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Chemical and antimicrobial analysis of husk fiber aqueous extract from *Cocos nucifera* L.

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***Cocos nucifera* L. (Arecaceae) is a widely distributed species around the tropical areas. Popular uses have been reported in the treatment of arthritis and diarrhea. This study evaluates the antimicrobial activity of husk fiber aqueous extract from *C. nucifera* and performed the identification of some biological active substances. The minimal inhibitory concentration (MIC) against human pathogen microorganisms was determined. Chromatographic and spectrometric procedures were also performed to isolate and identify the components present in the extract. In the MIC assay of crude aqueous extract, only the methicillin sensible and the resistant (MRSA) *Staphylococcus aureus* strains were susceptible at 156 µg/mL. The ethyl acetate partition taken from crude extract was more promising (MIC of 78 µg/mL). No fungal growth inhibition was observed. Catechin, epicatechin, two procyanidin dimers and condensed tannins were found in the organic phase. In addition, gallic and ellagic acids were detected for the first time in C*. *nucifera* husk fiber. Gallic acid showed MIC of 39 µg/mL and minimal bactericidal concentration (MBC) at 78 µg/mL. Ellagic acid was not active against the tested strains, as well as catechin and epicatechin. Additionally catechin, epicatechin, two procyanidin dimers and condensed tannins were also detected. The antimicrobial activity observed was selective to *S. aureus* strains.**

Key words: Antimicrobial analyses, *Cocos nucifera*, arecaceae, ellagic acid, gallic acid, procyanidins, *Staphylococcus aureus* strains.

INTRODUCTION

The coconut (*Cocos nucifera* L. family Arecaceae) is a well distributed fruit tree all around the world, providing food, especially in the tropical and subtropical regions and for its many uses it is often called the “tree of life”. There are 12 different crops of nuts under the name of coconut palm (DebMandal and Mandal, 2011). *C. nucifera* is widely distributed over the Brazilian northeastern coast, where is known as “Coco-da-Bahia”.

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Popular medicinal uses (against arthritis and diarrhea) of coconut husk fiber have been reported (Esquenazi et al., 2002; Alviano et al., 2004; Rinaldi et al., 2009), but the knowledge of its potential benefit or adverse effects in human beings is still very preliminary. Previous studies showed that aqueous *C. nucifera* husk fiber extracts present important biological activities such as antimicrobial, antiviral, antinociceptive, anti-inflammatory, antioxidant and antineoplasic properties (Esquenazi et al., 2002; Alviano et al., 2004; Rinaldi et al., 2009; Akinyele et al., 2011; Dua et al., 2013). Coconut husk fiber is rich in polyphenolic compounds. The *C. nucifera* husk fiber aqueous extracts are mainly composed by catechin, epicatechin and condensed tannins (B-type procyanidins) (Esquenazi et al., 2002). Plant phenols represent an important group of natural antioxidants and some of them are potent antimicrobial compounds (Chakraborty and Mitra, 2008). In general, polyphenols can prevent chronic diseases by their antioxidant, free radical scavenger and metal chelator properties (Daglia, 2012).

The industrial use of this plant generates large amounts of husk fiber as industrial reject, featuring an environmental problem. Based on our continuous interest in searching for medicinal plants from Brazilian Flora with antimicrobial activity and in expanding the knowledge about the phytochemical profile of *C. nucifera*, the purpose of this study was to investigate the antimicrobial activity of aqueous extracts and fractions from the husk fiber of the *C. nucifera* against bacteria (*Staphylococcus aureus*, *S. aureus* MRSA) and fungi (*Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum* and *Fonsecaea pedrosoi*).

MATERIALS AND METHODS

The coconut (*C. nucifera* Linn, Arecaceae), commonly known as “Coco-da-Bahia” variety, was collected in Aracaju, state of Sergipe, Brazil and a voucher specimen was deposited in Herbarium of Universidade Federal de Sergipe (ASE 13.631).

The solvents methanol, acetonitrile and ethyl acetate were all of high performance liquid chromatography (HPLC) grade and purchased from Tedia Brazil. Standards of gallic acid, ellagic acid, (+)-catechin, (-) epicatechin and Sephadex LH-20 were purchased from Sigma-Aldrich.

Microorganisms

The microorganisms tested were the Gram-positive bacteria *S. aureus* (ATCC 6548), *S. aureus* MRSA (BMB9393) from Hospital Universitário Clementino Fraga Filho, UFRJ), *Lactobacillus casei* (ATCC 4646) and the Gram-negative *Escherichia coli* (ATCC 8739); the yeast *Candida albicans* Seropyte B (ATCC 36802) and *Cryptococcus neoformans* Serotype A (T_1-444) from yeast collection from UNIFESP; the filamentous fungi *Trichophyton rubrum* (T544) and *Fonsecaea pedrosoi* (5VPL) from fungal collection of Hospital Universitário Clementino Fraga Filho, UFRJ.

