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Reduction of the DNA damages, Hepatoprotective Effect and Antioxidant Potential of the Coconut Water, ascorbic and Caffeic Acids in Oxidative Stress Mediated by Ethanol

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ABSTRACT

Hepatic disorders such as steatosis and alcoholic steatohepatitis are common diseases that affect thousands of people around the globe. This study aims to identify the main phenol compounds using a new HPLC-ESI$^+$-MS/MS method, to evaluate some oxidative stress parameters and the hepatoprotective action of green dwarf coconut water, caffeic and ascorbic acids on the liver and serum of rats treated with ethanol. The results showed five polyphenols in the lyophilized coconut water spiked with standards: chlorogenic acid (0.18 μM), caffeic acid (1.1 μM), methyl caffeate (0.03 μM), quercetin (0.08 μM) and ferulic acid (0.02 μM) isomers. In the animals, the activity of the serum γ-glutamyltranspeptidase (γ-GT) was reduced to 1.8 I.U/L in the coconut water group, 3.6 I.U/L in the ascorbic acid group and 2.9 I.U/L in the caffeic acid groups, when compared with the ethanol group (5.1 I.U/L, $p<0.05$). Still in liver, the DNA analysis demonstrated a decrease of oxidized bases compared to ethanol group of 36.2% and 48.0% for pretreated and post treated coconut water group respectively, 42.5% for the caffeic acid group, and 34.5% for the ascorbic acid group. The ascorbic acid was efficient in inhibiting the thiobarbituric acid reactive substances (TBARS) in the liver by 16.5% in comparison with the ethanol group. These data indicate that the green dwarf coconut water, caffeic and ascorbic acids have antioxidant, hepatoprotective and reduced DNA damage properties, thus decreasing the oxidative stress induced by ethanol metabolism.

Key words: Coconut water, ethanol, oxidative stress, phenolic compounds.

INTRODUCTION

Cocos nucifera L., also known as "life tree" (Chan and Elvitch 2006), is a monocotyledon plant that belongs to the Palmae family. Although it is not genuinely Brazilian, it became popular in the country's seacoast due to its easy cultivation and adaptation to the local soil and climate (Lopes and Larkins 1993). Coconut water is consumed as a refreshing (Santoso et al. 1996), tasty and

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nutritious drink, which can be a good source of minerals and is a natural isotonic (Borse et al. 2007) with similar composition to that of saline. Its polyphenolic composition has been associated with the inhibition of acyclovir-resistant herpes simplex virus type 1 (Esquenazi et al. 2002), cell proliferation (Kirszberg et al. 2003), leishmanicidal effects (Mendoça-Filho et al. 2004), free radical scavenging activities (Alviano et al. 2004) and antiglycation, hypoglycemic and nephroprotective activities in an experiment with rats with alloxan-induced diabetes (Pinto et al. 2015).

Studies that analyze coconut water (CW) detected the presence of *trans*-zeatin-O-glucoside, dihydrozeatin-O-glucoside (Ge et al. 2004), *ortho*-topolin (Ge et al. 2005b), kinetin, kinetin riboside (Ge et al. 2005a) and 14-O-(3-O-[beta-D-galactopyranosyl-(1-->2)-alpha-D-galactopyranosyl-(1-->3)-alpha-L-arabinofuranosyl]-4-O-(alpha-L-arabinofuranosyl)-beta-d-galactopyranosyl)-trans-zeatin riboside (Kobayashi et al. 1995, 1997). Both (+)-catechin and (-)-epicatechin were found in the coconut husk fiber and water (Chang and Wu 2011). Previous work done by our group showed the presence of caffeic acid (approximately 0.025 mg/mL CW) in lyophilized green dwarf coconut water and IMR-90 fibroblast protection against H2O2-induced stress after coconut water supplementation. We also found that green dwarf coconut water has higher polyphenols and ascorbic acid content between six types of coconut waters (Santos et al. 2013). All these features increase the nutritive property of green dwarf coconut water.

The role of dietary polyphenols on human health has been intensively research over the last 15 years. Polyphenols are universally present in plant-derived food products. These metabolites have been associated with the health benefits that plant-derived food provide to humans when consumed as part of a balanced diet. In human nutrition, phenolic compounds have received special attention, mostly because these compounds are considered to be potential antioxidants (Martin and Appel 2010). In addition to that, *in vitro* studies have shown that phenolic compounds have anticancer and chemopreventive properties in human lung tissue (Lin and Weng 2006). It has also been shown that quercetin and naringenin flavonoids lower the risk for lung cancer (Marchand et al. 2000).

Globally, harmful use of alcohol causes approximately 3.3 million deaths every year (or 5.9% of all deaths), and 5.1% of the global burden of disease is attributable to alcohol consumption (WHO 2014). There is extensive knowledge related to the causal relationship between alcohol consumption and more than 200 health conditions, including harmful use of alcohol and the incidence and clinical outcomes of infectious diseases such as tuberculosis, HIV/AIDS and pneumonia (WHO 2014). In 2004 Brazil was the 4th consumer of alcohol in the world, with 195 million liters per year (Meloni and Laranjeira 2004). The ingestion of ethanol alone or its combination with other factors cause liver injuries like steatosis, alcoholic steatohepatitis, alcoholic hepatitis, and cirrhosis (Kessova et al. 2003). The pathogenesis of liver diseases caused by alcohol abuse comes from the interaction of several factors, including the generation of oxidants and reactive metabolites from ethanol oxidation, which, as a result, cause other metabolic derangements (Terrence 2009). Corroborating this, several authors demonstrated that the generation of free radicals can be involved in the pathogenesis and progression of several disorders (Yuan 2009, Ippoushi 2009) and the substances which scavenge free radicals can act by modulating or inhibiting the appearance of these diseases (Saravanan et al. 2006).

