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ANTITUMORAL, ANTIOXIDANT AND ANTIMICROBIAL MOLECULES FROM *COMBRETUM RUPICOLA*

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ABSTRACT

This investigation describes the anticancer, antioxidant and antimicrobial properties of the extracts of *Combretum rupicola,* a native plant from Northeast of Brazil. Methanolic, ethyl acetate and chloroform extracts from leaves of *C. rupicola* were evaluated in relation to their potential in inhibiting the cell growth and cytotoxic properties against nine human cancer cell lines (MCF-7, NCI-ADR/RES, OVCAR-3, PC-3, HT-29, NCI-H460, 786-O, UACC-62, K-562). In addition, antioxidant activity of these extracts was measured using DPPH as radical scavenging assay and the antimicrobial activities against nine pathogens were tested by agar diffusion method. Preliminary results showed that the this plant demonstrates to have moderate activity against bacteria but, on the other side, the extracts showed significant anticancer activities against four cell lines and the most significant activity was observed against MCF-7 (65.9 µg mL^{-1}), highest inhibitory concentration IC_{50} 0.22 µg mL^{-1} for antioxidant activity. These founds are the first scientific reports on secondary metabolites with biological activities of *C. rupicola*, suggesting the potential of this botanic species for pharmaceutical industry.

KEYWORDS: ***Combretum rupicola*, antioxidant, anticancer, antimicrobial, Fernando de Noronha Island**

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INTRODUCTION

Natural products continue to play a major role in drug discovery and development, and medicinal plants have been a rich source of many compounds with large production of bioactive molecules and help to prevent the development of serious diseases [1, 2, 3]. Combretaceae family comprises species of trees, shrubs and lianas, with distribution in tropical and subtropical regions and is widely used for medicinal purposes in Africa, Asia, South America and India [4, 5]. The largest genera is *Combretum* and is known for many applications including the treatment of abdominal disorders, bacterial infections, cancer and heart diseases, skin diseases, sore throats, gastric problems [5, 6, 7]. *Combretum rupicola*, rare Combretaceae species found in the National Marine Park of Fernando de Noronha (03°51'13''S and 32°25'25''W, PE, Brazil) by the English naturalist H.N. Ridley, 1890 [8]. In accordance with previous studies, it is considered endemic to this island and there is no scientific record about medicinal and ethnopharmacological use. Phytochemical studies carried out with some species belonging to the genus *Combretum* showed a wide range of tannins, flavonoids, terpenoids, phenanthrenes and stilbenoids [9]. The overall aim of our research was to evaluate extract from *Combretum rupicola* for antimicrobial, antioxidant and anticancer activities with the motivations of discovering compounds and for the purpose of validating its ethnomedical use. This research is the first scientific report over secondary metabolites of *Combretum rupicola*.

MATERIALS AND METHODS

(i) Plant Material

Fresh leaves of *Combretum rupicola*, were collected from the National Marine Park of Fernando de Noronha, April/2010, and identified by the botanist *Msc*. Cosme Santos (State University of Feira de Santana, BA). A voucher of the specimen was deposited in HUEFS (Herbarium of the State University of Feira de Santana, Bahia State, Brazil), Department of Botany under the number 161260.

(ii) Preparation of plant extract

For investigation of the biologic potential of chemical compounds from *C. rupicola,* leaves were dried out at room temperature, and after the samples (30 g) were powdered, and extracted with ethyl acetate P.A. (1 L), mixed and macerated at room temperature (27 °C) for approximately 24 h, followed by chloroform P.A. (1 L), and finally, by addition methanol P.A. (1 L). The solvent was fully evaporated under reduced pressure, and the extract was concentrated and stored at -20 °C until use.

(iii) Biological screening

(iv) Antimicrobial activity

Eight common human pathogens were used to evaluate the antimicrobial activity of crude extracts from *C. rupicola*. The pathogens (obtained from the Culture Collection of the Environmental Microbiology Laboratory, Embrapa Environment, Jaguariúna, SP, Brazil) included the bacteria *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus schleiferi*, *Bacillus pumilus, Salmonella typhi*, and *Agrobacterium tumefaciens* and two phytopathogenic fungi *Pythium aphanidermatum* and *Sclerotinia rolfsii*. For antimicrobial evaluation, agar cup disc diffusion method was followed [10]. Minimal inhibition concentration (MIC) was determined by microdilution method according to NCCLS [11]. Different concentrations of the crude extracts were tested against each microorganism, in the plates with 96-well microtiter plate, which was incubated at 28ºC for 24 h. The microbial growth was determined for revelation with 50 µL of 1 chloride 2,3,5-triphenyl-2H-tetrazolium (Sigma). Chloramphenicol was used as antibacterial agent and nistatine was used as standard antifungal agent. The experiments were carried out in triplicate.

