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PHYTOCHEMICAL SCREENING AND SYNERGISTIC INTERACTIONS BETWEEN AMINOGLYCOSIDES, SELECTED ANTIBIOTICS AND EXTRACTS FROM THE BRYOPHYTE *OCTOBLEPHARUM ALBIDUM* HEDW (CALYMPERACEAE)

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Abstract - This work is the first to describe the modulation of antibiotic activity of the bryophyte *Octoblepharum albidum* Hedw extract. The antibacterial activity of ethanolic extract of *O. albidum* (EEOa), alone and in association with aminoglycosides, was determined against six bacterial strains by a microdilution test. The results showed a similar inhibitory activity of EEOa against *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 33018 (MICs 512 μg/mL). The synergistic effect of the extracts and aminoglycosides was also verified. The most pronounced effects were obtained with EEOa + gentamicin against *E. coli* and EEOa + kanamycin against *K. pneumoniae* with MICs reduction (128 to 32 μg/mL). The data from this study are indicative of the antibacterial activity of the bryophyte *O. albidum* extracts and its potential in modifying the resistance of aminoglycosides analyzed.

*Key words***:** *Octoblepharum albidum* Hedw, Bryophyta, antibacterial activity, modification of resistance, aminoglycosides.

INTRODUCTION

The search for new antibacterial agents is important due to the progressive increase in resistance of clinically important pathogens to known classes of antibiotics. New vegetal sources presenting antimicrobial activity and low toxicity could be a viable alternative (Costa et al., 2008). Of particular importance are natural products sourced from plants that have been evaluated not only for direct antimicrobial activity, but also as a resistance-modifying agent of antibiotics (Gibbons, 2004).

Natural products of plants have been shown to exhibit antibacterial properties and a capacity for modifying resistance. These effects can be synergistic or antagonistic (Rodrigues et al., 2009; Sousa et al., 2011). Synergism has been defined as a phenomenon in which two different compounds are combined to enhance their individual activities. If the combination results in worsening effect, it is called antagonism (Rani et al., 2009). Compounds with such activity are classified as modifiers of antibiotic activity, and can represent progress in attempts to overcome resistance mechanisms to aminoglycosides (Coutinho et al., 2008).

Aminoglycosides are potent bactericidal antibiotics targeting the bacterial ribosome. Several mechanisms have evolved in bacteria that confer

them with antibiotic resistance. These mechanisms can chemically modify the antibiotic, render it inactive through physical removal from the cell, or modify the target site so that it is not recognized by the antibiotic. They exhibit concentration-dependent bactericidal activity, and intermittent doses overcome bacterial adaptive resistance (Freeman et al., 2009; Gilbert, 1991).

Bryophytes are small plants characterized by their simple organization and little or no organized vascular tissue, although some species present a specialized conduction system (Raven et al., 2007). Bryophytes are the second largest group of land plants in the world today with over 20,000 species (Shaw and Goffinet, 2000). In Brazil, 3,200 species have been catalogued and, in the northeastern region, they occur mainly in Ceara and Pernambuco states (Pôrto, 1996).

Glime et al. (2007) reported the increase of the use of bryophytes in the treatment of diseases. In China, the mosses *Rhodobryum giganteum* and *R. roseum* have been used in the treatment of cardiac affections; the tea of *Sphagnum* has been used to treat ocular diseases for a very long time; *Polytrichum commune* has been used against inflammation and fever. In North America, the mosses *Barbula unguiculata, Bryum capillare* and *Octoblepharum albidum* are applied for fever and pain conditions.

There are reports of studies performed in the last century regarding the chemical composition of bryophytes (Zinsmeister et al., 1991). However, in the past two decades, biologists, chemists and pharmacologists have been interested in this group of plants. The phytochemistry of bryophytes has been neglected for a long time because they are very small and difficult to collect in large amounts as pure samples (Herz, 1982).

The aim of this work was to analyze phytochemically the extract of the bryophyte *Octoblepharum albidum* Hedw and to investigate its potentiation of the antibiotic activity of aminoglycosides. This activity has not been reported so far for this species.

MATERIALS AND METHODS

Plant material

Octoblepharum albidum Hedw was collected in Crato county, Ceará, Brazil (07º 16.915 S, 39º26.270W and 07º 16.959S e 39º.26.343Wº), between June and July 2008. The plant material was identified by Professor Kátia Cavalcante Pôrto from the Department of Biological Sciences of Federal University of Pernambuco. A voucher specimen (#3752) was deposited in the Herbarium Caririense Dárdano de Andrade Lima (HCDAL) - Department of Biological Sciences (URCA).

