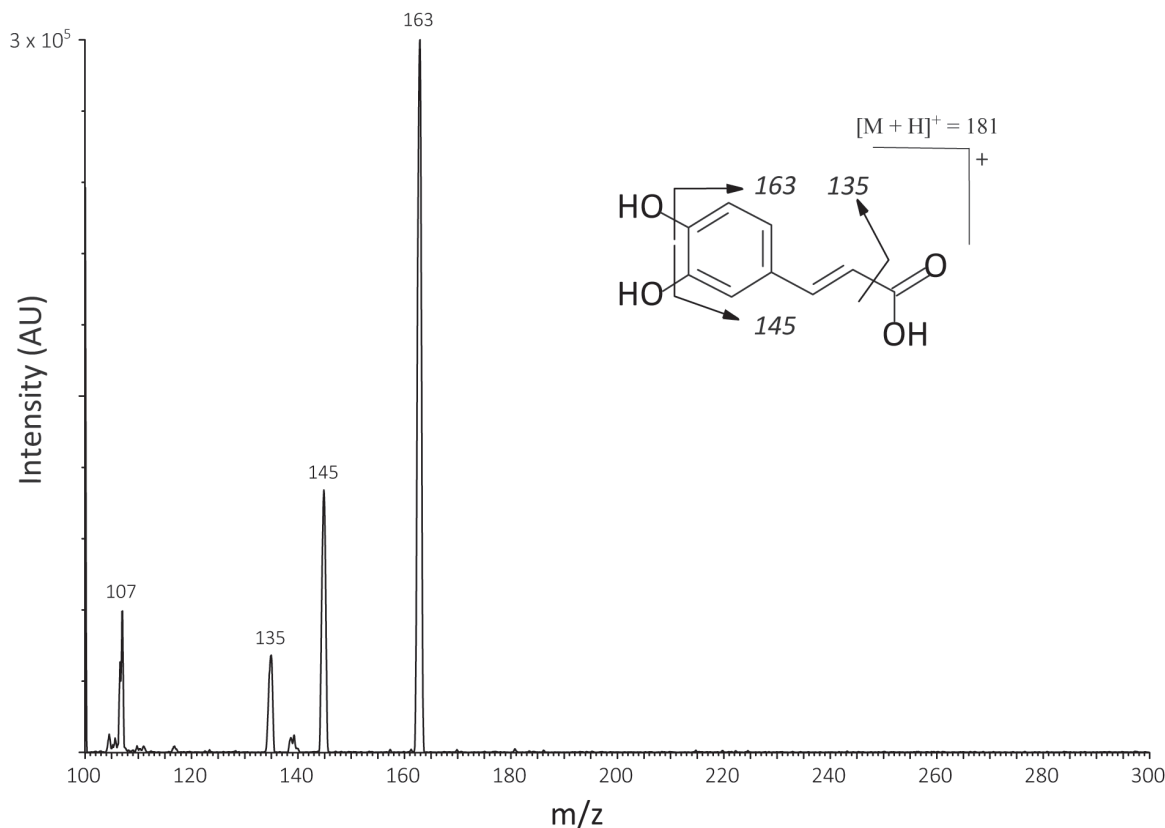
Before that works showed the presence of chlorogenic acid, a derivative of caffeic acid in the mesocarp of *Cocos nucifera* (Chakraborty and Mitra 2008). Considering that this portion is

in close contact with the coconut water, we were to investigate if caffeic acid is a compound of coconut water. First, we developed a sensitive methodology by HPLC/MS/MS in  $ESP^+$  (electron