Preparation of *Cocos nucifera* L. crude extract and fractions

535 g of coconut husk fiber was extracted by infusion as described previously (Esquenazi et al., 2002) yielding 8.0% of aqueous crude extract. After lyophilization, the crude extract obtained was dissolved in water and partitioned with ethyl acetate. After solvent evaporation, 2.3 g of the ethyl acetate crude extract was subjected to step gradient (water and methanol) mode separation on a Sephadex LH-20 column.

Isolation and identification of phenolic compounds

HPLC semi-preparative purification was performed with a RP-18 column (SUPELCO, 250 x 10 mm, 5 µm particles) under isocratic elution with methanol/water (2:8). Electrospray ionization-mass spectrometry (ESI-MS) analysis of the isolated compounds was made in a Brüker MicrOTOF II spectrometer operating in negative ion mode, scan range: 50 to 3000 amu, capillary voltage at 3.8 KV, dry gas flow at 4.0 $L.min^{-1}$ and heated capillary temperature at 180°C. The ultraviolet (UV) profile absorption and time retention similarities were also evaluated.

Minimal inhibitory concentration (MIC)

The broth microdilution method was carried out to evaluate the antibacterial and antifungal activities as recommended by Clinical Laboratory Standard Institute (CLSI) M7-A4 for bacteria, M38-A2 for filamentous fungus and M27-A3 for yeast (CLSI, 2008). In 96-well plates, after two-fold serial dilution of samples, wells were inoculated with 10 µL of a bacterial suspension (obtained by 1:20 dilution of 0.5 McFarland scale suspension) in Mueller Hinton or inoculated with 100 µL of the fungal suspension (1:1000 dilution of 0.5McFarland scale suspension) in Roswell Park Memorial Institute buffered with Morpholinopropanesulphonic acid (RPMI-MOPS pH 7.2). The conditions of incubation were 24 h at 37°C for bacteria and 48 h at room temperature for fungi. Pure medium was the negative control and positive one comprised inoculated growth medium incubated under the same conditions. The results were based on visual growth of microorganisms, which were confirmed with 30 µL of resazurin (Sigma-Aldrich) added aseptically to the plate-wells and incubated at 37°C for 1 h. The MIC was defined as the minimum concentration of the antimicrobial agent test which represented complete growth inhibition. Additionally, the plate-wells with no growth observation were re-inoculated in sterile medium without substance presence to evaluate if the concentrations presented microbicidal effect (MBC) or not.

RESULTS AND DISCUSSION

Extraction and partition procedures

The aqueous extract from *C. nucifera* L. husk fiber was obtained by infusion. The resulting extract was partitioned between ethyl acetate and water. The HPLC/DAD profiles of the organic, aqueous phases and crude extract were determined (Figure 1) under conditions originally proposed by Peng et al. (2001). Under such conditions, a characteristic chromatogram is obtained where compounds eluted until 40 min comprise monomers and oligomers of procyanidins including catechin and epicatechin. The large peak at 55 to 60 min belongs to a mixture of polymers formed by more than five monomeric units.