Thus, the aims of this study were to establish a methodology based on HPLC-MS/MS to identify the main phenol compounds of green dwarf coconut water and to evaluate the effects of green dwarf coconut water, ascorbic and caffeic acids on

parameters of oxidative stress and liver damage induced by ethanol in Wistar rats.

MATERIALS AND METHODS

VEGETABLE MATERIALS

The green dwarf coconuts were obtained from *Empresa Brasileira em Pesquisa Agropecuária* (EMBRAPA) – *Tabuleiros Costeiros* – Aracaju - SE/Brazil. We chose young samples (3-4 months), in good state (without bruises or visible alterations) and from the same tree. After that, the coconuts were properly cleaned with water, soap and 70% ethanol and stored at room temperature.

EXTRACTION AND QUANTIFICATION OF PHENOL COMPOUNDS

The coconut water from five green dwarf coconuts (500 ± 50 mL) was lyophilized, which resulted in 25.3 ± 2.4 g of highly hygroscopic mass, with a yield of 8.3 ± 1.2%. A seventy-five mg/mL of lyophilized coconut water (LCW) in acetonitrile (ACN) 10% with formic acid 0.1% solution was prepared and centrifuged at 3600 *g* for 10 minutes for debris precipitation. The supernatant was filtered using a 0.22 µm *Durapore* membrane and 150 µL of it was injected in high-pressure liquid chromatography coupled with a mass spectrometer -HPLC/MS/MS (Santos et al. 2013). All the samples were assayed in triplicate and the results were expressed as the mean ± standard deviation as 'mg phenolic compounds/mg LCW'.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY (HPLC-ESI$^+$-MS/MS)

HPLC-ESI$^+$-MS/MS analyses in the positive mode were carried out using a Quattro II mass spectrometer (Micromass, Manchester, U.K.) in the selected reaction monitoring mode (SRM). A Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of an auto-sampler (SIL-10AD/VP), an automated switching valve (FCV-12AH), two pumps (Class LC 10AD), an SPD-10AV/VP UV detector controlled by a communication bus module (SCL-10A/VP-CBM 10A), and a Class-VP software were used for sample injection and cleanup of the analytical column (Kinetex C-18 XB 100 mm x 4.6 mm i.d., 2.6 µm, 100 Å, Phenomenex, Torrance, CA). The sample was eluted from the column with flow of 0.175 mL/min and a linear gradient of (A) formic acid 0.1% in water and (B) acetonitrile with 0.1% formic acid as follows: 0 min – 10% of B, 25 min – 90% of B, 30 min – 90% of B, 43 min 10% of B – 50 min.

The standard compounds were individually dissolved in methanol and diluted at chosen concentrations in a mixed solution with ACN 10% with formic acid 0.1% for quantification. This solution was injected into the HPLC-ESI$^+$-MS/MS for simultaneous monitoring of the following *m/z* (Table I).

TABLE I
Polyphenols standards with respective fragmentation pattern, collision energy and retention time.

Compound	Monitored m/z	Collision energy (eV)	Retetion time (RT) min.
Chlorogenic acid	355 → 163	30	22.32
Caffeic acid	181 → 135	25	23.84
Rutin	611 → 303	25	24.15
Ferulic acid	195 → 145	20	26.31
Methyl caffeate	195 → 163	20	28.40
Cinnamic acid	149 → 103	20	30.54
Quercetin	303 → 153	40	30.87
Naringenin	273 → 153	30	30.91

The data were processed using the software MassLynx 4.1 (Waters, Milford, USA).

The transitions of the compounds were obtained through fragmentation of standards. The calibration curves were diluted in three replicates on the same day and interday to evaluate the precision of the method. The curves were accepted when $r^2 \geq 0.99$ (Hsieh et al. 2005). The limit of quantification (LOQ) was obtained by analysis of progressively lower analyte concentrations still S/N = 10. The coefficient of variation was calculated by standard deviation/average. The RSD% was determined as (SD/measured mass concentration) × 100. All analytic methods were validated according to guidelines for validation of analytical procedures (ICH 2005) by the International Councilfor Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).

METHOD VALIDATION

The different concentrations of standards were analyzed in order to obtain the optimal concentration ranges by LC–MS/MS. The linear calibration curves of standard with three replicates for the intraday assay on the same day and for the inter-day assay on three different days, respectively. All calibration curves (peak-areas versus mass concentrations) of the standards chlorogenic acid; caffeic acid; rutin; ferulic acid; methyl caffeate; quercetin; naringenin and cinnamic acid were carried out with the least correlation coefficients of 0.9981 and 0.9997, respectively (Hsieh et al. 2005). The limit of quantification (LOQ), defined as S/N of 10 were determined by analyzing progressively lower analyte concentrations until the respective S/N ratios were obtained. The coefficient of variation was calculated by standard deviation/average. The RSD% was determined as: (SD/measured mass concentration) × 100%. The relative error (RE) % was determined as: [(measured mass concentration _ actual mass concentration)/(actual mass concentration)] × 100%.

ANIMALS AND TREATMENT PROTOCOL

Male Wistar rats weighing 250 ± 50 g were obtained from the *Bioterio Central* of the Federal University of Sergipe. The animals were kept under controlled temperature of 25ºC ± 2ºC, light/dark cycle of 12 h, water *ad libitum* and with food privation of 8 hours daily. All procedures were in accordance with the Ethics in Animal Research Committee – CEPA/UFS (03/2010). After one week of acclimatization, the animals were divided into 6 (six) groups (n=5):

