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IN VITRO ANTIBACTERIAL EFFECT OF EUTERPE OLERACEA MART. AND THEOBROMA GRANDIFLORUM HYDROALCOHOLIC EXTRACTS

(Efeito antibacteriano In vitro de extratos hidroalcoólicos de Euterpe oleracea Mart. E Theobroma grandiflorum)

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RESUMO: Este estudo avaliou o potencial antimicrobiano *in vitro* destas espécies contra estirpes de bactérias Gram-positivas e Gram-negativas. Os extratos hidroalcoólicos foram preparados a partir das folhas, polpa e sementes secas, de *Euterpe oleracea* Mart. e *Theobroma grandiflorum* por percolação contínua com álcool etílico 70%. A atividade antimicrobiana foi avaliada frente a quatro microrganismos pelo método de difusão em disco e ensaio de concentração inibitória mínima (CIM). A atividade antimicrobiana mostraram que extratos de polpa e sementes de açaí possuiram inibição significativa, respectivamentem em *Clostridium perfringens* (320 e 640 CIM), *Staphylococcus aureus* (80 e 320 CIM) e *Pseudomonas aeruginosa* (640 e 2560). Extratos de cupuaçu não mostraram nenhum efeito sobre todas as bactérias. O uso fitoterápicos de açaí pode ser uma alternativa sustentável, viável e acessível para o tratamento antimicrobiano. Novos estudos devem ser realizados buscando melhores resultados nos extratos de açaí e novas formulações do extratos de cupuaçu.
Palavras-chave: açaí, antimicrobiano, cupuaçu, fitoterápicos

ABSTRACT: This study evaluated the *in vitro* antimicrobial activity of these species against strains of Gram-positive and Gram-negative bacteria. The hydroalcoholic extracts were prepared from dried leaves, pulp and seeds of *E. oleracea* Mart. and *T. grandiflorum* by continuous percolation with 70% ethyl alcohol. The antimicrobial activity was evaluated against four microorganisms by the agar disc diffusion method and the minimal inhibitory concentration (MIC) assay. The antimicrobial activity showed that the açai pulp and seeds possessed significant inhibition in *Clostridium perfringens* (320 and 640 MIC), *Staphylococcus aureus* (80 and 320 MIC) and *Pseudomonas aeruginosa* (640 and 2560 MIC). Cupuassu extracts showed no effect on any bacteria. The use of açai extract products can be a sustainable, viable and an accessible alternative for antimicrobial treatment. New studies should be conducted to determine better results for acai herbals and new formulations of cupuassu extracts.
Key Words: Açai, antimicrobial, cupuassu, phytotherapy

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INTRODUCTION

Due to existing cases of antimicrobial resistance against a diverse number of bacteria, new alternatives to combat these pathogens are being investigated. In recent years, phytotherapy research has increased, opening a new possibility as a viable complementary treatment for bacterial infections (Arantes *et al.*, 2014). Besides being a new solution against resistant pathogens, these agents are readily accepted, due to the fact that they are generally less toxic to the host organism and to the environment (Souza *et al.*, 2013).

The Amazon Forrest has the largest plant diversity in the world with various plants with known antimicrobial properties (Suffredini *et al.*, 2006; Ranilla *et al.* 2012). However, more than 80% of the plants still have to be studied and their medicinal properties elucidated (Silva & Franco 2010). Açai (*Euterpe oleracea* Mart.) and cupuassu (*Theobroma grandiflorum*) are among the most studied plants in Brazil (Carvalho *et al.*, 2009; Santos *et al.*, 2011). Studies have demonstrated the versatility of producing different pharmaceutical compounds using these plants using raw material, due to the diversity of the existing active compounds (Hanada *et al.*, 2010; Gonçalves *et al.*, 2012).