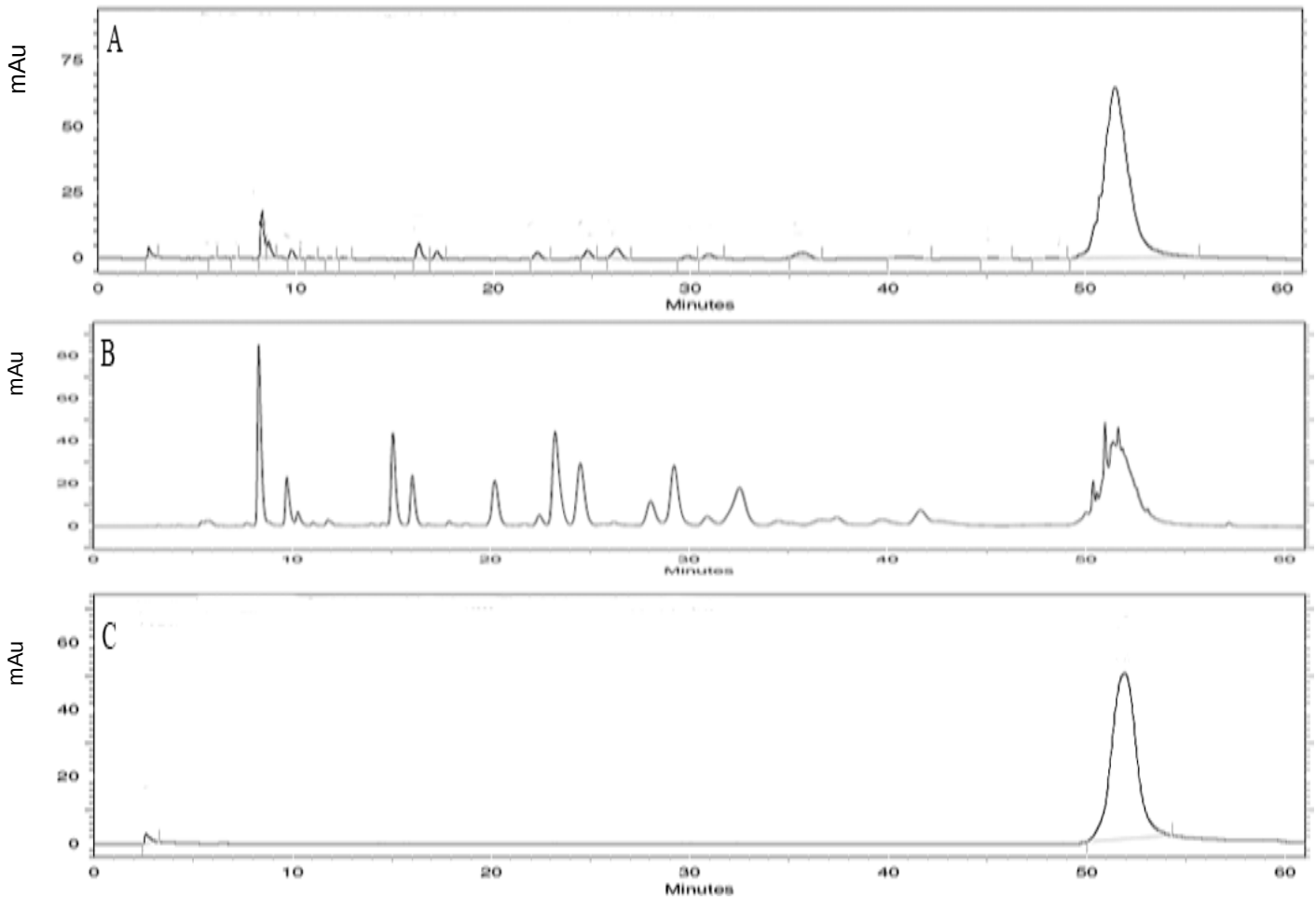

Figure 1. HPLC traces at 280 nm. **A.** Aqueous crude extract from *C. nucifera* L. **B.** Ethyl acetate partition. **C**. Aqueous fraction partition comprising only polymeric tannins.

Phytochemical analyses of substances present on ethyl acetate phase

Chromatogram of the ethyl acetate phase with captions indicates the compounds already identified (Figure 2). The isolation of some compounds was made, when necessary, by the use of Sephadex LH-20 chromatography complemented by semi-preparative HPLC. Other peaks were identified by co-injection of commercial standards and comparison with literature records. Characterization of the compounds was made through extensive utilization of UV absorption and off-line ESI/MS spectra (Table 1 and Figure 2).

Antimicrobial analyses of substances present on ethyl acetate phase

The ability of *C. nucifera* aqueous crude extract to inhibit pathogen microorganisms was evaluated by broth microdilution test (Table 2). As observed, only the methicillin sensible and the resistant (MRSA) Gram (+) *Staphylococcus aureus* strains were susceptible to this extract. No fungal growth inhibition was observed. The MIC of the aqueous crude extract determined against *S. aureus* was 156 µg/ml, and the same value was observed against MRSA. This crude extract exhibited also, in this same concentration, a microbicidal effect indicating that the antimicrobial action at this concentration affected irreversibly the microorganism viability. Under similar conditions, methicilin exhibited MIC and MBC values of 0.25 µg/ml and vancomycin presented MIC and MBC values of 2 µg/ml to MRSA. Our results confirm the antimicrobial profile described previously (Esquanazi et al., 2002) where only *S. aureus* strains had susceptibility to the aqueous crude extract of *C. nucifera*, while no inhibition has been shown against the fungal strains assayed (*Fonsecaea pedrosoi*, *Candida albicans* and *Cryptococcus neoformans*) (Table 2). The authors used the agar diffusion test, where diffusion of substance on