i) Saline (Sal), which received saline orally (v.o.) during the whole experiment;
ii) Ethanol (Eth), received 2.5 mL/Kg of ethanol 35% (w/v in water) twice a day v.o for 4 days according to Wang and Cederbaum, (2006), with modifications;
iii) Coconut Water + Ethanol (CW+Eth), which ingested 3 mL/Kg of coconut water, followed by (after one hour) 2.5 mL/Kg of ethanol 35%, both twice a day v.o for 4 days;
iv) Ethanol + Coconut Water (Eth+CW), 2.5 mL/Kg of ethanol 35%, followed by (after one hour) 3 mL/Kg of coconut water, both twice a day v.o for 4 days;
v) Ascorbic acid (L-Threoascobic acid - 25 µg/mL) + Ethanol (Asc+Eth), 3 mL/Kg in which ascorbic acid was administered and, one hour later, 2.5 mL/Kg of ethanol 35%, both twice a day v.o for 4 days;
vi) Caffeic Acid (3, 4 Dihydroxycinnamic acid) - 100 µg/mL solution in Tween 20 (5%) + Ethanol (Caf+Eth), ingested 3 mL/Kg of caffeic acid solution followed by ethanol 35% one hour later, both twice a day v.o for 4 days.
vii) For each day of treatment was used a different coconut from a single bunch.

PLASMA AND LIVER SAMPLES

Twenty-four hours after the last day of the treatment, with overnight food privation, blood

samples were collected by EDTA containing tubes from the abdominal aorta under anesthesia with xilazine chloridrate 5% (25 µL/50 g body weight) and ketamine 10% (50 µL/50 g body weight) and then immediately centrifuged to obtain the plasma samples. The liver was removed, washed with ice-cold physiological saline and frozen in liquid nitrogen.

ANALYSES OF PLASMA AND LIVER

Thiobarbituric acid-reactive substances (TBARS) assay

Lipid peroxidation was determined based on the amount of thiobarituric acid-reactive substances (TBARS) formed (Hartwig et al. 1993). The lipid peroxidation produced substances, especially malondialdehyde (MDA), which reacted with the **thiobarbituric** acid (TBA), generating a pink chromophore that absorbed light between 530-535 nm for spectrophotometer, using an extinction coefficient of 157.000 M-1 cm-1.

Dosage of protein

The dosage of protein was measured according to the method by Bradford (1976), based on the connection between Coomassie Blue G-250 and protein forming a chromophore of intense blue color.

Catalase activity (CAT)

CAT activity was measured using an adapted method by Beutler (1975). Hydrogen peroxide decomposition was recorded at 240 nm with a spectrophotometer. The activity was expressed in micromoles of H_2O_2 per minute per milligram of protein, or $U \times g^{-1}$ of protein.

Aspartate aminotransferase (AST) and alanine aminotransferase transaminases (ALT)

AST and ALT activities in plasma were measured using the method by Reitman and Frankel (1957). The pyruvate and oxalacetate were measured by producing a substance with intense color in alkaline medium (hidrazone). The absorbance was read at 505 nm for spectrophotometer.

Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG)

The GSH level was measured using the method described by Jollow et al. (1974) and GSSG by Bartoli et al. (1978). The ratio between them was calculated by GSSG/GSH.

HDL, Total Cholesterols and Triglycerides

Total, HDL cholesterol and triglycerides levels were measured by colorimetric assay using commercial Katal's kits purchased from Katal Biotecnológica Indústria e Comércio (MG – Brazil).

Activity γ-glutamyltranspeptidase (γ-GT)

It was measured by colorimetric assay using the commercial kits from Doles (GO – Brasil).

DNA extraction and enzymatic hydrolysis

DNA was isolated using the modified chaotropic NaI method, as previously described (Wang et al. 1994). Briefly, the tissues (500 mg) or the cellular pellets were homogenized in 10 mL of a lysis solution (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, 0.1 mM desferroxamine, and 1% (v/v) Triton X-100 at pH 7.5). After centrifugation at 1500 *g* for 10 min, the pellets were suspended again in 10 mL of the lysis solution and centrifuged one more time at 1500 *g* for 10 min. The pellets were then suspended in 6 mL of 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 0.15 mM desferroxamine, and 350 μL of 10% SDS. The enzymes RNAse A (30 μL, 10 mg/mL) and RNAse T1 (4 μL, 20 U/μL) in 10 mM Tris- HCl

buffer (pH 7.4) containing 1 mM EDTA and 2.5 mM desferroxamine were added, and the reaction mixture was incubated at 37°C. After 1 h, 300 μL of proteinase K (20 mg/ mL) was added, and the reaction was incubated at 37°C for 1 h. After centrifugation at 5000 *g* for 15 min, the liquid phase was collected, 1 mL of a solution containing 7.6 M NaI, 40 mM Tris-HCl (pH 8), 20 mM EDTA, and 0.3 mM desferroxamine was added, and then 5 mL of isopropanol was added. The contents of the tube were mixed well by inversion until a white precipitate appeared. The precipitate was collected by centrifugation at 5000 *g* for 15 min, washed with 5 mL of 60% isopropanol, centrifuged at 5000 *g* for 15 min, washed with an additional 5 mL of 70% ethanol, and centrifuged at 5000 *g* for 15 min. The DNA pellet was solubilized in 500 μL of desferroxamine (0.1 mM). The DNA concentration was measured with a spectrophotometric device at 260 nm and its purity was assessed by using $A_{260}/A_{280} > 1.75$. The DNA (100 µg) was diluted in 200 µL of deferral 0.1 M, followed by addition of 4 µL ammonium acetate buffer 1 M with 5 units of nuclease P_1, and incubated at 37°C for 30 min. One hundred fifty microliters of 1 M Tris-HCl (pH 7.4) and 3 units of Escherichia coli acid phosphatase were then added, followed by incubation at 37°C for 1 h. The sample was centrifuged and the collected aqueous layer was then analyzed by HPLC-electrochemical detection (ECD).

