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Research Article

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Assessment of antimicrobial and cytotoxic activities of crude extracts from *Cocos nucifera* Linn.

Cristiane do Nascimento Tavares Figueira¹, Jirliane Martins dos Santos¹, Fabiani Tenório Xavier Póvoas², Max Denisson Maurício Viana², Magna Suzana Alexandre Moreira², João Xavier de Araújo Júnior¹, Ednaldo Cavalcante de Araújo³, Eliane Aparecida Campesatto², Izabelle Cristina Acioly de Omena¹ and Maria Lysete de Assis Bastos^{1*}

¹Research Laboratory in Wound Care, School of Nursing and Pharmacy, Federal University of Alagoas, Brazil
²Laboratory of Pharmacology and Immunity, Institute of Biological Sciences and Health, Federal University of Alagoas, Maceió – AL, Brazil
³Department of Nursing, Federal University of Pernambuco, Recife – PE, Brazil

ABSTRACT

The evaluation of antimicrobial activity has been the subject of research, mainly due to the presence of bacterial multidrug resistance of conventional treatments. This research aimed to investigate the antimicrobial and cytotoxic activity of ethanolic crude extracts from leaves, leaf sheath, and stem of Cocos nucifera Linn (green dwarf coconut tree) in vitro. The antimicrobial activity was evaluated against gram-positive bacteria S. aureus and Gram-negative bacterias E. coli and P. aeruginosa and the Candica albicans fungus, through the methods of agar diffusion and identification of minimum inhibitory concentration through microdilution. The cell viability assay was performed using the J774 cell line by MTT. The sheath and leaf extracts of C. nucifera L. inhibited bacterial growth of S. aureus and P. aeruginosa with the minimum inhibitory concentration of 1.5×10^6 CFU by the extracts at 10%. The extracts showed no cytotoxicity at the concentrations of 200 and 100 µg/mL; the ethanolic extract from sheath did not exhibit cytotoxicity at 1000 µg/mL. The results show partial antimicrobial and cytotoxicity activities.

Key words: Anti-Infective Agents, Basic Research, Nursing, Pharmacognosy.

INTRODUCTION

The World Health Organization has encouraged the use of plants with therapeutic properties. In Brazil, the Ministry of Health (MOH) has invested in the applicability of unconventional therapies in the routine of health services, with the establishment of the National Policy on Integrative and Complementary Practices, to boost universal access to the Unified Health System (SUS) [1] and through creation of the National List of Plants with Therapeutic Properties of Interest to SUS, composed by species with the potential to generate products for SUS [2].

Thereby, Brazilian researchers have performed studies on plant species in the search for diverse biological activities. As an example, there is the evaluation of extracts from plantain (*Musa sapientum*) [3], through phytochemical screening, identifying its healing and antimicrobial action; of the ethanol extract from the "fluffy Ipe" (*Zeyheria tuberculous*), identifying its *in vitro* antimicrobial activity for *Streptococcus pyogenes*, *Staphylococcus epidermidis* and *Staphylococcus aureus;* the methanol extract from *Zabele* (*Croton pulegioides* Baill.) obtained from the root, stem and leaves, which demonstrated activities towards the *Bacillus subtilis*, *Staphylococcus aureus*, *S. epidermidis* and three species of fungus of the genus *Candida* [4].

The evaluation of antimicrobial activity has been ongoing subject of research in view of the bacterial multidrug resistance of conventional treatments [4], which has stimulated the acquisition of new compounds.

The aims of this study were to investigate the antimicrobial and cytotoxic potential of ethanol crude extracts from the leaves, leaf sheath and stem bark of *C. nucifera* Linn (green dwarf coconut tree) in vitro.

