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Full Length Research Paper

Anti-*candida* biofilm properties of Cameroonian plant extracts

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Candida infections can be superficial, invasive or disseminating. The virulence of _Candida_ species has been attributed to several factors, including the promotion of hyphae and biofilm formation, adherence to host tissues, and response to environmental changes and morphogenesis. Resistance to many clinically used antifungal agents has led to the need to identify new compounds and drugs for therapeutic use. Therefore, the objective of this study was to evaluate the anti-candida and anti-biofilm activities of some Cameroonian plant extracts against _Candida albicans_ and _Candida glabrata._ The biofilm biomass of _C. albicans_ and _C. glabrata_ was quantified using the violet crystal protocol. A microbroth dilution method was used to determine the minimum inhibitory concentrations (MICs), and a biofilm enumeration assay was employed to determine the minimum biofilm inhibition concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs) of the extracts. The absorbance value of the biofilm biomass of _C. albicans_ was 0.14±0.01 and that of _C. glabrata_ was 0.51±0.06. _Eugenia uniflora_ and _Terminalia mantaly_ aqueous leaf extracts showed MICs of 0.3125 and 0.625 mg/mL for _C. glabrata_, while the MICs for _C. albicans_ were 10 and 0.625 mg/mL, respectively. The MBIC and MBEC of _C. glabrata_ of _E. uniflora_ aqueous leaf extracts were 0.125 and 0.5 mg/mL, respectively, and 0.45 and >1.8 mg/mL, respectively for _T. mantaly_. The results of this study demonstrated the _in vitro_ anti-biofilm potential of _T. mantaly_ and _E. uniflora_ aqueous leaf extracts against _Candida_ biofilm. Nonetheless, further analyses of a larger number of _Candida_ isolates and plant extracts are needed to validate these findings.

Key words: Anti-*candida*, anti-biofilm, *Eugenia uniflora, Terminalia mantaly.*

INTRODUCTION

Candida species are the most common fungal pathogens in humans and the causative agents of superficial and systemic candidiasis, giving rise to severe morbidity in millions of individuals worldwide (Ruhnke, 2014; Silveira-

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Gomes et al., 2011; Pfaller and Diekema , 2007). The incidence of infections is increasing among compromised patient groups such as cancer patients on chemotherapy, patients receiving broad-spectrum antibiotic treatment, and HIV-infected individuals (Neeta and Uttamkumar 2011; Ye et al., 2004). Vaginal candidiasis is quite common in women and approximately 75% present this infection once in their lifetime. *C. albicans* is the most prevalent fungal pathogen in humans. Mucosal infections of *Candida albicans* are often benign, but systemic infections are usually fatal (Al-Ahmadey and Mohamed 2014; Foxman et al., 2013). Although *C. albicans* is the most frequent cause of infection, non-*albicans* species infections are on the rise (Mohandas and Ballal, 2011). Thus, *Candida glabrata* was reported to be the second most common agent of vaginal candidiasis; however, the increasing incidence of cases of vaginal candidiasis caused by non- *c. albicans* species has not yet been well established (Al-Ahmadey and Mohamed, 2014; Esmaeilzadeh et al., 2009). In the Littoral Region of Cameroon (Nylon District Hospital), the prevalence of oral and vaginal candidiasis in 2012 was 52.6 and 29.7%, respectively (Njunda et al., 2012). The prevalence of oral candidiasis among HIV patients in the study population of the Mutengene Baptist Hospital in the South West Region in 2013 was 66.7% (Njunda et al., 2013). It has been reported that the mortality rate of invasive infections is 40% (Klevay et al., 2009; Pfaller and Diekema, 2007; Bertagnolio et al., 2004) and *C. albicans* is estimated to be responsible for 50-60% of the cases of invasive candidiasis (Perlroth et al., 2007; Pfaller and Diekema, 2007).

One of the factors contributing to the virulence of *Candida* is the formation of surface attached microbial communities known as biofilms (Seneviratne et al., 2008). Biofilm formation helps the microorganisms evade host defenses, exist as a persistent source of infection and develop resistance against antifungal agents. The resistance of biofilm forming C*andida* spp. to antifungal agents represents a major challenge especially in the design of therapeutic and prophylactic strategies (Golia et al*.,* 2011). Aside from increasing the resistance to the available antifungal compounds, the toxicity of some of these compounds is high (Shreaz et al., 2011; Georgopapadakou and Walsh, 1994). Some major antifungals are limited to a few chemical classes such as Amphotericin B, a polyene fungicidal agent that has been implicated in hepatotoxicity and nephrotoxicity, coupled with decreasing efficacy (Pan et al., 2009; Dismukes, 2000; Arthington-Skaggs et al., 2000). Hence, the need for inexpensive, effective and less toxic antifungals is imperative.

Medicinal plants have been the major health care measure of resource-poor populations worldwide (Duraipandiyan and Ignacimuthu, 2011; Tharkar et al., 2010). According to the WHO, 80% of the world's population uses natural remedies and traditional medicines (WHO, 2001, 2003). This is particularly common in Africa, as well as in most low-income countries, where a high proportion of the population still resorts to traditional medicine for primary health care. Cameroon has a rich biodiversity, with ~8,620 plant species (Mbatchou, 2004; Earth Trends, 2003), some of which are commonly used in the treatment of several microbial infections (Kuete and Efferth, 2010). Some plant extracts have demonstrated positive response during pharmacological investigations (Suresh et al., 2010; Patel and Coogan, 2008).