Analysis of 8-oxo-2'-desoxiguanosina (8-oxodGuo) with HPLC-electrochemical detection (ECD)

Samples (100 µg) of DNA digests were injected on the HPLC-EC system, consisting of a LC-10AD Shimadzu pump connected to a Phenomenex Luna C-18 reverse-phase analytical column 250 x 4.6mm 100°A 5µm particle size. The isocratic eluent was 25 mM potassium phosphate buffer, pH 5.5 and 8% methanol at a 0.8 mL/min flow rate, under 16ºC. Coulometric detection was provided by a Coulochem II detector (ESA, Chemsford, MA). The potentials of the two electrodes were set at 130 and 280 mV. Elution of unmodified nucleosides was simultaneously monitored by UV spectrometer set at 254 nm. The molar ratio of 8-oxodGuo to dGuo in each DNA sample was determined based on coulometric detection at 280 mV for 8-oxodGuo and absorbance at 254 nm for dGuo in each injection (Matos et al. 2000). CLASS-LC 10 software was used for processing the data. Stock standard solutions were prepared by weighing proper quantities of 8-oxodGuo and 2'-deoxyguanosine (dGuo).

STATISTICAL ANALYSIS

The values were expressed by mean ± SD for five animals. ANOVA was used for the statistical analysis, followed by Dunnett's test. The values were significant for $p<0.05$.

RESULTS AND DISCUSSION

Polyphenols are compounds commonly found in plants, and their association with human health has been increasingly evident (Dai and Mumper 2010). However, only a few methods are available to identify these components in natural products accurately. Knowing the range of different phenolic compounds found in different plant species, the development of a rapid, sensitive and specific method to identify such substances is **essential** for understanding their function. Here, we describe the development and validation of a methodology (Table SI – Supplementary Material) for simultaneous quantification of phenolics compounds in coconut water by high pressure liquid chromatography couple to mass spectrometry in tandem with electronspray source ionization in positive mode (HPLC-ESI+-MS/MS) without extensive sample preparation. Eight different phenol compounds were properly detected and quantified within 51

min (Figure 1) of the chromatographic run. In addition, it is able to detected, even in complex sampling such as LCW extract, with only one injection: Chlorogenic acid, Caffeic acid, Ferulic acid, Methyl caffeate, Quercetin (Figure 2).

The methodology permits simultaneous quantification of eight phenolic compounds mixed in solution and five in complex matrices of natural products like LCW. It fulfills the needs of various industries for characterizing and quantifying natural products. Here, a 75 mg/mL solution of LCW in ACN 10% with formic acid 0.1% was prepared and 150 µL was injected in the HPLC system to be analyzed by LC-MS/MS, as described in the Material and Methods section. The analytical result allowed excellent baseline and resolution of the interested peaks of the LCW (Figure 2).

The representative chromatogram for the LCW (75 mg/mL) is shown in Figure 2. Thus, for confirming all phenolic compounds found in the lyophilized coconut water a spike with authentic standards (Figure 3) was performed adding 0.18 µM Chlorogenic acid, 1.1 µM caffeic acid, 0.02 µM ferulic acid, 0.03 µM methyl caffeate and 0.08 µM quercetin to the sample of LCW. All these compounds were found to be antioxidants *in vitro* (Santos et al. 2013, Raneva et al. 2001, Chakraborty and Mitra 2008) and may be responsible for the protective effect against many pathological processes that offer a wide range of biological activities, including the antioxidant, antimicrobial and anticarcinogenic ones attributed to coconut water (Scalbert et al. 2005).

Alcohol is widely consumed around the world (WHO 2014). In Brazil 10% of all health problem are attributed to alcohol consumption (Meloni and Laranjeira 2004), and in Russia it is considered a public health problem, which is responsible for 52% of deaths from men aged 15–54 years (Zaridzea et al. 2009). Conversely, the WHO European Region is home of the most ill and premature death caused by alcohol in the world. About more than 20% of European population aged 15+ years are under high alcohol level consumption (WHO 2016). The generation of oxidants and reactive metabolites from ethanol oxidation is one of the accepted mechanisms of the pathogenesis of hepatic disorders (Terrence 2009). Our results showed that consuming the equivalent to one vodka glass (200 mL in human been) twice a day for 4 days is enough to alter several metabolic parameters of the rats.

In the liver, we observed a significant increase of TBARS formation from the ethanol group in relation to the saline group ($p<0.05$), and decrease in the Eth+CW and ascorbic acid treated groups (Table II and Figure 4). The caffeic group also demonstrated TBARS increase, probably due to its metabolism, as proposed by Moridani et al. (2002), which involves GSH conjugation, and could cause citotoxicity effects. Despite that, all tested groups had a significant decrease of DNA damage in comparison with the ethanol group ($p<0.05$). As showed in Figure 7, the level of 8-oxo-2'-deoxyguanosine increased 85.2% in the ethanol group in comparison with the control group. The reduction of the DNA damages (8-oxodGuo) in the liver was of 36.2% and 48.0% in the coconut water groups, 42.5% in the caffeic acid group, and 34.5% in the ascorbic acid group, in comparison with the ethanol group ($p<0.05$).

In the plasma, the increase of γ-GT, one specific marker of hepatic function, indicates the harmful effect of ethanol with only 4 days of treatment (Table II). The results obtained in our study showed that, with the administration of coconut water and caffeic acid, these levels were reestablished to the same of the saline group. The data of γ-GT are in accordance with the work by Pérez-Alvarez et al. (2001), who used Wistar rats to prove the hepatoprotective effect of caffeic acid, whose activity is directly linked to the hydroxyl in 4 aromatic ring position. The hepatoprotective action was proven by the reduction of transaminase ALT (other hepatic maker) in the plasma of all