EXPERIMENTAL SECTION

Plant Material

Leaves, leaf sheath, and stem bark of *Cocos nucifera* L were collected from a five-years-old coconut tree in the restinga region - situated on sandy marsh land, near the Mundaú Lagoon and the Atlantic Ocean (geographical coordinates S 509°44'082 " and W 35°49'957"), in the Massagueira district, municipality of Marechal Deodoro, south coast from Alagoas, at 6 Km from the capital Maceio. Samples of plant material (leaf and inflorescence) were referred for recognition to the Institute for the Environment of the State of Alagoas (IMA), where exsiccates were deposited under N° . 55.997.

Obtaining plant extracts

Plant sample, after drying at room temperature and trituration with knife mill, was weighed in a semi-analytical scale and packaged in glasses of 3 L for extraction by maceration using hydrated ethylic alcohol (EtOH) at 98% as solvent in the ratio 2:1.

The procedure was performed in triplicate $(1^{st}$ with thirty days, 2^{nd} and 3^{rd} with five days of submersion) for maximum extraction. At the end of the 3^{rd} extraction, vegetable residues were discarded [5]. After each extraction stage, the solutions were concentrated in a rota-evaporator. For each cycle, 300 mL were added and the centered extracts were placed in sterile glass bottles and weighed on analytical balance. The extracts were left uncapped for drying in a hothouse at 40° C.

From these materials, three ethanolic crude extracts (leaves, leave sheath, and stems) were obtained in addition to an ethanol/methanol extract (leaves) in the ratio 2:1.

Antimicrobial assays

The antimicrobial *in vitro* biomonitoring was performed by disk diffusion and drilling in agar. Sequentially, the MIC (Minimum Inhibitory Concentration) of the extracts was determined by broth microdilution.

Microorganisms used were standard strains of Gram-positive bacteria – *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pyogenes* (CCCD S012); Gram-negative bacteria – *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (CCCCD-P001), and *Pseudomonas aeruginosa* (ATCC 27853); and the fungus *Candida albicans* (ATCC 10231). All the tests were performed in triplicate.

The results of the tests were expressed from diameter of the halo of hibition of microbial growth, according to the following criteria: inhibition zone $\geq 75\%$ (active); inhibition $\geq 25\%$ and < 75% (moderately active); inhibition < 25% (inactive) [6].

Disk diffusion

Bacterial inoculums were prepared with the microorganisms at a concentration of approximately 10^8 CFU/mL corresponding to reading standard 0.5 McFarland and seeded on Mueller-Hinton agar for bacteria and Sabouraud-Dextrose for fungi. In each plate, three testing disks impregnated with 20 μ L of test sample were deposited, one negative control disk, and another positive control.

From a stock solution of 1000 mg of the extracts, which was solubilized with 2 drops of Cremophor + 1 mL 0.9% saline solution, an aliquot of 50000 μ g/mL was separated. Sterile disks of filter paper of 6 mm in diameter were impregnated with 20 μ L of stock solution corresponding to 1000 μ g/disk. As positive control for the fungi, disks impregnated with 20 μ L of miconazole were used [7]. In the assays with bacteria, standardized disks of vancomycin (30 μ g/disk), oxacillin (1 μ g/disk) gentamicin (10 μ g/disk), and ciprofloxacin (5 μ g/disk) were used. The negative control was done with disks containing the solvent ethanol PA used in the solubilization of the samples.

After diffusion of the extracts, the plates were poured and incubated at 35°C for 24 hours for bacteria and at 28°C for 48 hours for fungi [8]. The inhibitory activity of the samples was evaluated by the formation of inhibition halo from growth of microorganisms around the disks, measured using a paquimeter.

Drilling in agar

 $50 \ \mu$ L at 10% of the extract were added to each well; two drops of Cremophor were solubilized in 1 mL of 0.9% saline solution, whereas Ceftriaxone (for Gram-positive bacteria), Ciprofloxacin (Gram-negative), and Miconazole for fungi were used as positive control. Then, the plates were incubated at 36°C and 28°C for 24 and 48 hours for bacteria and fungi, respectively. After this period, the halos of inhibition were measured with the aid of a paquimeter.