Therefore, the main objective of the present study was to evaluate the anti-candida biofilm properties of several plant extracts by determining the minimum inhibitory concentrations (MIC), minimum biofilm inhibition and minimum biofilm eradication concentrations (MBEC).

MATERIALS AND METHODS

Plant material and extraction

Leaves, twigs, stem bark and stems of different plants were collected at Mount Kalla in Yaoundé (Central region) and Dschang (West region) Cameroon on the 11th of September 2011 and 2014, and voucher specimens were deposited at the National Herbarium of Cameroon, Yaoundé. The plant parts were individually dried at room temperature and then ground to fine powder. Five hundred grams (500 g) of each sample were macerated with regular stirring in 2 L of 95% ethanol or distilled water for 72 h. The filtrate was evaporated using a rotary evaporator (Rotavapor BÜCHI 011). The plant residues were dried and macerated in distilled water for 72 h and the filtrate dried at room temperature (25-28°C) using a fan. The extraction yields were calculated as percentage relative to the starting plant material.

Biofilm quantification

The biofilm forming ability was assessed by quantification of total biomass by violet crystal (VC) staining. Thus, after washing, biofilms were fixed with 200 µl of methanol 99%, which was removed after 15 min. The microtitre plates were allowed to dry at room temperature, and 200 µl of VC (1% v/v) were added to each well and incubated for 5 min. The wells were then gently washed with sterile, ultra-pure water and 200 µl of acetic acid (33% v/v) were added to release and dissolve the stain. The absorbance of the solution obtained was read in triplicate in a microtitre plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 590 nm. The experiment was repeated three times (Silva et al., 2009).

Screening of plants extracts for MICs

Two clinical *Candida* isolates (*C. albicans* and *C. glabrata*) were collected from patients with vaginal candidiasis in the Hospital Clinic of Barcelona. The inoculum of each yeast isolate and strain was prepared from a 2-day-old culture on Sabouraud Dextrose Agar (SDA) at 37°C. The suspension was adjusted to 1 x 10^3 cells/mL using yeast nitrogen base (YNB) medium from 0.5 McFarland standards. The broth micro-dilution method was used to assess yeast susceptibility to extracts using YNB medium supplemented

with 5% glucose.

Briefly, each extract (200 mg/mL in 5% DMSO) was serially diluted in YNB supplemented with 5% glucose in 96-well plates. Eighty microlitres of inoculum standardized at 1×10^3 colony forming units (CFU)/mL was added to each well to achieve a final volume of 230 µL. The final concentrations tested ranged between 0.039 and 40 mg/mL for the crude extracts. The positive control consisted of microorganisms growing without extract. After 48 h of incubation at 37°C, the MIC was determined as the lowest concentration of the crude extract in the broth medium that inhibited visible growth of the microorganisms tested. All tests were performed in duplicate. Wells without inoculum or extract were included in each plate to control background sterility and growth. The extracts with the greatest activity were chosen to continue the experimental part of the work.

Determination of the MBIC and MBEC using the Calgary protocol

The isolates were cultured overnight in SDA medium. After preparation of 0.5 McFarland in broth medium, 200 µL were added to each well of a flat-bottom 96-well microtitre plate (MBEC ™ Biofilm Inoculator Innovotech product panel P and G panel lot: 14040004).

For the MBIC, flat-bottom microtitre plates containing two-fold dilutions of plant extract in 150 µl of YNB per well (antibiotic challenge plate) were used. The plant extracts included *Eugenia uniflora* aqueous leaf extract (1-0.125 mg/mL) and *Terminalia mantaly* aqueous leaf extracts (1.8-0.225 mg/mL) in *C. glabrata*, and (3.6-0.45 mg/mL) in *C. albicans*. Eighty microlitres of a subculture adjusted to $1x10^3$ CFU/mL was added to all the wells, except for those of the negative control, covered with the pegs lid inthe biofilm growth plate, and incubated for 18-20 h at 37°C.

For the MBEC, *Candida* biofilms were formed by immersing the pegs of the cover lid into this biofilm growth plate, followed by incubation at 37°C for 20 h-24 h without shaking. The peg lids were rinsed three times in sterile water, placed onto new flat-bottom microtitre plates containing two-fold dilutions of plant extract in 150 µl of YNB per well (antibiotic challenge plate), and incubated for 18-20 hours at 37°C. The plant extracts included *E. uniflora* aqueous leaf extract (1-0.125 mg/mL) and *T. mantaly* aqueous leaf extracts (1.8-0.225 mg/mL) in *C. glabrata*, and (3.6-0.45mg/mL) in *C. albicans.*

After antibiotic incubation, the peg lids were washed three times with sterile water and placed into extract-free YNB fresh medium in a new flat-bottom microtitre plate (biofilm recovery plate). To transfer the biofilms from the pegs to the wells, each plate was sonicated at room temperature for 20 min (using a Bransonic 220; BransonCo., Shelton, Conn.). The peg lid was discarded and replaced by a standard lid. The sonicated culture media of each well of the microtitre plate was spread on YNB agar plates and incubated at 37°C for 24 h. Adequate biofilm growth for the positive control wells was defined as the number of colonies obtained after 24 h of incubation. The positive control contained microorganisms and culture medium, and the negative control included only medium. The results were expressed as the number of CFU counted in each extract concentration and per strain.