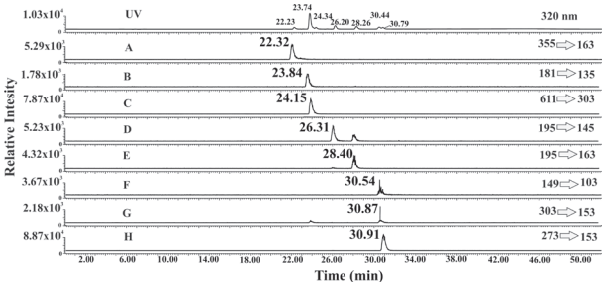

Figure 1 - Simultaneous quantification of phenolic compounds. A mix of these compounds was made in ACN 10% with formic acid 0.1% and injected in the LC/MS/MS to analyze the system in a Kinetex C-18 XB (100 mm x 4.6 mm i.d., 2.6 µm of particle size, 100 Å, Phenomenex, Torrance, CA), under 20°C and 0.175 mL/min flow. As for the mobile phase, it was used (a) formic acid 0.1% and (b) ACN with formic acid 0.1% following linear elution gradient: 0 min –10% of (b), 25 min – 90% of (b), 30 min – 90% of (b), 43 min 10% of (b) – 50 min (stop), coupled with Quattro II mass spectrometer (Micromass, Manchester, UK) in positive mode (ESI^+), 3.5 V on capillary, 25 V on cone, 15 psi of nebulizer gas, 350 L/h of dry gas, 300ºC desolvation temperature and 100ºC block source temperature, A – 4 pmol Chlorogenic acid; B – 80 pmol Caffeic acid; C – 4 pmol Rutin; D – 16 pmol Ferulic acid; E – 16 pmol Methyl caffeate; F – 2000 pmol Cinnamic acid; G – 12 pmol Quercetin; H – 4 pmol Naringenin.

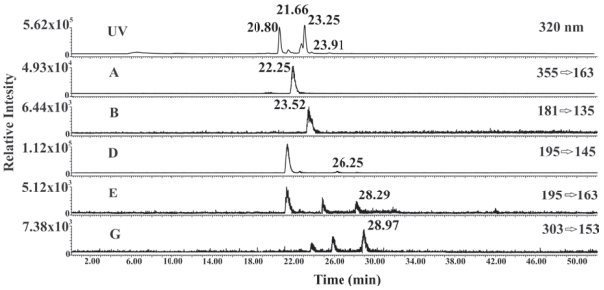

Figure 2 - Chromatogram of the lyophilized coconut water, by LC/MS/MS. Injection of 75 mg/mL solution of LCW in ACN 10% with formic acid 0.1%. The lyophilized CW was analyzed by Kinetex C18 XB (100 mm x 4.6 mm i.d., 2.6 µm of particle size, 100 Å, Phenomenex, Torrance, CA), at 20°C, injection volume of 150 µL and flow rate of 0.175 mL/min. As for the mobile phase, it was used (a) formic acid 0.1% and (b) ACN with formic acid 0.1% following linear elution gradient: 0 min – 10% of (b), 25 min – 90% of (b), 30 min – 90% of (b), 43 min 10% of (b) – 50 min (stop), coupled with Quattro II mass spectrometer (Micromass, Manchester, UK) in positive mode (ESI^+), 3.5 V on capillary, 25 V on cone, 15 psi of nebulizer gas, 350 L/h of dry gas, 300ºC desolvation temperature and 100ºC block source temperature. UV-320 nm; compounds detected - A: Chlorogenic acid; B: Caffeic acid; D: Ferulic acid; E: Methyl caffeate; G: Quercetin.

TABLE II
Concentration of TBARS and catalase activity in liver and ALT, AST, γ-GT in serum of rats treated by ethanol.

Groups	TBARS (nM/g Protein)	CAT (mU/g Protein)	ALT (U/mL plasma)	AST (U/mL plasma)	γ-GT (U/mL plasma)
Saline	1.6 ± 0.1	16.0 ± 0.4	105.0 ± 9.0	271.0 ± 2.0	2.3 ± 0.3
Ethanol	2.0 ± 0.2*	18.2 ± 0.3*	102.5 ± 7.0	266.0 ± 1.6	5.1 ± 0.7*
CW+Eth	1.7 ± 0.1	6.0 ± 0.1**	69.1 ± 6.5**	194.4 ± 1.9	2.7 ± 1.0**
Eth+CW	1.3 ± 0.1**	9.0 ± 0.3**	41.7 ± 6.0**	188.0 ± 1.6	1.8 ± 0.4**
Asc + Eth	1.2 ± 0.1**	5.0 ± 0.2**	67.2 ± 5.0**	228.3 ± 2.0	3.6 ± 1.2
Caf + Eth	2.6 ± 0.1	11.0 ± 0.3	77.3 ± 9.0**	230.6 ± 2.0	2.9 ± 0.6**

Eth – 2.5 mL/Kg of solution 35%; CW+Eth – 3 mL/Kg of coconut water followed by 2.5 mL/Kg ethanol; Eth+CW – 2.5 mL/Kg of ethanol followed by 3 mL/Kg of coconut water; Asc + Eth – 3 mL/Kg of solution 25 µg/µL followed by 2.5 mL/Kg of ethanol; Caf + Eth – 3 mL/Kg of solution 100 µg/µL followed by 2.5 mL/Kg of ethanol. The data are average ± S.D. for five animals. Means values were significant different for $p<0.05$; (*) different when compared with the saline group $p<0.05$; (**) different when compared with the ethanol group $p<0.05$. For multiple comparisons, ANOVA, followed by Dunnett's test, were applied.