Minimum Inhibitory Concentration (MIC)

The microdilution protocol in Muller-Hinton broth was performed with some adaptations. Thereby, a volume of 200 μ L of each plant extract was prepared at a concentration of 2000 μ g/mL, using 10% DMSO as solvent for the solubilization of the vegetable samples. Subsequently, the extracts were filtered using Millipore filters 0.45 μ m and then inoculated in columns 1 to 9 of line A. The other wells were filled with 100 μ L of Mueller-Hinton broth (two times concentrated) [9]. The vegetable extracts which showed antimicrobial activity for the majority of microbial strains and halos of inhibition equal to or greater than 9 mm in the assay of drilling in agar were subjected to the test.

Cell viability assay

It was used a colorimetric technique using the bromide solution of 3-(4, 5-dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide (MTT), which is based on checking of mitochondrial activity and integrity interpreted as a measure of cell viability. Tests were conducted with the crude extract at the concentrations of 2000, 1000, 200, and 100 μ g/mL, respectively corresponding to 2.0, 1.0, 0.2, and 0.1% of DMSO.

Inflammatory macrophages obtained from the peritoneal cavity of Swiss mice were used, four days after injection of 1 ml of sterile Thioglycollate Medium of Na + 4%. After this period, the peritoneum was washed with 5 mL PBS. These adherent-phenotype macrophage line was cultured in DMEM supplemented with 10% FBS at 37°C with 95% humidity and 5% CO₂. Briefly, cell suspensions containing 5.0×10^5 cells/mL were seeded in a 96-well plate in triplicate and incubated at 37°C for 24 h [10-11]

Once this time had elapsed, 100 μ L of supernatant were removed of each well and 20 μ L of MTT at 5 mg/mL were added. After 4 hours of incubation at 37°C in humid atmosphere containing 5% CO², the culture medium was completely removed and 100 uL of a solution containing isopropanol and 10X Triton were added to dissolve the Formanzan crystals. The group of dead cells (positive control) was obtained 1 hour before addition of MTT by cell lysis with the addition of 2 uL of 100X Triton. The cell viability of the cultures treated with the substances was compared to the pattern of death obtained in control cultures [11]

RESULTS AND DISCUSSION

In vitro antimicrobial assays for disk diffusion

The four crude extracts were evaluated against the bacterias *S. aureus*, *P. aeruginosa*, and *E. coli*; and the fungus *C. albicans* at a concentration of $1000 \mu g/disk$ by disk diffusion technique, whose results are shown in Table 1

The sheath extract showed strong antimicrobial activity against *S. aureus* with a growth inhibition percentage of 76.5%. This value against the Gram-positive bacterium is considered high when correlated with existing parameters [12-13]. Moreover, it is significant because, despite being part of the normal microbiota of the human body, *S. aureus* is a pathogen that acts in a range of infections, for instance, in surgical wounds, more severe in immunocompromised individuals, and this issue is intensified by emerging resistant strains [14].

Among the four extracts tested against the Gram-negative bacterium *P. aeruginosa*, the leaf extract EtOH/MeOH (2:1) was considered active with a inhibition percentage of 76% (inhibition halo \geq 75%), whereas the ethanolic extract of the sheath was moderately active with inhibition of 29.4% (inhibition \geq 25% and < 75%). Ethanolic extracts of the stem and leaf showed no halo, hence displaying no activity (inhibition < 25%) as explained in Table 1.

Another classification restricted to the size of the halo by the extract states that the crude extracts from leaves EtOH/MeOH and leaf sheath EtOH of *C. nucifera* L. were active against *P. aeruginosa* (halos \ge 9 mm) and only the sheath extract was active for *S. aureus*. This classification was adopted in a study that tested the extract of *Anadenanthera macrocarpa* (angico) against *S. aureus* and obtained sensibilities with inhibition halos between 19 and 25 mm [15].