(v) Antioxidant activity

The antioxidant activity of the crude extracts was analyzed using DPPH (2,2-diphenyl-1-picrylhydrazyl), which is a radical scavenging assay. This was carried out according to Milardovic et al. (2006) [12] and Santos et al. (2011) [13]. Radical scavenging activity was expressed as percentage and was calculated by using the following formula: % scavenging = $[(A_{control} - A_{sample})/A_{control}] \times 100$. For each sample, the result was presented as an IC_{50} (sample concentration that produced 50% scavenging of the DPPH radical).

(vi) Cytotoxicity assay

The anticancer activity was monitored by *in vitro* cytotoxic activity assay against UACC-62 (melanona), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR- 3 (ovarian), PC-3 (prostate), HT-29 (colon), 786-O (renal), NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance) and K-562 (leukemia) cancer cell lines. A 48-h sulforhodamine B cell viability assay was performed to determine growth inhibition and cytotoxic properties of crude extract from *C. rupicola* [14]. Extracts were tested against the nine cell lines over a 3 –log concentration range (0, 0.25, 2.5, 25 and 250 µg mL^{-1}). According to Holbeck (2004) [15], three endpoints can be calculated for each cell line. In the present research, we used the TGI endpoint to compare the potential and specificity of the extracts. Doxorubicin was used as a positive control.

RESULTS AND DISCUSSION

1. Antimicrobial activity

The methanolic, chloroform and ethyl acetate extracts (10 mg $disc^{-1}$) obtained from leaves of *Combretum rupicola* were evaluated according to their antimicrobial activity by the diffusion method (table 01). It has demonstrated that methanolic extract is more potent, showing a higher degree of antimicrobial activity, with a zone of inhibition of 14 mm by the disc diffusion method in comparison to other extracts. Moreover, the growth of *Staphylococcus aureus* (14 mm) was highly inhibited. In the same way, this extract had showed high antifungal activity against *P. aphanidermatum* and *Sclerotinia rolfsii*. Moderate effects were observed when the extracts were obtained with ethyl acetate, with zone inhibition of 10 mm, and activity against the phytopathogenic bacterium *Agrobacterium tumefaciens*. The chloroform extract showed activity against only three clinical pathogens. Out of the seven bacterial strains tested, only *Pseudomonas aeruginosa* was resistant to all extracts. The antimicrobial activity of the organic solvents extracts showed varying magnitudes depending on the susceptibility of the tested microorganism. Eloff (1999) [16] quantified the antibacterial activities of leaf extracts from 27 members of Combretaceae family and Fyhrquist et al. (2002) [6] found activity in extracts of the roots and stembark of *Combretum* and *Terminalia* species used in Tanzania [17, 18, 19].

Table 1
In vitro antimicrobial activity of crude extracts from Combretum rupicola.

Test Organisms	Diameter of inhibition zone (10 mg.disc^{-1} (mm))		
	Chloroform	Ethyl acetate	Methanol
	Pathogen Bacteria		
Staphylococcus schleiferi	7	8	12
Staphylococcus aureus	7	8	14
Bacillus pumilus	7	7	12
Enterococcus faecalis	-	-	10
Pseudomonas aeruginosa	-	-	-
Salmonella typhi	-	-	12
Agrobacterium tumefaciens	-	10	-
	Phytopatogenic Fungi		
Pythium aphanidermatum	-	+	+
Sclerotinia rolfsii	-	-	+

(-) no activity, (+) activity with halo of 10 mm

2. Antioxidant activity

A methanol solution of DPPH free radical was found to be stable for more than 60 min. by spectrophotometry at 517 nm in this solution. The radical scavenging effects of *C. rupicola* inedit medicinal plant extracts were then measured spectrophotometrically for DPPH free radical. The control intensity (absorbance of extracts) was taken as 100%, and the percentage intensity was calculated. The methanolic extract of *C. rupicola* scavenged 50% DPPH free radical revealed strong antioxidant activity, with highest inhibitory concentration IC_{50} 0,22 µg mL^{-1} this value was lower than those of positive standards trolox (IC_{50} 4 µg mL^{-1}). The ethyl acetate extract of the plant revealed moderate antioxidant activity (IC_{50} 239 µg mL^{-1}) and the chloroform extract displayed the lowest inhibitory concentration (IC_{50} 266 µg mL^{-1}). The results denote the presence of antioxidant principles in the extracts and methanol extracted the highest number of antioxidant compounds based on DPPH analysis. It can be associated to its small molecular size and the affinity with both polar and non-polar compounds [20]. Plants may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinines, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity [9, 21]. Some species from the Combretaceae family have been found to have antioxidant activities, as *C. erythrophyllum* from southern Africa which was isolated the antioxidant compounds 5-hydroxy-7,4'-dimethoxyflavone [22]. In this study, *C. rupicola* is a good candidate for the isolation of antioxidant active compounds due to the elevated number of compounds with a high degree of activity.