Clostridium perfringens is a group of obligatory anaerobic bacteria highly pathogenic to both animal and humans. Due to the inadequate use of antimicrobials, cases of antibiotic resistance have been observed for tetracycline, clindamycin, lincomycin and erythromycin, worldwide (Gholamiandehkordi *et al.*, 2009; Gamboa-Coronado *et al.*, 2011). *Escherichia coli* is a bacteria of great medical importance as it is present in the normal flora of the gastrointestinal tract of the animals, causing different diseases. This species possesses great genetic plasticity and as such it can develop resistance to diverse antimicrobial drugs (Hoban *et al.*, 2011; Tadesse *et al.*, 2012). *Pseudomonas aeruginosa* is a bacteria that causes opportunistic infections, presenting diverse clinical manifestations in humans and animals, such as pneumonia, enteritis, otitis. This organism can present natural or acquired resistance to a great number of antibiotics used in clinical practices. (Neves *et al.*, 2011). *Staphylococcus aureus* is a pathogen encountered on the skin and animal cavities and is also of great importance due to opportunistic infections that it may cause. It

possesses a notable ability to develop resistance to medications shortly after it's used (Weese & van Duijkeren, 2010).

The objective of this study was to evaluate the effectiveness of different formulations of phytotherapeutic agents as antimicrobials, produced from leaves, fruits and seeds from açai and cupuassu.

MATERIAL AND METHODS

The açai (*Euterpe oleracea* Mart.) leaves, fruits and seeds were collected at the Biological Science Institute of the Federal University of Para, city of Belem, Brazil in July 2014. The cupuassu (*Theobroma grandiflorum*) leaves, fruits, and seeds were collected at Ver-o-Peso Market, in the same city and time period. The material was identified by its standard botanical description (Henderson, 1995). After collection, the material was dried at room temperature and subsequently grounded in a mill. This was followed by inspection and removal of foreign bodies and the product was subsequently stored in a dark recipient, protected from light and hermetically sealed, up to the time to be used in the production of the extracts.

The hydroalcoholic extracts were obtained utilizing an amber flask and sealing all its lateral extensions with aluminum foil. Seventy-two grams of each plant part were placed in separate flasks with 720 ml of 70% ethyl alcohol (v/v). Subsequently, the content was stored for 30 days in the refrigerator at 4^0C and stirred periodically. This step was followed by a filtration with the aid of a vacuum pump and a funnel with filter paper into a Kitasato flask.

After obtaining the extracts, the same were concentrated with a rotary evaporator under reduced pressure at a temperature lower than 30°C and subsequently lyophilized. The amount of açai extract obtained from the leaves (EFoA), fruits (EFrA) and seeds (ESA) were: 4.24%; 4.08% and 4.34% respectively. For cupuassu, the amount of extract obtained from the leaves (EFoC), fruits (EFrC) and seed (ESC) were 4.01%; 3.87% and 4.38% respectively.

The extracs were diluted in distilled water at a concentration of 10mg/ml, according to Matos (1997) to determine the following metabolites: phenols, tannins and anthocyanins, anthocyanidins, flavonoids, catechins, leucoanthocyanidins, steroids, triterpenes, saponins, resins and alkaloids. The Folin-Ciocalteu assay was

utilized to determine the dosage of phenolic compounds according to the methodology adapted from McDonald *et al.* (2001). The absorbance of the extracts was compared to the standard of gallic acid (1 µg/ml a 50 µg/ml).

The cytotoxic activity of the extracts was evaluated according to the adapted method from Meyer *et al.* (1982). Metanauplius eggs were placed in an aquarium with artificial seawater at 38g/L, under constant aeration and controlled temperature (28ºC). The larvae hatched 48h after incubation, and ten metanauplius were transferred to a 24-well plate containing the diluted extracts with DMSO (1% v/v) in concentrations varying from 31.75 to 8000 µg/ml. The negative control group was prepared with only artificial seawater plus DMSO (1% v/v), and quinidine sulfate was used for the positive control. All tests were done in triplicates. After 24 h of contact, the number of live larvae was counted and the larvae that remained immobile after more than 15 sec of agitating the plate were considered dead. From this value the lethal concentration (LD_{50}) was calculated. The toxicity criteria of the extracts was established according to Déciga-Campos *et al.* (2007) with values of >1000 µg/mL (non-toxic), ≥ 500 ≤ 1000 µg/mL (weekly toxic) and < 500 µg/mL (toxic).