These results are compatible with those reported in studies in which the antibacterial activity against Gram-positive bacteria presented, in general, larger inhibition zones than the Gram-negative bacteria due to the increased

susceptibility of Gram-positive bacteria, since they only have an outer layer of peptidoglycan which is not an efficient barrier [16-17].

Mieroenconiemo	Average of halos of inhibition (mm)				
	Crude Extracts (1000 µg/disk).				
(ATCC)	Loof EtOH	Loof EtOU/MoOU 2:1	Leaf Sheath	Stem	Control
(AICC)	Leaf EIOH	Leaf ElOH/MEOH 2.1	EtOH	EtOH	
S. aureus (25923)	0.0	0.0	13.0	0.0	17 ^C
P. aeruginosa (27853)	0.0	26.0	10.0	0.0	34 ^A
E. coli (25922)	0.0	0.0	0.0	0.0	34 ^A
C. albicans (10231)	0.0	0.0	0.0	0.0	21 ^D
^A Ciprofloxacin	540 BC offria	xone 30ug ^{, C} Gentamicin 1	Oug ^D Micona	701e 5040	

Table 1. Halos of inhibition of microbial growth by crude extracts of C. nucifera L. by disk diffusion (1000 µg/disk)

Ciprofloxacin 5µg; "Ceftriaxone 30µg; "Gentamicin 10µg," Miconazole 50µg.

None of the extracts from C. nucifera L. inhibited the Gram-negative E. coli, which is consistent with the results obtained, reporting that Gram-negative bacteria such as E. coli and P. aeruginosa may offer barrier in permeability, due to the chemical composition of their cell walls [18].

Our findings corroborate a survey conducted in India, which used another part of the same plant species, in which the antimicrobial activity of the root of C. nucifera was similarly demonstrated against the same microorganisms tested here [19].

The antimicrobial activities displayed by crude extracts of leaves and sheath of C. nucifera L. are relevant and stimulate the search for bactericidal activity against other Gram-positive and Gram-negative microorganisms; since the discovery of biocomponents from plants with antimicrobial activity can lead to optional therapies.

In vitro antimicrobial assays for drilling in agar

The leaf extract from C. nucifera extract by EtOH/MeOH (2:1) inhibited the growth of S. aureus (Figure 1) with an average of the inhibition halos of 21 mm; similarly to the positive control - Ceftriaxone with 26.5 mm. It also showed bactericidal activity against P. aeruginosa (Figure 2) with halo of 12 mm, while the positive control, Ciprofloxacin, displayed a halo of 34 mm.



Figure 1: Halo of 21 mm of inhibition growth of S. aureus extract by EtOH / MeOH (2: 1) from the leaves of C. nucifera L. (right arrow). Halo of 26mm of positive control: Ceftriaxone 30µ (left arrow)



Figure 2: Halos of 12 mm (A) of growth inhibition of P. aeruginosa from the leaves extract by EtOH / MeOH of C. nucifera L. PC: Ciprofloxacin control 5ug (B)

The results against the Gram-negative bacteria were promising (12 mm inhibition halo) because P. aeruginosa has resistance conferred by enzymatic degradation of the drug, low expression of membrane proteins, resistance to different antibiotics (such as cephalosporins and carbapenems), alteration of the target site alternative metabolic pathways and decrease of the intracellular concentrations of the antimicrobial drug (decreased permeability, change in transport systems in the cell, active inflow – elimination of the drug by over-expression of efflux pumps) [20].

Tests conducted with the ethanolic extracts from leaf, leaf sheath, and stem at 10% against *S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans* showed no antimicrobial activity in the test of drilling in agar (Table 2).