3. Cytotoxicity assay

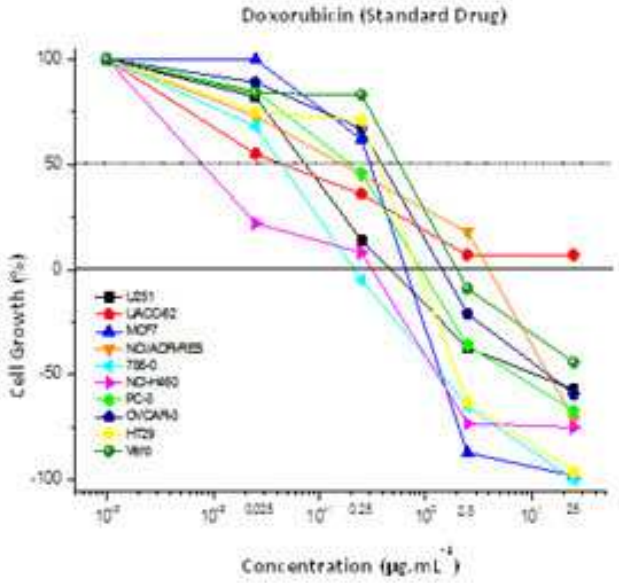
Anticancer activity of the extracts was assayed using routine tissue culture techniques for nine cancer cells line using the SRB assay. The cytotoxicity profile of *Combretum rupicola* was compared with doxorubicin and showed significant power of action and only ethyl acetate extract of *C. rupicola* exhibited antiproliferative activity for four cell lines, with TGI varying from 65 to 194 µg mL^{-1}. The most significant activity was observed against MCF-7 (TGI 65.9 µg mL^{-1}, breast cancer) according to table 02. For VERO cell (non-cancer cell-line), the TGI estimated was 68.9 µg mL^{-1}. The methanol and chloroform extract showed no cytotoxic activity for the cells used in this assay. Despite the cytotoxic activity of the crude extract of *C. rupicola* being lower than that of the positive control (doxorubicin), the present results reveal the antitumor potential of this species.

Table 2
Antiproliferative activity of crude extract from Combretum rupicola leaves

Cell lines	TGI (µg/mL) Ethyl Acetate	Doxorubicin
U251	194.0	0.68
UACC-62	-	>25
MCF-7	65.9	0.56
NCI=ADR/RES	-	2.3
NCI-H460	-	0.15
PC-3	111.1	1.0
OVCAR-3	-	1.8
HT-29	244.9	0.85
786-O	131.7	0.73
VERO	68.9	3.2

U251 (glioma); UACC-62 (melanoma); MCF-7 (breast); NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance); 786-0 (renal); NCI-H460 (lung, non small-cells); PC-3 (prostate); OVCAR-3 (ovarian); HT29 (colon); VERO (kidney epithelial cells). (-) no activity >250.

In all tests performed in this study the compounds present in methanol extracts and ethyl acetate were the most potent in the activities examined in this study (figure 1). This reflects the characteristic chemistry of solvents used presenting profile of medium to high polarity, it is known that the Combretaceae family have a wide group of broad spectra of activity as observed in this study and possibly the chemical profile of extracts obtained from *Combretum rupicolum* this study were triterpenes, polyphenols, arjunolics acids, stilbenes, alkaloids, chromatographic data (data not shown) are obtained and the extracts fractionated to isolate and characterize chemically possible the biomolecule of interest.



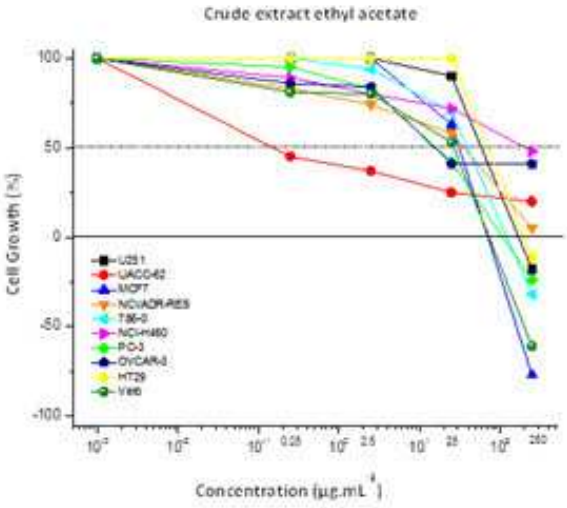


Figure 1

Percent inhibition of cell growth of tumor cell lines according to the concentrations at 48 hours. U251 (glioma); UACC-62 (melanoma); MCF-7 (breast); NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance); 786-0 (renal); NCI-H460 (lung, no small-cells); PC-3 (prostate); OVCAR-3 (ovarian); HT29 (colon); VERO (kidney epithelial cells).

CONCLUSION

The results presented in this communication confirm the possibility of ethnomedicinal use of *Combretum rupicola* species to antioxidant, anticancer and antimicrobial properties, which are comparable with the reference drugs. There are unavoidable limitations associate with *in vitro* tests, such as the lack of physiologically representative environment and absence metabolic activation. Therefore it is necessary to confirm promising efficacy observed in laboratory test with activity in an animal model to proceed with development of a potential new anticancer or antimicrobial or antioxidant preparation. In addition, due to the remarkable bioactive activity of *C. rupicola*, further studies are in progress in this laboratory for the isolation and identification of the bioactive components.

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