The antioxidant activity was determined according to Blois (1958). The samples at various concentrations (10 to 640 µg/mL) were added to a 152 µM solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol. After incubation at 37°C for 30 min, the absorbance of each solution was determined in spectrophotometer at 517 nm. The antioxidant activity of the samples was expressed as IC_{50} (inhibitory concentration), which was defined as the concentration, in µg/m, of the sample required to inhibit the formation of DPPH radicals by 50%. In this experiment, ascorbic acid was used as positive control.

All microorganisms were obtained from the American Type Culture Collection (ATCC), two Gram-positives strains, *Clostridium perfringens* (ATCC 12919) and *Staphylococcus aureus* (ATCC 29213) and two Gram-negatives, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853).

All bacteria were inoculated in BHI agar and incubated at 37ºC for 24h. After which, subculturing was done with 50µl of the initial inoculum that was transferred to Mueller-Hinton broth, which was incubated at 37ºC for 1h until a discrete turbidity was observed:

corresponding to 0.5 McFarland turbidity standard equivalent to $1.5x10^6$ UFC ml^{-1}. The agar diffusion test was done according to the method described by Karaman *et al.* (2003). With the aid of a sterile swab, the microbial inoculum was streaked onto the surface of the Muller-Hinton agar. The agar was poured at a depth of 4 mm within a Petri dish. Filter paper discs of 6 mm in diameter were impregnated with 10 µl of extract. Using the serial dilution method, a total of eight different concentrations (10–2.560 µg/ml) of the plant extracts were obtained. The disks were deposited at a distance of 30mm from each other and the periphery of the petri dish to reduce overlaying of the inhibition zone. The negative controls were prepared utilizing the same solvents used to dissolve the extracts. For the positive control Ciprofloxacin (40 µg/disk) and Gentamicin (30 µg/disk) were utilized. The plates were incubated at 37ºC for 24h. Each test in this experiment was done in triplicates. The antimicrobial activity was evaluated by measuring the inhibition zone formed around the disks. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extract able to cause the death of the inoculum.

The microbial susceptibility index (MSI) was adapted from Eloff (2004) method, to compare the relative susceptibility among the microbial strains. MSI values ranged from 0 (resistant to all extracts) to 100 (susceptible).

$$MSI = \frac{100 \times \text{number of extracts effective against each microbial strain}}{\text{Number of total products}}$$

RESULTS AND DISCUSSION

The study of the Amazonian flora with the aim of discovering new antimicrobial agents is of great importance. *In vitro* tests serves as a preliminary step for the validation of novel phytotherapy products, as they allow the characterization of possible active compounds present within the plants and the evaluation of their biological properties (Silva *et al.*, 2006; Paulo *et al.*, 2009).

In both extracts the presence of the following metabolites were detected: phenols, tannins, steroids, triterpenes, resins, catechins, saponins and alkaloids. However, leucoanthocyanidins were not detected by the tests used. In the açai extract, anthocyanins and anthocyanidins were detected, however these compounds were not find in measurable quantities in the extracts produced from cupuassu. The data observed is consistent with what is available in the recent literature (Galotta

& Boaventura, 2005; Rodrigues *et al.*, 2006; Carvalho *et al.*, 2009; Rodrigues *et al.*, 2010; Sousa *et al.*, 2011). The total phenol value in gallic acid equivalents of the extracts from the leaves, fruits and seed from the açai plant were: 78.2 ± 0.987 $mg.g^{-1}$; 318.2 ± 0.755 $mg.g^{-1}$ and 185.2 ± 0.91 $mg.g^{-1}$ respectively. Whereas, cupuassu presented: 64.8 ± 1.258 $mg.g^{-1}$; 224.8 ± 0.58 $mg.g^{-1}$ and 148.8 ± 0.413 $mg.g^{-1}$ respectively.