Microorganisms (ATCC)			Crude Extracts at 10%			Desitive	
	Leaf	Leaf EtOH/MeOH		Leaf Sheath	Stem	Control	
	EtOH	2:1		EtOH	EtOH		
S. aureus (25923)	0.0	21	.0	0.0	0.0	26.5 ^A	
P. aeruginosa (27853)	0.0	12	.0	0.0	0.0	34 ^B	
E. coli (25922)	0.0	0.	.0	0.0	0.0	34 ^B	
C. albicans (10231)	0.0	0.	.0	0.0	0.0	21 ^C	
Acti	2.0	P		C	0		

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		· · · · · A · · ·				

^ACeftriaxone 30µg; ^BCiprofloxacin 5µg; ^C Miconazole 50µg.

The low or even absent inhibitory activity of the extracts against the Gram-negative bacterium *E. coli* may be related to the structural differences these bacteria present in comparison with Gram-positive bacteria. These bacteria have a cell wall with dual membranes in the form of a complex envelope, which gives them greater resistance and protection [21].

The selective activity in Gram-negative bacteria was also observed in a study that tested extracts from leaves and branches of *Ilex paraguariensis* and identified that *P. aeruginosa* and *Proteus mirabilis* were sensitive to the extract and resistance to *E. coli*, raising the possibility that there may be features specific from *E. coli* that make it resistant to those extracts [22].

The results of the antimicrobial testing by drilling in agar are encouraging since the crude extracts from leaves and sheath of *C. nucifera* showed a growth inhibitory effect on Gram-positive bacteria and selective for Gram-negative.

Minimum Inhibitory Concentration (MIC)

Microdilution assays in eppendorfs and 96-well plates with active extracts in the diffusion in agar assays – leaf and sheath of *C. nucifera* L. at 10% (20 mg) – were conducted to test the sensibility of the microorganisms *S. aureus* and *P. aeruginosa* (Table 3).

Microorganisms	Crude Extracts				
ATCC	Leaf EtOH	Leaf EtOH/MeOH 2:1	Leaf Sheath EtOH	GC	NC
S. aureus (25923)	00	Active 10% (10 mg)	Active 10% (10 mg)	+	-
P. aeruginosa (27853)	00	Active 20% (20 mg)	Active 20% (20 mg)	+	-

Table 3 - Evaluation of the minimum inhibitory concentration (MIC) of microbial growth of crude extracts from C. nucifera Linn.

GC - Growth control and NC - Negative Control = SC - sterility control.

There was growth inhibitory activity of *S. aureus* by ethanolic extracts of leaf sheath and leaves EtOH / MeOH (2: 1) of *C. nucifera* L, in concentrations of 10% to 5% (20 to 10 mg/mL). These sensitivities should be explored in order to combat this Gram-positive bacterium which has an important marker of pathogenicity for being positive coagulase – ability to clot plasma that is mainly responsible for infections [23] and that from the beginning of the antimicrobial era demonstrates ability to resist to the action of antibiotics and some anti-infectives chemotherapeutics [16].

Compared with other antimicrobial studies on *S. aureus*, including one made in 2009 that evaluated the antimicrobial and antifungal potentials of *Coutarea latiflora* (Rubiaceae) by microdilution technique and that they found that the MIC of the extract in methanol for *S. aureus* was between 500 and 700 mg/mL [24]. It was observed that the MIC presented by *C. nucifera* extracts is high, but on a scale that classifies plants showing MIC values less than 600 mg/mL as active, between 600 and 1500 mg/mL as moderately active, and MIC values greater than 1600 mg/mL as weakly active [25].

In this score, the sheath extract in EtOH and leaf extract in EtOH/MeOH (2:1) were active against *P. aeruginosa*. This pathogen is associated with infection and pulmonary insufficiency, particularly in individuals with cystic fibrosis, which makes it responsible for the major cause of morbidity and mortality in this group [6].

There is no consensus on the acceptable concentration levels of plant extracts in comparison with standard antibiotics. These results of antimicrobial activity at concentrations of 20 mg/mL for *P. aeruginosa* and 10 mg/mL for *S. aureus* are corroborative as antibiotic alternatives, given the accelerated metabolism and reproduction of the bacteria associated with the genetic material exchange mechanism which enables them, over time, to develop forms of intrinsic resistance to physical cell structure and mutations [26].