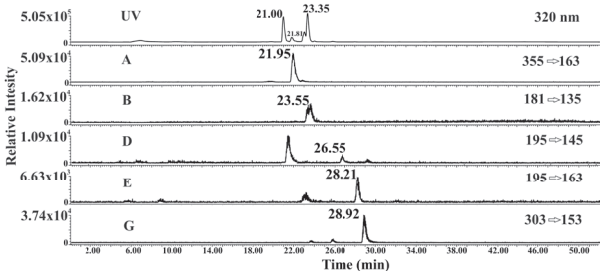

Figure 3 - Chromatogram of the lyophilized coconut water by LC/MS/MS spiked with standards. Injection of 75 mg/mL solution of LCW in ACN 10% with formic acid 0.1% plus mixed standard compounds solution. The lyophilized CW was analyzed by Kinetex C18 XB (100 mm x 4.6 mm i.d., 2.6 µm of particle size, 100 Å, Phenomenex, Torrance, CA), at 20°C, injection volume of 150 µL and flow rate of 0.175 mL/min. As for the mobile phase, it was used (a) formic acid 0.1% and (b) ACN with formic acid 0.1% following linear elution gradient: 0 min – 10% of (b), 25 min – 90% of (b), 30 min – 90% of (b), 43 min 10% of (b) – 50 min (stop), coupled with Quattro II mass spectrometer (Micromass, Manchester, UK) in positive mode (ESI^+), 3.5 V on capillary, 25 V on cone, 15 psi of nebulizer gas, 350 L/h of dry gas, 300ºC desolvation temperature and 100ºC block source temperature. UV-320 nm; Added compounds and respective concentrations - A: 0.18 µM Chlorogenic acid; B: 1.1 µM Caffeic acid; D: 0.02 µM Ferulic acid; E: 0.03 µM Methyl caffeate; G: 0.08 µM Quercetin.

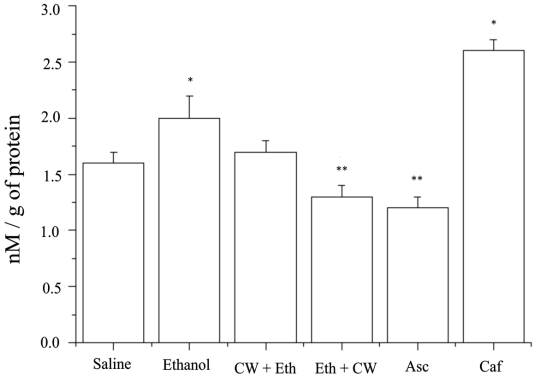

Figure 4 - Effect of CW, ascorbic and caffeic acid in the liver lipid peroxidation of rats treated by ethanol. Eth – 2.5 mL/Kg of solution 35%; CW+Eth – 3 mL/Kg of coconut water followed by 2.5 mL/Kg ethanol; Eth+CW – 2.5 mL/Kg of ethanol followed by 3 mL/Kg of coconut water; Asc + Eth – 3 mL/Kg of solution 25 µg/µL followed by 2.5 mL/Kg of ethanol; Caf + Eth – 3 mL/Kg of solution 100 µg/µL followed by 2.5 mL/Kg of ethanol. The data are average ± S.D. for five animals. Means values were significant different for $p<0.05$; (*) different when compared with the saline group $p<0.05$; (**) no difference for saline group but different of ethanol group $p<0.05$. For multiple comparisons, ANOVA, followed by Dunnett's test, were applied.

treated groups in relation to the saline ($p<0.05$). The ethanol group did not present differences for this enzyme (Table II). Still, in the plasma, the caffeic acid treated group reestablished the glutathione levels when compared with the control group ($p<0.05$), corroborating with Raneva et al. (2001), who demonstrated that caffeic acid did not exhibit toxic effects and showed antioxidant activity in plasma. This effect was similar to the ascorbic acid, a well-known antioxidant (Choi et al. 2003) (Figure 6).

Additional data showed that lipidic profile did not show significant differences ($p<0.05$) in LDL, HDL and total cholesterol, with exception of the triglycerides, which increased significantly ($p<0.05$) in the ethanol group and decreased in all treated groups ($p<0.05$) (table III). This might have happened because the ethanol metabolism increased the $NADH/NAD^+$ ratio changing the redox state balance in hepatocytes (Zakhari 2006), which mobilizes fatty acids for accumulation instead oxidation, resulting on fatty liver condition that lead to impairments of others metabolic pathways increasing the serum triglycerides. Thus, beyond antioxidant property of coconut water, ascorbic and caffeic acid modulating positively triglycerides levels (Madrigal-Santillán et al. 2015), impairment on glucose and lipid metabolism could be improved by glucose, ascorbic and caffeic acid present in coconut water, leading to reduction on triglyceride serum content (Braun et al. 1996, Jung et al. 2006).

Reduced glutathione (GSH) and its oxidized counterpart, GSSG, serve as the major low-molecular-weight redox buffer in eukaryotic cells. Owing to its high abundance (5–10 mM), glutathione has a pivotal role in keeping cytosolic proteins reduced and in protecting cells against reactive oxygen species. The reduced state of glutathione is maintained by the enzyme glutathione reductase, which in turn oxidizes the general cellular electron donor NADPH (Winther and Jakob 2013). Regarding the animals in all

TABLE III
Effect of coconut water, ascorbic and caffeic acids in the lipid profile of rats treated by ethanol.

Groups	Total Cholesterol (mg/dL plasma)	Cholesterol HDL (mg/dL plasma)	Cholesterol LDL (mg/dL plasma)	Triglycerides (mg/dL plasma)
Saline	43.3 ± 3.4	13.9 ± 2.8	17.0 ± 5.0	62.3 ± 2.2
Ethanol	50.8 ± 0.7	19.9 ± 11.7	8.6 ± 3.0	111.6 ± 3.1*
CW+Eth	41.7 ± 5.3	29.8 ± 15.9	11.4 ± 2.4	25.0 ± 0.5**
Eth+CW	40.3 ± 3.4	16.5 ± 3.4	22.1 ± 1.6	8.3 ± 0.6**
Asc + Eth	54.8 ± 5.1	33.4 ± 8.6*	16.6 ± 4.0	23.5 ± 1.7**
Caf + Eth	47.0 ± 6.2	20.7 ± 10.2	21.3 ± 8.0	13.3 ± 3.8**