Phenolic compounds are natural antimicrobials with a high level of antioxidants (Oliveira *et al.*, 2008) where this activity is greater when the pH is above 5.0. The antimicrobial effect of tannins has been confirmed in several studies. Its activity is due to enzyme inhibition in a direct action to the membrane, competing for metallic ions, essential for microbial metabolism (Shohayeb *et al.*, 2013), whereas the antimicrobial activity of sterols occurs due to the alteration in the pH of the media and the alkylation of important proteins of the microorganisms (Schinor *et al.*, 2007). Anthocyanins are known for their medicinal properties, such as its antimicrobial properties due to their antioxidant action (Rocha *et al.*, 2011). Saponins, catechins and triterpenes possess relative bactericidal activity, especially against Gram-positive bacteria (Avato *et al.*, 2006; Castilhos *et al.*, 2007; Mencherini *et al.*, 2007).

The açai pulp showed the lowest DPPH scavenging potential with IC_{50} values of 52.7µg/ml (Table 1) followed by the açai seed extract with IC_{50} values of 64.8µg/ml. Ascorbic acid (Control) exhibited an IC_{50} of 2.1µg/ml. Polyphenols have been associated with the anti-oxidant activity in fruits and vegetables, including açai and cupuassu, the flavonoids were found to be the major polyphenols in these plants. Many studies have demonstrated that flavonoids have strong anti-oxidant activities and others biological properties (Kang *et al.*, 2010). In these plants, this compound is found primarily within the pulp of the fruit/plant. Other polyphenols that are also responsible for antioxidant activity are tannins and lignins (Gülçin, 2012).

Table 1. Antioxidant activity and cytotoxicity against *Artemia salina* of *Euterpe oleracea* Mart. (açaí) and *Theobroma grandiflorum* (cupuassu) hydroalcoholic extracts.

Samples	DPPH assay IC50 (µg/ml)[a]	Cytotoxicity LC50 (µg/ml)[a]
Açaí		
Leaves	165.2 (141.5-214.5)	2033
Pulp	52.7 (41.5-65.1)	1053
Seeds	64.8 (55.3-76.7)	1310
Cupuassu		
Leaves	145.8 (111.2-185.3)	3507
Pulp	101.2 (89.7-140.1)	3003
Seeds	135.7 (100.9-180.1)	2876
Ascorbic Acid[b]	2.1 (1.7-2.5)	-
Tymol[b]	-	1.5 (0.8-2.7)

[a] The results in parentheses are the 95% confidence limits; [b] Positive controls.

To establish the toxicity of *A. annua*, the lethal-dose test against *A. salina* was used as a highly reliable assay to

determine the toxicity of phytotherapeutics agents (Fernández-Calienes Valdés *et al.*, 2009). The brine shrimp bioassay is a simple, rapid and low cost test that can be performed *in vitro* (Lieberman, 1999). It is important to analyze the toxicity of **new therapeutic agents** to verify the possible side effects when the product is tested *in vivo*. The results of the toxicity test are shown in Table 1. None of the extracts were toxic with values exceeding 1000 µg/ml.

The antimicrobial activity of the plant extracts is shown in Table 2. The extracts produced from the açai fruits and seeds, demonstrated antimicrobial activity against *C. perfringens*, *S. aureus* and *P. aeruginosa*. However, the extract produced from the açai leaves presented antimicrobial activity only against *S. aureus*. The extracts produced with the different parts of the cupuassu were not effective against any bacteria. None of the products had any effect against *E. coli*.

Table 2. Minimum inhibitory concentration (MIC) values of parts of *Euterpe oleracea* Mart. and *Theobroma grandiflorum* hydroalcoholic extracts (µg/ml).