Antimicrobial activities of plants against these pathogens were also investigated using *Peperomia pellucida* (herb-of-jabuti) and *Portulaca pilosa* (kiss-me-quick), which showed inhibition at concentrations from 500 to 62.5 mg/mL, and the extract from *Miconia rubiginosa* (Melastomataceae), which obtained for *S. aureus* MIC higher than 400 mg/mL [27].

Cell viability assay

Toxicity is a limiting factor for the release and use of drugs and, therefore, toxicity analysis associated with the biological activity of a compound is essential for determining, its application and to establish the therapeutic index [28-29].

In Figure 3, it is possible to observe the result of the cytotoxicity assay at the concentrations of 1000, 200, and 100 μ g/mL, because the controls of medium and lysis showed excellent. The negative control (2% DMSO) used in the extracts at 2000 μ g/mL caused cell lysis (p < 0.001), so the experiments CNL1, 2, 3, and 4 at 2000 μ g/mL were unfeasible.

The leaves extracts in EtOH/MeOH (2:1) EtOH from leaves, stem, and sheath were tested at the concentrations of 1000, 200 and 100 μ g/mL and correlated to the negative controls DMSO at 1%, 0.2%, and 0.1%, respectively.

At the concentrations of 200 and 100 μ g/mL, the extract in EtOH from leaves (CNL1), leaf in EtOH/MeOH 2:1 (CNL2), sheath (CNL3) and stem (CNL4) showed no statistically significant citotoxicity against macrophages of the J774 lineage (p < 0,01).



Figure 3 - C. nucifera L. extracts (CNL) activity at concentrations of 2000, 1000, 200 and 100

Mean * (p < 0.05), ** (p < 0.01) and *** (p < 0.001). $\mu g/mL$ in the J774 lineage macrophages. The data represent the mean and standard error of the at the concentration of 1000 $\mu g/mL$, sheath extracts did not show statistically significant mitochondrial cytotoxicity, while leaves and stems presented toxicity (p < 0.001). The extracts from leaves and stems did not exhibit statistically significant cytotoxicity at the concentrations of 200 and 100 $\mu g/mL$, whereas the sheath extract was nontoxic from 1000 $\mu g/mL$.

CONCLUSION

The crude ethanolic extracts from sheath and EtOH/ MeOH (2:1) extracts from leaves of *Cocos nucifera* L inhibited bacterial growth of *S. aureus* and *P. aeruginosa* in the diffusion tests on agar plates.

The minimum inhibitory concentration of the growth of *S. aureus* by the crude extracts from leaf in EtOH/MeOH (2:1) and the ethanolic extract from sheath of *Cocos nucifera* L was 10 mg (5%). The minimum concentration able to inhibit microbial growth of the Gram negative bacterium *P. aeruginosa* was 20 mg (10%); presented by the

EtOH/MeOH (2:1) crude extracts from leaf and ethanolic extract from the sheath of *Cocos nucifera* Linn. The extract from stem at 10% did not inhibit bacterial growth of *S. aureus*, *P. aeruginosa* and *E. coli*. None of the extracts showed activity at 10% against *E. coli* or *C. albicans* at a concentration of 1.5×10^6 CFU.

The crude extracts of *C. nucifera* L showed no cytotoxicity at the concentrations of 100 and 200 μ g/mL against the J774 lineage cells (48 hours). The crude extract in EtOH from sheath exhibited no statistically significant mitochondrial cytotoxicity (p < 0.05) at the concentration of 1000 μ g/mL; while the leaves and stem showed toxicity at this concentration (p < 0.001). The cytotoxicity assays at 2000 μ g were unfeasible by cytotoxic activity of the solvent 2% DMSO.

The antibacterial activities presented strengthen the viability of the continuation of antimicrobial trials with active plant parts (leaves and leaf sheath), which suggests the realization of antibacterial screening.

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