Eth – 2.5 mL/Kg of solution 35%; CW+Eth – 3 mL/Kg of coconut water followed by 2.5 mL/Kg ethanol; Eth+CW – 2.5 mL/Kg of ethanol followed by 3 mL/Kg of coconut water; Asc + Eth – 3 mL/Kg of solution 25 μg/μL followed by 2.5 mL/Kg of ethanol; Caf + Eth – 3 mL/Kg of solution 100 μg/μL followed by 2.5 mL/Kg of ethanol. The data are average ± S.D. for five animals. Means values were significant different for $p<0.05$; (*) different when compared with the saline group $p<0.05$; (**) different when compared with the ethanol group $p<0.05$. For multiple comparisons, ANOVA, followed by Dunnett's test, were applied.

groups treated with ethanol, especially the caffeic acid group, it was observed a significant ($p<0.05$) decrease of liver GSH levels, when compared with the saline group, as well as low levels of GSSG (Figure 5). Since all groups treated with ethanol had their GSH levels lower than saline group on liver, explanation for this can be attributed to the antioxidant role of GSH on reactive oxygen species (ROS) following elimination of it (Das et al. 2008, Waly et al. 2010). Also, caffeic acid metabolism which involves oxidation of CYP2E1 pathway, formation of o-quinone compounds and glutathione conjugation (Moridani et al. 2002) plus the oxidative metabolism of ethanol, both, causing decreases in the GSH level (Wang and Cederbaum 2006). However, there are alternative mechanisms with less toxicity pathways for the caffeic metabolism as, for instance, excretion by glucuronidation (Rechner et al. 2001) or o-methylation (Moridani et al. 2002, Wang et al. 2010). The treatment with coconut water was efficient in recuperating the ratio GSSG/GSH suggesting a protective effect of coconut water in oxidative stress mediated by ethanol as cysteine content in coconut water can be the responsible for GSH restoration (Atkuri et al. 2007) (Figure 5).

Reactive oxygen species induce different injuries to DNA, including strand breaks, interstrand and DNA–protein cross-links, apurinic/apyrimidinic sites, and base modifications (Halliwell 1993). One of the many oxidative damages induced in the DNA by $^{\bullet}OH$ is 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodGuo) (Halliwell 1993), which represents 30–50% of total base-modification products induced in different experimental models. Important to note that others ROS are able to induce 8-oxodGuo on DNA, more specifically oxygen singlet or any capable of guanine electron abstraction, thus 8-oxodGuo is an interesting biomarker for ROS damage on DNA (Dizdaroglu et al. 1991, Glaucia et al. 2003, Valavanidis et al. 2009). There is extensive experimental evidence that oxidative damage permanently occurs to lipids of cellular membranes, proteins, and DNA. In the present paper, we have reassessed the 8-oxodGuo level in liver DNA as a biomarker of DNA oxidative damage induced by ethanol and we observe a decrease of 8-oxodGuo DNA concentration in the rats treated by coconut

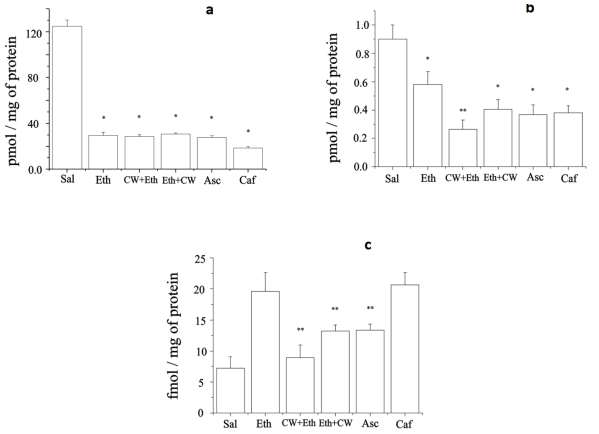

Figure 5 - Effect of CW, ascorbic and caffeic acid of GSH and GSSG levels in liver of rats treated by ethanol. A – GSH concentration, B - GSSG concentration, C – GSSG/GSH ratio. Eth – 2.5 mL/Kg of solution 35%; CW+Eth –3 mL/Kg of coconut water followed by ethanol 2.5 ml/Kg; Eth+CW – ethanol 2.5 mL/Kg followed by coconut water 3 mL/Kg; Asc + Eth – 3 mL of solution 25 µg/µL followed by ethanol 2.5 mL/Kg; Caf + Eth – 3 mL of solution 100 µg/µL following by ethanol 2.5 mL/Kg. The data are average ± S.D for five animals. Means values were significant different for $p<0.05$; (*) different when compared with saline group $p<0.05$; (**) different when compared with ethanol group $p<0.05$. For multiple comparisons, ANOVA, followed by Dunnett's test, were applied.

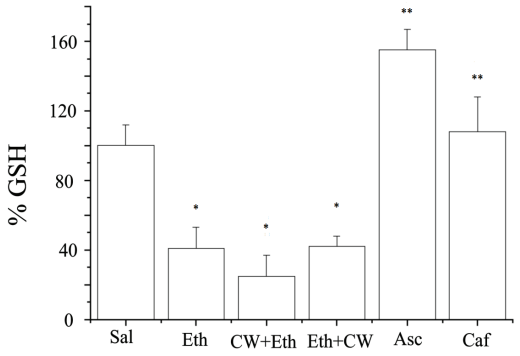

Figure 6 - Effect of CW, ascorbic and caffeic acid in GSH percentage in plasma of rats treated by ethanol. The results are express in percentage in relation to Saline group. Eth – 2.5 mL/Kg of solution 35%; CW+Eth – 3 mL/Kg of coconut water followed by 2.5 mL/Kg ethanol; Eth+CW – 2.5 mL/Kg of ethanol followed by 3 mL/Kg of coconut water; Asc + Eth – 3 mL/Kg of solution 25 µg/µL followed by 2.5 mL/Kg of ethanol; Caf + Eth – 3 mL/Kg of solution 100 µg/µL followed by 2.5 mL/Kg of ethanol. The data are average ± S.D for five animals. Means values were significant different for $p<0.05$; (*) different when compared with saline group $p<0.05$; (**) different when compared with ethanol group $p<0.05$. For multiple comparisons, ANOVA, followed by Dunnett's test, were applied.