Samples/Bacteria	*Clostridium perfringens*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Açai				
Leaves	-	-	-	1280
Pulp	320	-	640	80
Seeds	640	-	2560	320
Cupuassu				
Leaves	-	-	-	-
Pulp	-	-	-	-
Seeds	-	-	-	-

-: No action

MSI values were useful in evaluating the susceptibility of the different strains of microbes to the tested material. *S. aureus* (ATCC 29213) strain was found to be the most susceptible organism (MSI of 50) (Table 3). On the other hand, *E. coli* (ATCC 25922) showed to be resistant to all products.

Table 3. Microbial susceptibility index (MSI) calculated for the different strains of microorganisms used for screening of *Euterpe oleracea* Mart. and *Theobroma grandiflorum* hydroalcoholic extracts.

Bacteria	Number of active extracts	MSI values
C. perfringens (ATCC 12919)	2	33.3
E. coli (ATCC 29213)	0	0
P. aeruginosa (ATCC 27853)	2	33.3
S. aureus (ATCC 29213)	3	50

Araújo *et al.*, (2013) demonstrated that extracts from the seed of the açai inhibit the growth of *S. aureus*. Melhorança-Filho & Pereira, (2012) studied the antimicrobial activity of the oils produced from the açai fruit and observed antimicrobial activity against *S. aureus*. This bacteria is highly sensitive to antibiotics and substances with a similar mode of action, and as such, it can be used as a biological indicator for substances with potential antimicrobial activity (Oliveira *et al.*, 2008). However, there are a few reports of the germicidal action of the extracts produced from the açai plant against gram-negative organisms. It is believed that this occurs due to the more complex structure and a greater lipid content of the cell wall of the plant, which could make the bacteria more resistant to phytochemicals (Efstratioi *et al.*, 2012).

Although Silva *et al.*, (2014) analyzed the bactericidal effectiveness of plants from the Brazilian Caatinga Biome and observed that when these products were diluted in water there were no bactericidal action against *E. coli* and *Enterobacter* sp. and low effectiveness against *Actinobacter* sp. and *Krebisiella* sp., when the same material was diluted in ethyl-alcohol, antimicrobial activity was demonstrated to all microorganisms. But, this procedure can only be done *in vitro*, because the diluent is toxic to experimental animals (Nogueira *et al.*, 2014). In our work the açai leave extract acted only on *S. aureus*, possibly due to the high sensitivity of the bacteria to its germicidal agents. This also occurred at low concentrations of the biocompounds, with the germicidal activity in this part of the plant. To increase the amount of phenolic compounds such as flavonoids and anthocyanins, and consequently improve the germicidal activity could be done by performing a cold digestion of the leaves in methanol (Rodrigues *et al.*, 2006).

Cupuassu is one of the most studied plant in the pharmaceutical industry, mainly for the production of nutraceutical capabilities (Carvalho *et al.*, 2009). However, there are no scientific studies that aimed to demonstrate its antimicrobial, activity. Thus, the present data is the first evidence study attempting to demonstrate their dose-dependent activity against a range of potentially harmful bacteria. The phytochemical characteristics showed that the produced extracts from açai did not possess some of the important natural antimicrobial compounds such as anthocyanins. Besides this, the Folin-Ciocalteu test showed a low total phenol content. The cupuassu seed possesses anthocyanin and anthocyanins, compounds not found in the present test. This may be due to the extraction method utilized, where acidified methanol (methanol:acetic acid = 99:1) should have been utilized instead of hydro alcohol (ethyl alcohol : water = 70:30) (Pugliese, 2010). Fermenting cupuassu before the extraction with methanol 70% (v/v) can free more phenolic content, principally flavonoids, which are compounds with antimicrobial properties which may cause an oxidation of the cell wall of the microorganisms (Genovese & Lannes, 2009). For the pulp of the fruit, it is recommended that freshly prepared pulp be used as it preserves a greater quantity of ascorbic acid, an element that possess a high antioxidant potential but is scarce in

cupuassu in comparison with other tropical fruits (Maiai *et al.*, 2010).

CONCLUSIONS

The use of açai plant extract can be a sustainable, viable and accessible alternative for antimicrobial treatment, however new studies should be performed to improve the present results. New formulations of cupuassu should also be investigated.

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