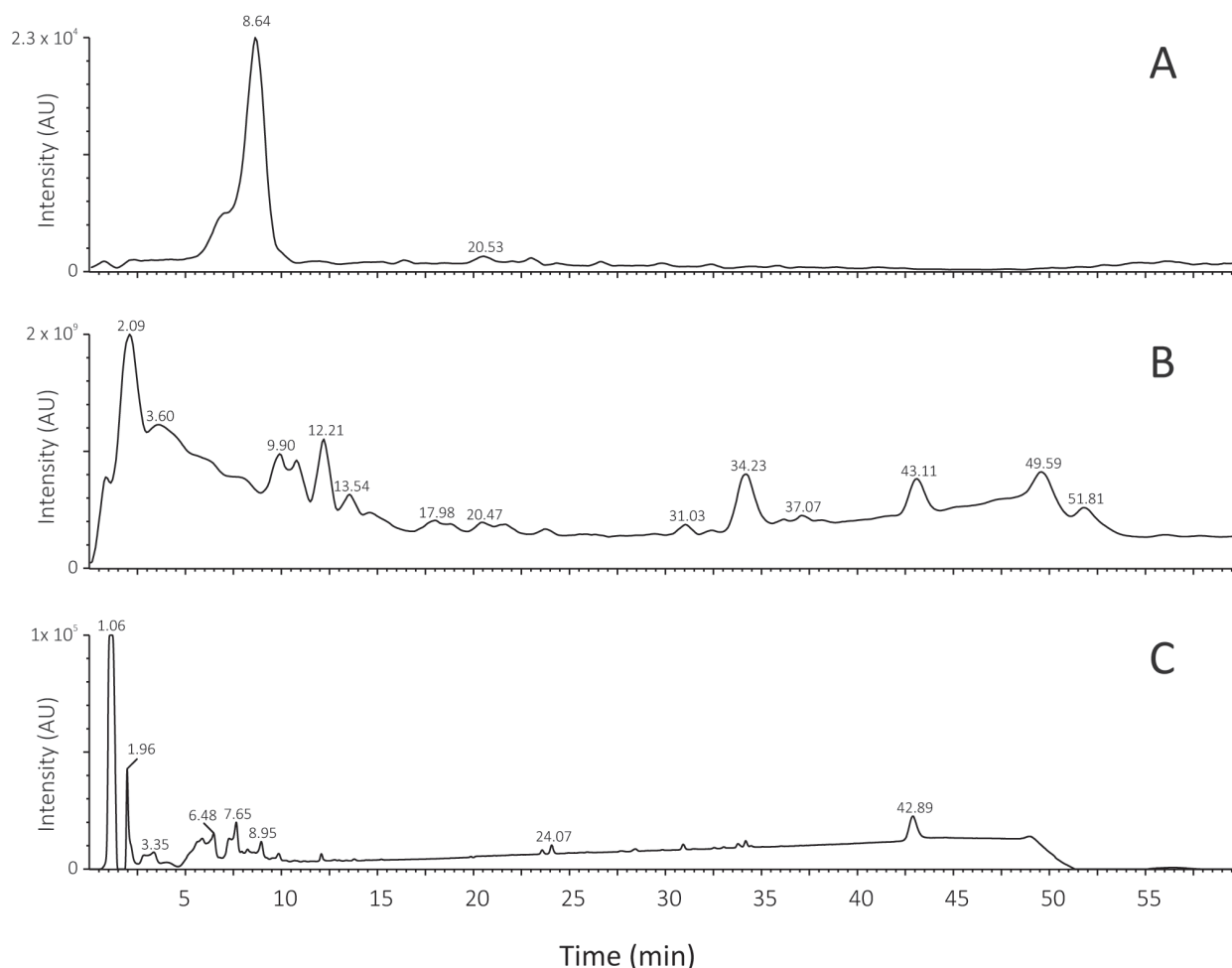
spray ionization in positive mode) to quantification of the caffeic acid. For this method, as showed in profile fragmentation of the Figure 3, the caffeic acid (standard Sigma) forming majority  $m/z = 181 \rightarrow 163$  at a retention time (RT) of the 8.2 minutes (dates no showed). In Figure 4, we can see that when this transition ( $m/z = 181 \rightarrow 163$ ) is monitored in the LCW (150  $\mu\text{L}$  of a solution 75 mg/L in MeOH 50%) we found a pick in same RT of the caffeic acid. For this results, we can prove that caffeic acid is present in coconut water in a concentration of  $1.078 \pm 0.013 \mu\text{g/mL}$ .