Phytochemical screening of *E. uniflora and T. mantaly* aqueous leaf extracts

Phytochemical analysis was done to identify the different components responsible for the activities observed according to the protocols described by Igwe (2004), Trease and Evans (1996) and Sofowora (1982).

RESULTS AND DISCUSSION

Plant extracts

The plant extracts used in the experiments were obtained as defined in the materials and methods section. Table 1 describes the plant collection site and date, and the extraction solvent used.

Biofilm quantification

The average value of *C. albicans* and *C. glabrata* biofilm was 0.14±0.01 and 0.51±0.06, respectively, and 0.13±0.02 for the negative control. Therefore, *C. albicans* was not considered in the biofilm inhibition studies.

Determination of the MIC

The aqueous leaf extracts of *E. uniflora* and *T. mantaly* showed the best MIC in *C. glabrata* with values ranging from 0.3-0.5 to 0.625-1 mg/mL, respectively. However, only the aqueous leaf extract of *T. mantaly* revealed the best activity in *C. albicans,* showing a MIC of 0.625 to 1.8 mg/mL. These extracts were selected for the determination of the MBIC and MBEC of the strains (Table 2).

Effect of *E. uniflora* and *T. mantaly* aqueous leaf extracts on biofilm inhibition and eradication in *C. glabrata*

Figure 1 shows the inhibition of biofilm formation of both extracts. *T. mantaly* aqueous leaf extract inhibited biofilm formation of *C. glabrata* at a concentration of 0.45 mg/mL, while *E. uniflora* aqueous leaf extract presented inhibition at a concentration of 0.125 mg/mL.

In *C. glabrata,* the MBEC of the *E. uniflora* aqueous leaf extracts ranged from 0.5-1 mg/mL. However, the eradication activity of the aqueous *T. mantaly* leaf extract was detected at concentrations >1.8 mg/mL (Figure 2).

Phytochemical studies

The different components presented in both extracts were flavonoids, saponins, tannins, glucosides, phenol, steroids, triterpenes and anthraquinones, among others. However, contrary to what was expected, anthocyanin was absent (Table 3).

DISCUSSION

Candida species are important opportunistic fungal

Table 1. Plant collection site and date, and extraction solvent.

Plant number	Plant name and identification number	Plant parts	Date and place of collection	Extraction solvents	
				Distilled water	Ethanol
1	*Eremomastax speciosa* No. HNC/136984	Leaves	2 August 2014 Dschang (West Region-Cameroon)	ES aqueous leaf	ESleaf EtOH
2	*Hisbiscus noldeae* No 9977SRFCAM	Leaves	2 August 2014 Dschang (West Region-Cameroon)	HN aqueous leaf	HN leaf EtOH
3	*Piper umbellatum* No 10391SRFCAM	Leaves Seeds	2 August 2014 Dschang (West Region-Cameroon)	PU aqueous leaf; PU aqueous seeds	PU leaf EtOH
4	*Polyathia longifolia* No	Twigs	14 July 2014 Mont kalla (Centre Region-Cameroon)	PL aqueous twigs	
5	*Uvariondendron calophyllum* 28734/SFR/CAM	Leaves, stem, stem bark and twigs	11 September 2011 Mont kalla (Centre Region-Cameroon)	UCl aqueous, UCst aqueous, UCtr aqueous, UCtw aqueous, UCst H20	UCtw EtOH, UCst EtOH,
6	*Vernonia amadalyna* No 35809HNC	Leaves	7 September 2014 Dschang (West Region Cameroon)	Bitter leaf aqueous	Bitter leaf EtOH
7	*Eugenia uniflora* *No 34063HNC*	Leaves	7 September 2014 Yaoundé (Centre Region-Cameroon)	F aqueous leaf	F leaf EtOH
8	*Psidium Guava* No 2884/SRFK/	Leaves	7 September 2014 Yaoundé (Centre Region-Cameroon)	Guava aqueous leaf	Guava leaf EtOH
9	*Dacryodes edulis* No 64929/HNC	Leaves	7 September 2014 Yaoundé (Centre Region-Cameroon)	Plum aqueous leaf	Plum leaf EtOH
10	*Mangifera indica* *No* 57347HNC	Leaves	7 September 2014 Yaoundé (Centre Region-Cameroon)	Mango aqueous leaf	
11	*Eryngium foetidium* No 17442/SRF/CAM	Leaves	7 September 2014 Yaoundé (Centre Region-Cameroon)	P aqueous leaf	
12	*Terminalia mantaly* *64212/HNC*	leaves and stem bark	7 September 2014 Yaoundé (Centre Region-Cameroon)	TeMsb aqueous, TMl aqueous	TM leaf EtOH
13	*Terminalia catappa* 51244/HNC	Leaves	7 September 2014 Yaoundé (Centre Region-Cameroon)	TCl aqueous	

pathogens due to the increasing occurrence of infections by these fungi, especially in patients with cancer, diabetes and HIV (Hamza et al., 2006). However, the antifungal agents used in the treatment of Candida infections and in biofilms can select drug-resistant microbes (Agarwal et al., 2008). The ability of these microorganisms to form biofilm together with the acquisition of new antimicrobial resistance, has led to new problems in treating infections caused by this pathogen. Thus, the WHO has recommended the evaluation of the effectiveness of plants against resistant