Preparation of extracts

An ethanolic extract of *O. albidum* (EEOa) was prepared using the cold extraction method (Matos, 1997). A total 84 g of samples of *O. albidum* were placed in a flask containing 2 L of ethanol (95%) for 48 h at an ambient temperature. A rotary vacuum evaporator (model Q-344B, Quimis, Brazil) and ultrathermal bath (model Q-214M2, Quimis) were used to remove the ethanol (under reduced pressure, 50ºC), obtaining 1.78 g of extract with yields of 2.11 (w/w).

Phytochemical screening

The phytochemical screening of the EEOa was performed according to Matos (1997). Specific qualitative tests were conducted to investigate the presence of saponins, tannins, alkaloids, flavonoids, triterpenes, steroids and coumarins. The tests were based on visual observation of changes in color or formation of precipitate after the addition of specific reagents.

Antibacterial assay and minimal inhibitory concentration (MIC)

The antibacterial test for minimal inhibitory concentration (MIC) determination of the EEOa was performed by a microdilution assay (CLSI, 2006). The assay was carried out with six bacterial species obtained from Fundação Oswaldo Cruz – FIOCRUZ:

Staphylococcus aureus ATCC 12692, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 33018, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 15442 and *Shigella flexneri* ATCC 12022.

Brain heart infusion broth (BHI 3.8%) was used for bacterial growth (24 h, 35 ± 2°C). The inoculums of each bacterial species in BHI broth were diluted in the same media to a final concentration of about 1 x 10^8 CFU/mL (0.5 nephelometric turbidity units – McFarland scale). The suspension was then diluted to 1 x 10^5 CFU/mL in BHI 10%. 100 µL of each dilution were distributed in 96-well plates with extracts, achieving 5 x 10^5 CFU/mL as the final concentration of the inoculums.

The initial solution of the EEOa was performed using 10 mg of extract dissolved in 1 mL of dimethyl sulfoxide – DMSO, to obtain an initial concentration of 10 µg/mL. From this concentration, several dilutions were made in distilled water in order to obtain a stock solution of 1024 µg/mL. Further serial dilutions were performed by addition of BHI broth to reach a final concentration in the range of 8-512 µg/mL.

The experiments were performed in triplicate and the microdilution trays were incubated at 35 ± 2°C for 24 h. Antibacterial activity of the extract was detected using a colorimetric method by adding 25 µL of resazurine staining (0.01%) aqueous solution in each well at the end of the incubation period. The MIC was defined as the lowest extract concentration able to inhibit bacteria growth, as indicated by resazurine staining (died bacteria cells are not able to change the staining color by visual observation – blue to red).

Antibiotic modifying test

In order to evaluate EEOa as a modulator in the resistance of antibiotics, the MIC of the antibiotics neomycin, kanamycin, amikacin and gentamicin against *P. vulgaris* ATCC 13315 and *S. aureus* ATCC 10390 strains was determined in the presence or absence of the extracts at sub-inhibitory concentrations (MIC x 1/8). The experiments were performed in triplicate by a microdilution assay, utilizing suspensions of 10^5 CFU/mL in BHI (10%) and antibiotic concentrations ranging from 0.0012-2.5 mg/mL (2-fold serial dilutions). The plates were incubated for 24 h at 37°C and controls using DMSO in MIC determination and antibiotic modulation activity tests were performed.

RESULTS AND DISCUSSION

Phytochemical screenings has revealed the presence of compounds such as tannins phlobaphenes, tannins pyrogallates, anthocyanins, flavones, flavonols, flavonones, aurones, proanthocyanidins, alkaloids and terpenes, as shown in Table 1. The antibacterial properties of the EEOa were verified by the inhibitory activity against six bacterial strains (Table 2). It was verified that *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 33018 (MICs 512 µg/mL) had greater sensitivity. The EEOa showed weak antibacterial activity against other bacteria strains (MICs >512 µg/mL). The MICs of the antibiotics towards the bacteria were in the range of 64 to 128 µg/mL (Table 3).