This study highlights the importance of coconut water as a nutritious food and its antioxidant properties. All varieties showed good antioxidant potential, highlighting the green dwarf which showed the best results in scavenging

DPPH radical and in protecting lipids against the formation of TBARS, as well as recording the highest levels of total phenols and vitamin C. We also observed that the industrialized processes, such as UHT and lyophilization drastically reduced the coconut water contents and, therefore, its therapeutic properties. Moreover, in cell culture the green dwarf water was effective in protecting fibroblasts against damage induced by hydrogen peroxide. Finally, the chromatographic analysis confirmed the presence of the caffeic acid ( $1.078 \pm 0.013 \mu\text{g/mL}$  or 539  $\mu\text{g}$  for units of coconut consumed,  $500 \pm 50 \text{ mL}$ ), which proved to be effective in inhibiting the *in vitro* TBARS formation. Although we achieved our goals, there is still a huge gap in the determination of coconut water constituents.



**Figure 3** - Profile fragmentation of standard caffeic acid. The analysis by mass spectrometry Quattro II (Micromass, Manchester, UK) with source ionization  $\text{ESP}^+$  monitoring of the  $m/z = 181 \rightarrow 163$ . Parameters of the mass spectrometer: Source temperature 100  $^{\circ}\text{C}$ ; Desolvation temperature 150  $^{\circ}\text{C}$ ; Capillary voltage 2.5 kV; Cone voltage 25 V, Collision energy 15 eV.



**Figure 4** - HPLC/MS/MS analysis of the lyophilized coconut water (LCW). It was analyzed 150  $\mu$ L of a methanolic solution (MeOH 50%) 75 mg/mL in column Luna C18 150 x 3 mm 5  $\mu$ m particular size, under 20  $^{\circ}$ C, flow 0.6 mL/min and using as mobile phase (A) Acetic acid 0,1% and (B) ACN with 0,1% acetic acid. The total flow (0.6 mL/min) was divided by splitter, going to mass spectrometry 0.135 mL/min. **A** - monitoring of the  $m/z = 181 \rightarrow 163$ ; **B** - Total scan to  $ESP^{+}$ ; **C** - chromatogram in 320 nm. Parameters of the mass spectrometer: Source temperature 100  $^{\circ}$ C; Desolvation temperature 150  $^{\circ}$ C; Capillary voltage 2.5 kV; Cone voltage 25 V, Collision energy 15 eV.

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#### RESUMO

A água de coco contém várias substâncias não caracterizadas e é amplamente utilizada na alimentação humana. Neste estudo, foram detectados e quantificados ácido ascórbico e ácido cafeico e polifenóis totais em diversas variedades de coco, e usando HPLC/MS/MS ( $25,8 \pm 0,6$   $\mu$ g/mL,  $1,078 \pm 0,013$   $\mu$ g/mL and  $99,7$   $\mu$ g/mL, respectivamente, em água de coco do anão verde, ou 10 mg, 539  $\mu$ g e 39,8 mg por unidades de coco consumido,  $500 \pm 50$  mL). O potencial antioxidante da água de coco das quatro variedades de

coqueiro (anão verde, anão amarelo, anão vermelho e amarelo malasiano) foi comparada com água de coco industrializada e água liofilizada da variedade anão verde. Todas as variedades foram eficazes - com destaque para as propriedades antioxidantes do anão verde, uma vez que é o mais comum usado - no sequestro do radical DPPH ( $IC_{50}=73 \mu L$ ), óxido nítrico (0,1 mL com IP de 29,9%) e inibindo a produção de espécies reativas com o ácido tiobarbitúrico (1 mL com IP de 34,4%) *in vitro*. Em cultura de células, a água de coco da variedade anão verde foi eficiente na proteção contra danos oxidativos induzidos por peróxido de hidrogênio.

**Palavras-chave:** Atividade antioxidante, ácido ascórbico, ácido cafeico, *Cocos nucifera*, polifenóis.

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