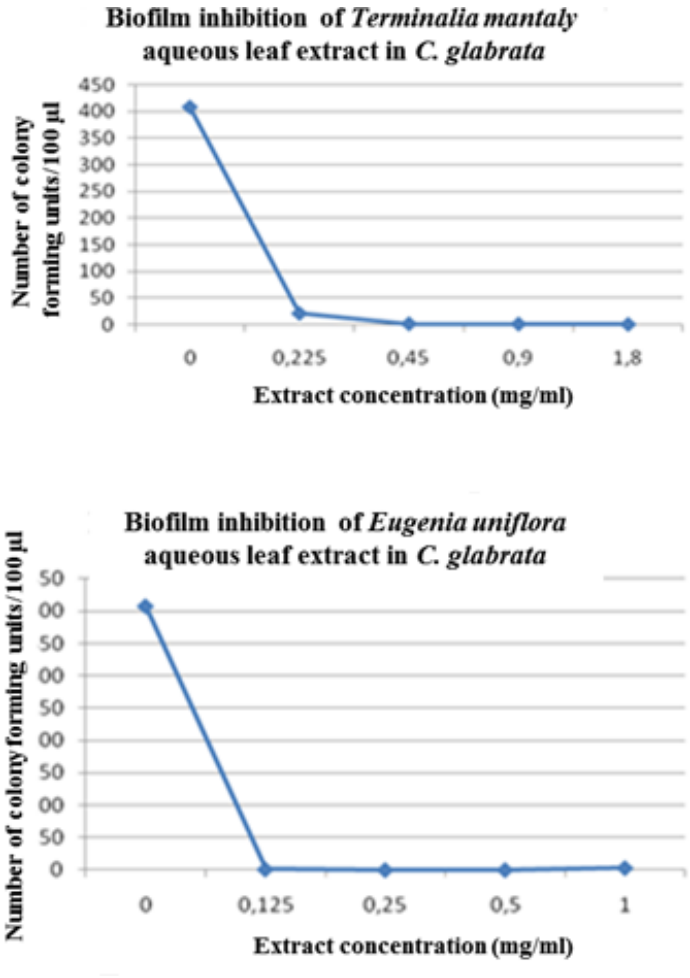

Figure 1. Biofilm inhibition concentration of *E. uniflora* and *T. mantaly* aqueous leaf extract in *C. glabrata.*

pathogens (Eisenberg et al., 1993). In this regard, new agents affecting the growth of biofilm-associated *C. albicans* and *C. glabrata* are greatly needed (Alviano et al., 2005). The present study was, therefore, carried out in order to evaluate the anti-biofilm activity of some Cameroonian plant extracts. Moreover, the exploration of additional natural resources for new antifungal agents with anti-biofilm activity could possibly reveal new antifungal agents with different modes of action or which affect different sites in *Candida* cells. This study summarizes the activity of the different extracts against *C. albicans* and *C. glabrata* in both the planktonic and biofilm state. This study's results show that the MIC of all the extracts ranged from 0.3 to >40 mg/mL, with the aqueous leaf extracts of *E. uniflora* and *T. mantaly* showing the best activity. Few studies have evaluated the antimicrobial activity of these extracts. The ethanoic extract of *E. uniflora* has antimicrobial activity against *Staphylococcus epidermidis* and *Staphylococcus aureus,* with MICs of 52 and 250 µg/mL, respectively (Bernardo et al., 2015). However, no assays using *Candida* species have been carried out. Other species within the genera Eugenia, such as *Eugenia dysenterica*, have shown antimicrobial activity against several *Candida* species with MICs ranging between 125 and 500 µg/mL (Correia et al., 2016). These values are similar to those found in the present study using *E. uniflora*.

Plants are used in local communities worldwide for the treatment of various diseases. *E. uniflora* has been used in the traditional medicine of some African countries to treat various ailments such as wounds, skin diseases, dysentery and fever. In Brazil, *E. uniflora* leaf infusion is used as an antipyretic, astringent and also for treating several stomach problems. In Surinam, the *E. uniflora* leaf decoction is drank as a cold remedy and as an antipyretic in combination with lemongrass (Auricchio and Bacchi , 2003; Wagner et al., 1999; Morton, 1987; Stone, 1970). Likewise, *T. mantaly* leaf is taken as a decoction

Table 2. Minimal inhibitory concentrations (MICs) (mg/ml) of the plant extracts studied.