water (CW), ascorbic acid and caffeic acids (Figure 7) or be reduced the DNA damages in liver to 36.2% and 48.0% for the coconut water groups, 42.5% in the caffeic acid group, and to ascorbic acid group in 34.5% in comparison with the ethanol group ($p<0.05$). These values are comparable to those previously quoted for different cells and organisms 0.5 to 2.0 8-oxodGuo/10^5 dGuo (Kasai et al. 1986, Rodríguez-Ariza et al. 1999, Matos et al. 2000, 2001).

In previous studies, we have shown that natural substances possess antioxidant activity. *In vitro*, gallic acid was efficient in scavenging nitric oxide (NO) (Maia et al. 2010). In cultured mammalian cells, the lycopene can protect against damage caused by Fe-NTA/ascorbate treatment (Matos et al. 2000), showing the protective effect *in vivo* of lycopene towards iron-induced DNA and membrane damage and histopathologic changes in rat liver (Matos et al. 2001) and that both lycopene and ß-carotene protected *in vivo* from iron-induced oxidative stress damage in rat prostate (Matos et al. 2006).

The caffeic acid is a catechol compound is widely found in several varieties of food. This substance was chosen because Chakraborty and Mitra (2008) demonstrated the presence of cholorogenic acid (an caffeic acid derivative) in the mesocarp, the fleshy portion which is in direct contact with water, and Seneviratne and Dissanayake (2008), observed caffeic acid in the copra oil, Santos et al. (2013) was the first to show the presence of caffeic acid (approximately 0.025 mg/mL CW) in lyophilized green dwarf coconut water, *Cocos nucifera* L. The methodologies used for analyzing the phenolic compounds in natural

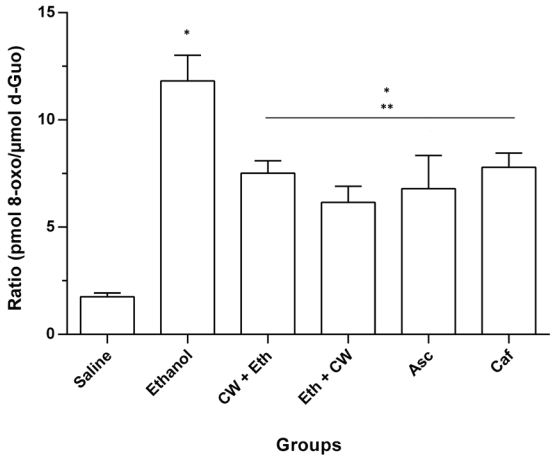

Figure 7 - Effect of CW, ascorbic and caffeic acid in the 8-oxo-2'-desoxiguanosinre DNA concentrations in liver of rats treated by ethanol. Eth – 2.5 mL/Kg of solution 35%; CW+Eth – 3 mL/Kg of coconut water followed by 2.5 mL/Kg ethanol; Eth+CW – 2.5 mL/Kg of ethanol followed by 3 mL/Kg of coconut water; Asc + Eth – 3 mL/Kg of solution 25 µg/µL followed by 2.5 mL/Kg of ethanol; Caf + Eth – 3 mL/Kg of solution 100 µg/µL followed by 2.5 mL/Kg of ethanol. The data are average ± S.D. for five animals. Means values were significant different for $p<0.05$; (*) different when compared with the saline group $p<0.05$; (**) difference with the ethanol group $p<0.05$. For multiple comparisons, ANOVA, followed by Dunnett's test, were applied.

products usually include a number of steps ranging from exhaustive solvent extraction, passing though extract clean up, concentration procedures, filtration and centrifugation of liquid samples. However, in many cases, a simple filtration is not effective for recovering a wide variety of polyphenols, and alternative strategies are needed (Robards 2003).

The concentration of the compounds found in coconut water can be an important index of its biological activity. The obtained data showed that consuming green dwarf coconut water is involved in the modulation of liver metabolism, suggesting a hepatoprotective effect and alterations in lipid parameters. Relevant biomarkers, such as factors involved in sensitization to ethanol-induced oxidative stress, are required to better elucidate the relationship between alcohol consumption, oxidative stress and carcinogenesis.

CONCLUSIONS

Herein we describe the development and validation of a simple, fast and sensitive methodology for the simultaneous quantification of eight different phenolic compounds in coconut waters HPLC-ESI^{+}MS/MS. The results obtained demonstrated the presence of chlorogenic acid, caffeic acid, methyl caffeate, quercetin as well as ferulic acid isomers in green dwarf coconut water. In animals, the administration of coconut water, caffeic and ascorbic acids were efficient in inhibiting the harmful effects of ethanol on the metabolism, which was proven by the decrease of 8-oxodGuo and TBARS concentrations in the liver and an increase of glutathione levels in the plasma by ascorbic and caffeic acid only. The data also suggest that the consumption of coconut water and caffeic acid, in the concentrations used here, has a hepatoprotective effect, confirmed by the γ-GT, ALT values. The next step now is to elucidate the mechanisms involved in this protection, such as the down regulation of CYP2E1 or the protection of DNA against oxidative damage. The beneficial effects of drinking coconut water as a healthy beverage or as medication must be further explored.

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SUPLEMMENTARY MATERIAL

TABLE SI - Precision values of the LC–MS/MS analyses for the standard phenolics compounds.