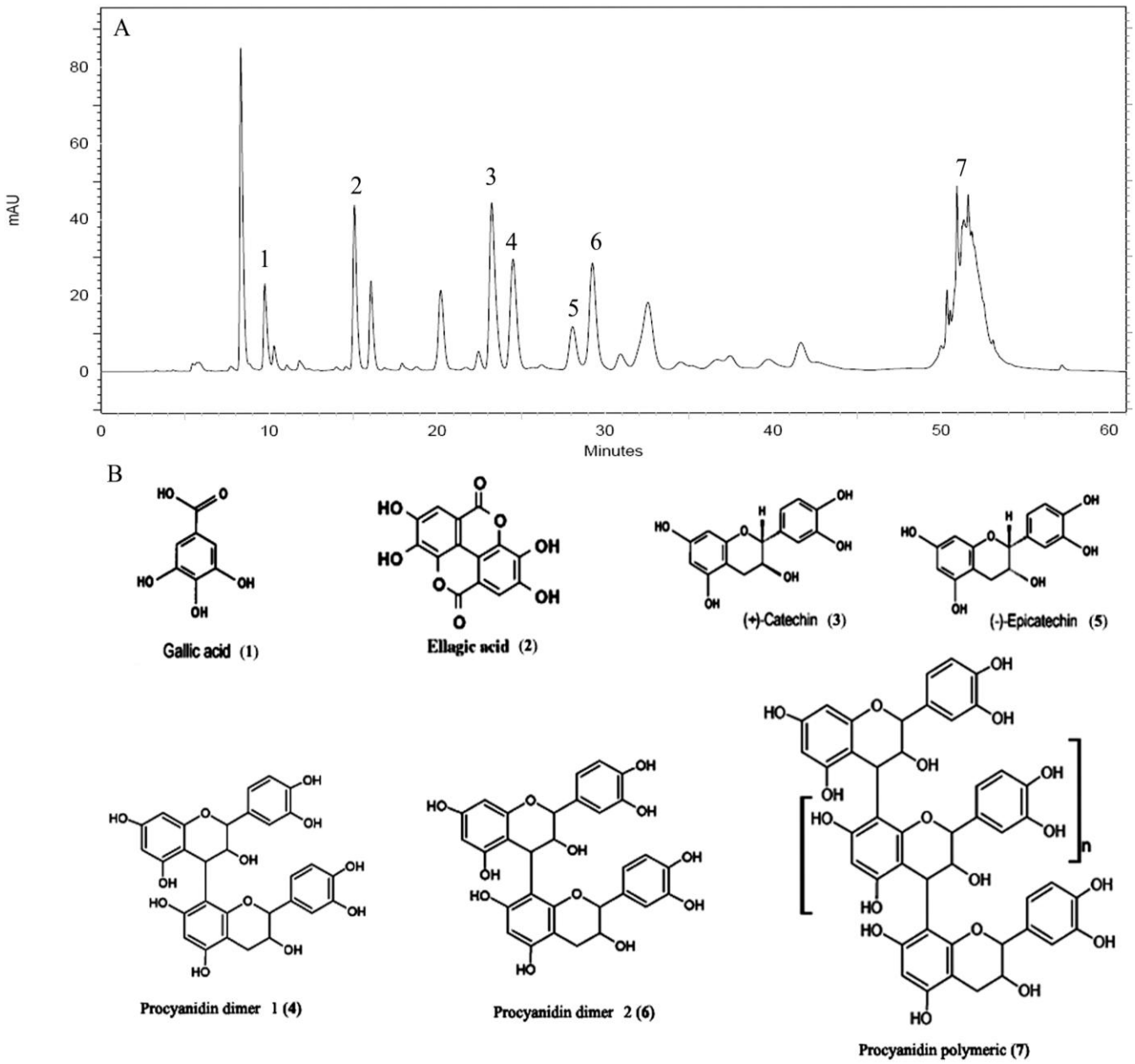

Figure 2. A) HPLC trace of ethyl acetate fraction at 280 nm. Captions: gallic acid **(1)**, ellagic acid **(2)**, catechin **(3)**, procyanidin dimer 1 **(4)**, epicathechin **(5)**, procyanidin dimer 2 **(6)** and polymeric procyanidin **(7)** were obtained; **B)** Structures of substances identified in the ethyl acetate fraction of *C. nucifera* L. aqueous husk fiber extract.

Table 1. Characterization of components by off-line ESI-MS spectra.

Compound	ESI/MS analysis of Deprotonated Ions $[M-H]^-$ *m/z*
Gallic acid	169
Ellagic acid	301
Catechin	289
Procyanidin dimer 1	577
Epicatechin	289
Procyanidin dimer 2	577

Table 2. Antimicrobial activity of the *Cocos nucifera* L. husk fiber crude extract, fractions and isolated substances against *S. aureus* strains.