Plant extracts	*C. albicans* MIC (mg/ml)	*C. glabrata* MIC (mg/ml)
Guava aqueous leaf	>40	>40
Plum aqueous leaf	20	10
HN aqueous leaf	>40	>40
F aqueous leaf	10	0.3125
Bitter aqueous leaf	40	40
PL aqueous twigs	40	>40
ES aqueous leaf	10	5
UCl aqueous	5	20
UCst aqueous	20	10
UCtr aqueous	20	5
PU aqueous leaf	>40	5
P aqueous leaf	10	10
PU aqueous seeds	10	40
Mango aqueous leaf	40	40
UCtw aqueous	10	5
TCl aqueous	>40	40
TeMsb aqueous	>40	0.3125
UCst H20	20	20
Guava leaf EtOH	>40	>40
ES leaf EtOH	>40	>40
Bitter leaf EtOH	>40	>40
UCst EtOH	>40	>40
F leaf EtOH	>40	20
HN Leaf EtOH	>40	>40
TM leaf EtOH	>40	5
PU leaf EtOH	>40	>40
UCtw EtOH	>40	>40
TMl aqueous	0.625	0.625

ES aqueous leaf: *Eremomastax speciosa* aqueous leaf extract; ES leaf EtOH: *Eremomastax speciosa* ethanoic leaf extract; HN leaf aqueous; *Hisbiscus noldeae* aqueous leaf extract; PU aqueous leaf: *Piper umbellatum* aqueous leaf extract; PU aqueous seeds: *Piper umbellatum* aqueous seeds extract; PU leaf EtOH: *Piper umbellatum* ethanoic leaf extract; PL aqueous twigs: *Polyathia longifolia* aqueous twigs extract; UCl aqueous: *Uvariondendron calophyllum* aqueous leaf extract; UCst aqueous, UCst H_20: *Uvariondendron calophyllum* aqueous stem extract; UCtr aqueous: *Uvariondendron calophyllum* aqueous trunk extract; UCst EtOH: *Uvariondendron calophyllum* ethanoic stem extract; UCtw EtOH: *Uvariondendron calophyllum* ethanoic twigs extract; Bitter leaf aqueous:*Vernonia amadalyna* aqueous leaf extract; Bitter leaf EtOH: *Vernonia amadalyna* ethanoic bitter?? leaf extract; F leaf aqueous: *Eugenia uniflora* aqueous leaf extract; F leaf EtOH*: Eugenia uniflora* ethanoic leaf extract; Guava leaf aqueous: *Psidium Guava aqueous leaf extract*; Guava leaf EtOH: *Psidium Guava* ethanolic leaf extract; Plum leaf aqueous: *Dacryodes edulis* aqueous leaf extract; Mango aqueous leaf: *Mangifera indica* aqueous leaf extract; P aqueous leaf: *Eryngium foetidium* aqueous leaf extract; TMl aqueous: *Terminalia mantaly* aqueous leaf extract; TeMsb aqueous: *Terminalia mantaly* aqueous stem bark extract; TM leaf EtOH: *Terminalia mantaly* ethanoic leaf extract;TCl aqueous: *Terminalia catappa* aqueous leaf extract.

and infusion in the treatment of many ailments such as gastroenteritis, arterial hypertension, diabetes, dental affections and cutaneous and genital infections (Coulibaly, 2006).

The MBICs and MBECs of these extracts were also determined. The results obtained showed that *T. mantaly* aqueous leaf extract inhibited biofilm formation of *C. glabrata* at a concentration of 0.45 mg/mL and was able to eradicate this biofilm at a concentration >1.8 mg/mL. On the other hand, *E. uniflora* aqueous leaf extract

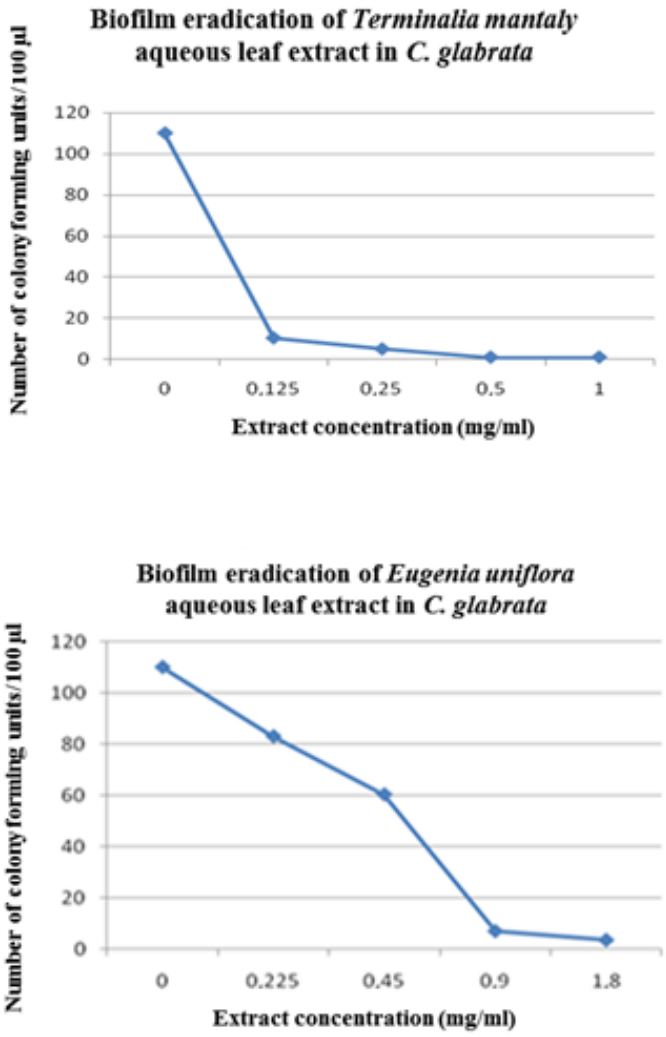

Figure 2. Biofilm eradication concentration of *E. uniflora* and *T. mantaly* aqueous leaf extract in *C. glabrata.*