Table 3 shows the MICs of the antibiotics and the synergic effects of the EEOa in association with antibiotics. The MICs for all antibiotics were reduced in the presence of the extract. The most pronounced effects were obtained with the EEOa+neomycin against *E. coli*, and the EEOa+kanamycin against *K. pneumoniae*, with MIC reduction of 128 to 32 µg/mL. In general, the extract interference on antibiotic action was correlated to the antibiotic type and bacteria strain. The control DMSO showed a MIC ≥ 1024 µg/mL and no modifying antibiotic activity.

The antibacterial properties and modulation of antibiotic activity can be due to the presence of compounds with known antibacterial activity, such as terpenoids, alkaloids, tannins and flavonols. Flavonoids, for example, have exhibited antioxidant, anti-inflammatory, anticarcinogenic and antimicrobial activities. Some lipophilic flavonoids can cause rupture of the plasma membrane of microorganisms (Tsuchiya et al., 1996).

Table 1. Phytochemical *screening* of *O. albidum* ethanolic extract.

Sample	Metabolites															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
EEOa	+	+	+	-	+	+	-	+	-	-	+	+	-	+	+	-

EEOa = Ethanolic Extract of *O. albidum*; 1 = tannins phlobaphenes; 2 = tannins pyrogallates; 3 = anthocyanins; 4 = anthocyanidins; 5 = flavones; 6 = flavonols; 7 = flavononols; 8 = flavonones; 9 = xanthones; 10 = chalcones; 11 = aurones; 12 = proanthocyanidins; 13 = catechins; 14 = alkaloids; 15 = terpenes; 16 = saponins + = presence; - = absence.

Table 2. Values of the minimal inhibitory concentration (MIC µg/mL) for the *O. albidum* ethanolic extract.

Strains	MIC (µg/mL)
Staphylococcus aureus ATCC 12692	> 512
Escherichia coli ATCC 25922	512
Klebsiella pneumoniae ATCC 33018	512
Pseudomonas aeruginosa ATCC 15442	> 512
Proteus vulgaris ATCC 13315	> 512
Shigella flexneri ATCC 12022	> 512

Table 3. Minimal inhibitory concentration (MIC - µg/mL) values for the aminoglycosides in the absence and presence of *O. albidum* ethanolic extract.

Antibiotics	*Escherichia coli* ATCC 25922		*Klebsiella pneumoniae* ATCC 33018	
	MIC alone	MIC combined	MIC Alone	MIC combined
		EEOa 64 µg/mL		EEOa 64 µg/mL
Amikacin	64	16	128	64
Neomycin	128	32	64	16
Gentamicin	64	16	64	32
Kanamycin	128	64	128	32

EEOa = Ethanolic extract of *O. albidum*

Tannins have been employed to combat diarrhea, arterial hypertension, rheumatism, bleeding, scars, burns, renal problems and inflammatory processes. Among all the elements found in the plants, alkaloids are the most powerful, exhibiting analgesic, spasmolytic, anti-inflammatory, anticarcinogenic and others activities.

One of the classes with the most active compounds is the terpenoids, which comprises different types of compounds that may be divided into more important chemical structure groups due to their high medicinal value. The mechanism of the activity of terpenoids is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds, with permeability enhancement. This property can facilitate the antimicrobial agents to penetrate into a cell, leading to an activity enhancement.

The production of antibacterial substances is a widespread phenomenon in bryophytes. In general,

bryophytes are not infected by bacteria and fungus, neither are they attacked by insects, snails and other small animals. Some bryophytes produce volatile simple terpenes or aromatic compounds that are responsible by a variety of odors.

On the other hand, a notable range of symbiotic relations is observed among bryophytes, bacteria and fungi. In some species, these relations are very important for the different stages of the life cycle, such as spore germination and gametophyte development. However, bryophytes produce a variety of antibiotic substances at the same time that they need the presence of other microorganisms. This apparent contradiction could be explained by the selective antibacterial activity of the extracts.

The improvement of antibiotic activity against the Gram-negative bacteria *E. coli* and *K. pneumoniae* demonstrated a significant result because gram-positive bacteria are more susceptible to natural products (Silva et al., 2007). Gram-negative bacteria present structural particularities that are difficult for antibiotic penetration, such as the lipopolysaccharide structures containing polysaccharides of different lengths that largely contribute to cell surface properties, such as membrane permeability and antibiotic susceptibility (Yokota and Fullii, 2007).

In conclusion, this study demonstrated that *O. albidum* extract has antibacterial activities. It is suggested that it could be used as a source of plant-derived aminoglycosides which possess a resistance-modifying antibacterial activity.

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