Microorganism	MIC and MBC (µg/ml)			
	S. aureus		*S. aureus* - MRSA	
	MIC	MBC	MIC	MBC
Crude extract	156	156	156	156
Ethyl acetate phase	78	78	78	78
Aqueous phase	156	156	156	156
Gallic acid	39	78	39	78
Ellagic acid	-	-	-	-
Catechin	-	-	-	-
Procyanidin dimer 1	1000	>1000	1000	>1000
Epicatechin	-	-	-	-
Procyanidin dimer 2	1000	>1000	1000	>1000
Methicillin	0,25	0,25	NT	NT
Vancomycin	NT	NT	2	2

MIC, Minimal inhibitory concentration; MBC, microbicidal concentration; NT, not tested; -, no activity observed. No growth inhibitions were observed against the other microorganisms tested.

agar slides surface may influence the activity observed. Nevertheless, our results were obtained applying a more reliable assay, in which the antimicrobial profile of *C. nucifera* aqueous extract was evaluated quantitatively.

The antimicrobial profiles of ethyl acetate and water partition phases were evaluated and the results show that the organic phase was more promising than the crude extract (Table 2). The organic phase presented a MIC value of 78 µg/ml, while the aqueous phase exhibited the same value as the crude extract (156 µg/ml). The antimicrobial activity of aqueous phase may be associated, to the presence of polymeric procyanidins, as evidenced in Figure 1C. In addition, the increasing antimicrobial activity observed for the ethyl acetate phase led us to consider a substantial contribution from the components eluted until 40 min (Figure 2).

The antimicrobial profiles of the substances and fractions isolated from *C. nucifera* against *S. aureus* and *S. aureus*-MRSA are summarized on Table 2. With the exception of catechin, epicatechin and ellagic acid, all the other substances displayed some activity against the tested microorganism strains. Gallic acid was the most active showing MIC=39 µg/ml and MBC=78 µg/ml. The two different procyanidin dimers isolated exhibited MIC=1000 µg/ml and MBC>1000 µg/ml.

Different mechanisms were proposed to explain antimicrobial activity of polyphenols. They are dependent of crucial extracellular microbial enzymes inhibition, growth inhibition by substrate deprivation or acting on direct metabolism through oxidative phosphorylation inhibition (Heinonen, 2007).

Gallic acid is a well-known antioxidant and antitumor agent (Daglia, 2012; Reedy et al., 2012). Its antimicrobial activity was also reported against Gram (+) bacteria including *S. aureus* (Saavedra et al., 2010). The authors have shown that the pure gallic acid was more efficient than a commercial antimicrobial product, where it was used in association with streptomycin, demonstrating a synergic effect.

Ellagic acid has been widely reported as antioxidant and antimalarial agent (Soh et al., 2009; Saul et al., 2011) although the inhibitory effect on *Helicobacter pylori* of ellagic acid has been cited as bactericidal concentration of 2 µg/mL (Martini et al., 2009). Our study did not show the antimicrobial activity against the microorganism tested, but its presence can be associated with the other bioactivities already described to the aqueous crude extract of *C. nucifera*. The mechanisms of antimicrobial action of phenolic acid include inhibition of extracellular enzymes crucial to microbial growth or direct action on oxidative phosphorylation, modifying the microbial metabolism (Kang et al., 2008).

In general, several biological activities have been associated to the presence of catechin and epicatechin, such as antitumoral, antinociceptive and antiprotozoal. Our results show no antimicrobial activity of these substances obtained from *C. nucifera*, but their presence may be important to explain other biological activities already described to *C. nucifera* aqueous crude extract.

The acute toxicity of *C. nucifera* L. aqueous crude extract evaluated by the oral administration in animal model showed no behavioral alterations on the treated mice given a LD_{50} of 2.3 g/kg. Furthermore, when used on a rabbit model, no ocular irritations or allergic reactions were induced. These results are consistent since the adverse effects have never being described by the popular use of this extract (Alviano et al., 2004).

Conclusion

The observed antimicrobial profile of *Cocos nucifera* husk

fiber extracts against *S. aureus* strains supports the use of the plant on folk medicine. Two unreported substances present in the *C. nucifera* husk fiber aqueous crude extract, gallic and ellagic acids besides procyanidins already reported were isolated and identified. In this regard, the elucidation of the composition of the extracts contributes to the knowledge on the mechanisms involved in the activity displayed by them, as already described by our research group. The results presented here provide a motivation for further investigation on the subject, since this plant has several medicinal popular uses.

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