Table 3. Phytochemical screening of aqueous leaf extracts of *T. mantaly* and *E. uniflora.*

Extracts	**Alkaloids**	**Flavonoids**	**Saponins**	**Tannins**	**Glucosides**	**Phenols**	**Steroids**	**Triterpenes**	**Anthocyanines**	**Anthraquinones**
Terminalia mantaly aqueous leaf	+	+	+	+	+	+	+	+	-	+
Eugenia uniflora aqueous leaf	-	+	+	+	+	+	+	+	-	+

+ Present, - absent.

inhibited biofilm formation of *C. glabrata* at a concentration of 0.125 mg/mL and eradicated mature biofilm at a concentration of 0.5 mg/mL. To the authors' knowledge, there is no previous study on the anti-biofilm activity of these plants. These activities could be due to the presence of tannins, steroids, triterpenes, flavonoid

glucosides, saponins and anthraquinones in the *E. uniflora* extracts as has been suggested previously (Fiúza et al*.,* 2008; Lorenzi and Matos, 2002). The presence of these components could act individually or in combination to produce the effects observed at the respective concentrations. Indeed, each of these constituents has a specific mode of action on the microbial strain. Thus, for example, tannins can act as antiseptic and antimicrobial agents and have antihaemorrhagic, antidiarrhoeic and wound-healing properties (Simões et al*.,* 2004). On the other hand, terpenoids have been reported to have the ability to interfere with biofilm formation without disrupting cellular growth (Hertiani et al*.,* 2010; Skindersoe et al., 2008).

Conclusion

The results of this study show that the studied extracts have antimicrobial activity and inhibit biofilm formation at the concentrations tested, suggesting that the bioactive compounds of these extracts are responsible for these activities. However, further studies are needed to verify which protein is inhibited and what chemical compounds in the extract are responsible for the activity observed. These compounds could be good candidates for the development of new anti-candida antibiotics, and tests with these compounds against other pathogenic microorganisms would also be of interest.

Conflict of Interests

The authors have not declared any conflict of interests.

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Antimicrobial activities and preliminary phytochemical tests of crude extracts of important ethnopharmacological plants from Brazilian Cerrado

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Screening of native plants with therapeutical effects constitutes a valuable way to enhance biological attributes of medicinal herbs and discover new drugs. Therefore, ethanol and methanol extracts from ten plants collected from Brazilian Cerrado were tested to inhibitory effects against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) through disc diffusion. The crude extracts that showed antibacterial activities from *Anacardium humile*, *Psidium guineense* and *Myracrodruon urundeuva*, were tested on the standard strain *S. aureus* ATCC 25923. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined and the interactions between the plant extracts and the main groups of secondary compounds present were investigated. The MICs of *P. guineense, M. urundeuva* and *A. humile* were all 4.1 μg/L for *S. aureus*. *P. guineense* and *A. humile* extracts tested against *P. aeruginosa* were 8.2 μg/L, whereas the extract from *M. urundeuva* had a MIC of 4.1 μg/L for the same strain. In addition, the observed MBCs were equivalent to the corresponding MICs. There were synergistic interactions in combinations of these plant extracts and tannins and flavonoids were identified in phytochemical analyses. These metabolites may be related to the biological activities that were found, indicating possible candidates for the development of strategies for treatment of infections caused by bacteria tested.

Key words: Crude plant extracts, biological activity, disc diffusion, traditionally use, popular medicine, Cerrado biome.

INTRODUCTION

The use of plants with therapeutic properties is an ancient practice and plants has been used as an important source of bioactive compounds (Pinto et al., 2002; Ncube et al., 2012; Khan et al., 2013; Li et al., 2016). Eating

herbs and leaves to relieve and to cure diseases was the earliest methodologies involving natural products (Viegas et al., 2006). It was through observation and experimentation that primitive peoples discovered the therapeutic properties of plants and disseminated this information from generation to generation (Turolla and Nascimento, 2006). The World Health Organization estimates that 80% of the world population uses medicinal plants as main resource in primary health care and they have been encouraging this practice (WHO, 2002). Medicinal plants and herbal medicines have an important role in therapy, as 25% of prescribed drugs worldwide are of natural origin, plants represent an important source of new biologically active compounds (Canton and Onofre, 2010). Selecting plant species for research and development based on allegations of a given therapeutic effect in humans may be a valuable shortcut to the discovery of pharmaceutical drugs (Schenkel et al., 2004; Viegas et al., 2006). The use of natural products as raw materials for the synthesis of bioactive substances has been widely reported (Schenkel et al., 2004; Viegas et al., 2006; Dias et al., 2012; Cragg and Newman, 2013).

During the last decades, the development of effective pharmaceutical drugs against bacterial infections has revolutionized medical treatment, resulting in a drastic reduction in mortality caused by microbial diseases (Dias et al., 2012; Cragg and Newman, 2013). However, the widespread use of antibiotics have caused bacteria to develop defenses, culminating in the emergence of resistance and imposing serious limitations on the options to treat infections, which represents a threat to public health (Silveira et al., 2006; Valgas et al., 2007). Because the use of plant extracts may constitute a viable alternative therapy to antibiotics (Nascimento et al., 2000), searching for new pharmaceutical drugs or prototypes from plant species has been suggested as a technological measure to solve the problem of multi-resistant bacteria (Silva et al., 2010).

In this context, this study aimed to test crude extracts of ten plants collected from the Brazilian Cerrado (Table 1) against the bacterial strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and 15 clinical strains of *S. aureus* isolated from patients at the Hospital Universitário Clemente de Faria (University Hospital) and from clinical laboratories at Montes Claros, State of Minas Gerais, Brazil. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBCs) of the extracts were determined by microdilution method. The interactions between the extracts of these plants using the disc diffusion methodology and the existence of the main secondary metabolite groups present in the crude extract with antibacterial activity were investigated.

MATERIALS AND METHODS

Ethnobotanical information

The plant material was collected between August and September 2006 at cities of Jaíba (15°20'16"S and 43°40'26"W), Glaucilândia (16°51'00"S and 43°41'49"W) and Claro dos Poções (17°04'48"S and 44°12'32"W) (States of Minas Gerais, Brazil). The botanical material was treated using identification and herborization techniques and was placed in the Montes Claros Herbarium of the Universidade Estadual de Montes Claros, city of Montes Claros, state of Minas Gerais, Brazil.

Screening test (Plant collection)

Leaves from the 10 different species (Table 1) were separated, oven-dried at 50°C, pulverized with a Willey type grinder and crushed on 1:5 ratio (200 g pulverized material per 1000 ml solvent) and two different solvents were used: ethanol and methanol for seven days at room temperature. After filtration, the filtrate was evaporated in a forced-air oven at 50°C for 24 h, and the residue was reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 300 mg/ml (Celloto et al., 2003). Bacterial inhibition tests were performed to *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) by disc diffusion protocol (NCCLS, 2003) using 6 mm diameter blank discs sterile containing 10 µl of the plant extracts. DMSO and 30 µg cefoxitin discs were used as negative and positive controls, respectively. The extracts that showed an inhibition zone were considered active and selected to next step.

Extraction of crude metabolite

Leaves from species with positive antibacterial activities from screening test were dried in oven at 50°C, pulverized in a Willey type mill and ground in ethyl alcohol PA at a 1:5 ratio (200 g pulverized material per 1000 ml solvent) at room temperature for 48 h with occasional stirring. To determine this milling time, a pilot study was conducted for milling times between 48 and 72 h and seven days. There was no difference in the diameter of the inhibition zone formed, so we chose the shorter time (48 h) to perform the tests. After filtration with cotton, the filtrate was evaporated in a rotary evaporator at 70°C, 135 rpm and a negative pressure of -50 Kpa, and the residue was reconstituted in DMSO at a concentration of 300 µg/ml (Celloto et al., 2003). The extract obtained was subjected to filtration with a 0.22 µm Millex filter. The extracts were maintained at room temperature on dark condition.

Antibacterial activity: Disc diffusion

Bacterial inhibition tests were performed using *S. aureus* ATCC 25923 Müller-Hinton agar was used for bacterial growth incubated for 24 h in a growth chamber set at 35°C. Bacterial suspensions were prepared from fresh cultures with 0.98% NaCl saline solution with a turbidity of 0.5 McFarland Scale ($1.5x10^8$ cells/ml) (NCCLS, 2003). The suspensions were used to inoculate on Müller-Hinton agar plates using a sterile swab. Antibacterial activity was verified

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Table 1. Plant species studied, name and common uses.

Botanical species	Family	Common name	Common uses	Voucher: Unimontes number	References
Anacardium humile	Anacardiaceae	Cajuzinho-do-campo, cajueiro-anão, cajuí, caju-do-cerrado	Antidiarrheal, antidiabetic, Anti-syphilitic, in skin disorders	200	Rodrigues and Carvalho (2001); Lorenzi and Matos (2002)
Astronium fraxinifolium	Anacardiaceae	Gonçalo-alves	Anti-rheumatic and in contusions	449	Lorenzi and Matos (2002)
Boehmeria arborescens	Asteraceae	Assa-peixe	Fever-reducing, in bronchitis, pneumonia, the flu and cough	454	Rodrigues and Carvalho (2001)
Brosimum gaudichaudii	Moraceae	Mama-cadela	Blood cleanser, in bronchitis, the flu, colds, skin spots and vitiligo, Anti-rheumatic, in chronic intoxications, poor blood circulation	455	Rodrigues and Carvalho (2001)
Eugenia dysenterica	Myrtaceae	Cagaita	Anti-diarrheal	453	Tassara (1996).
Luehea divaricata	Malvaceae	Açoita-cavalo	Antidysenteric, anti-hemorrhagic, anti-rheumatic, in ulcers, burns and gangrenous wounds	450	Rodrigues and Carvalho (2001)
Myracrodruon urundeuva	Anacardiaceae	Aroeira, aroeira-preta, aroeira-do-campo, aroeira-verdadeira, urundeúva	Anti-inflammatory, healing agent, antiseptic for ulcers, in skin disorders, urinary tract problems and respiratory tract problems	448	Nunes et al. (2006); Fenner et al. (2006); Lorenzi and Matos (2002)
Psidium guineense	Myrtaceae	Brazilian guava	Anti-diarrheal, diuretic	456	Rodrigues and Carvalho (2001); Morton (1987)
Solanum lycocarpum	Solanaceae	Wolf apple	Emollient, anti-rheumatic, tonic for the flu, colds and asthma, anti-spamodic, anti-epileptic, in abdominal and renal cramps and urinary tract disorders	452	Rodrigues and Carvalho (2001); Lorenzi and Matos (2002)
Solanum paniculatum	Solanaceae	Jurubeba	Antidiabetic, fever-reducing, in jaundice, hepatitis, wound healing, in gastritis, anemia and uterine tumors	451	Rodrigues and Carvalho (2001); Lorenzi and Matos (2002)

by disc diffusion (NCCLS, 2003b) protocol using 10 µl of these 10 extracts dissolved in DMSO with concentration of 300 mg/ml added on 6 mm diameter blank sterile discs. Negative and positive controls were conducted with DMSO and 30 µg of chloramphenicol added on blank disk, respectively. The plates were incubated in growth chamber at 35°C for 24 h. After incubation, the inhibition zone formed around the colonies was measured (Table 2). The effect of the response dose of the prior effective extracts and its inhibitory activities against the same strains were determined according to disc diffusion, using increasing concentrations (5, 10, 15, 20, 25, 30, 35, 40 and 50%) of

Table 2. Inhibition zones[1] by disc diffusion of extracts from plants extracted with different solvents against ATCC bacterial strains*.

Plant species	Ethanol extract			Methanol extract		
	S. aureus	***E. coli***	***P. aeruginosa***	***S. aureus***	***E. coli***	***P. aeruginosa***
Myracrodruon urundeuva	15	0	14	15	0	15
Eugenia dysenterica	0	0	0	0	0	0
Solanum lycocarpum	0	0	0	0	0	0
Solanum paniculatum	0	0	0	0	0	0
Astronium fraxinifolium	0	0	0	0	0	0
Brosimum gaudichaudii	0	0	0	0	0	0
Luehea divaricata	0	0	0	0	0	0
Boehmeria arborescens	0	0	0	0	0	0
Anacardium humile	6	0	8	6	0	7
Psidium guineense	15	0	14	15	0	15
Cefoxitin (30 µg) [2]	24	22	22	25	22	22

*Tests performed in triplicates. [1]Diameters in mm. [2]Positive control antibiotic.

Table 3. Inhibition zones[1] of extracts plants and the positive control by NCCLS (2003b) disc diffusion protocol* against S. aureus ATCC 25923.

Extract concentration (µg/L)	Zone of inhibition measurement (mm)		
	P. guineense	***M. urundeuva***	***A. humile***
30	0	0.375	0
60	0.333	6.563	1.166
90	1.375	9.604	5.146
120	5.104	10.833	7.396
150	6.813	11.604	9.333
180	9	12.417	9.854
210	9.604	12.792	10.396
240	10.521	13.146	10.896
270	11.083	13.5	11.313
300	12.875	14.896	12.875
Chloramphenicol (30 µg)[2]	20.854	20.625	20.458

*Tests performed in triplicates.

the extracts diluted in DMSO that were done in three replicates for each test (Table 3).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

To determine the MICs) of the crude extracts, microdilution test was used following the standard protocol M7-A6 (NCCLS 2003a). Concentrations of extracts ranging from 1024 to 0.5 mg/ml were tested. Dilutions were performed with 96-well plates, which were added 0.1 ml of bacterial suspensions from fresh cultures in 0.98% NaCl saline solution with a turbidity of 0.5 McFarland Scale (1.5×10^8 cells/ml), and incubated at 35°C for 24 h at 0.2 ml final volume. The resazurin was used as indicator of microbial growth and MIC was defined as the lowest concentration which there was no microbial growth, indicated by blue resazurin. The dilutions that did not show growth were then streaked to evaluate the MBC. MBCs were determined in triplicate using the same CLSI recommended for MIC instructions. BHI was used for MBC bacterial quantification of all extract samples during 24 h at 35°C.

Interactions between plant extracts

Synergistic interactions (agonism or antagonism between plant extracts) against standard strain *S. aureus* ATCC 25923 were evaluated following procedures from several reports (Ahmad and Aqil, 2007; Aqil et al., 2005; Zhao et al., 2001). The extracts were combined two by two varying concentrations and each combination was tested adding on 6 mm diameter blank discs following a disc diffusion assay protocol. Control tests were conducted with discs soaked in DMSO or 30 µg chloramphenicol and all plates were incubated at 35°C for 24 h. After incubation, the inhibition zones formed around colonies were measured. Three replicates were performed for each test.

Phytochemical analyses

The characterization of the main groups of plant substances with antibacterial activity was performed with dry and pulverized leaves using qualitative chemical reactions that resulted in the development of colors and/or precipitates characteristic for each compound

Table 4. MICs and MBCs from crude extracts1 against ATCC strains (Staphylococcus and Pseudomonas)*.

Plant sample	Plant portion	*S. aureus* MIC MBC	*P. aeruginosa* MIC MBC
A. humile	Leaves	4.1	8.2
P. guineense	Leaves	4.1	8.2
M. urundeuva	Leaves	4.1	4.1
Control	Chloramphenicol[2]	-	-

*Tests performed in triplicates. [1]Concentration (µg/L). [2]Concentration (30 µg/ml).

groups. The material was subjected to chemical processes of identification for the following classes of chemical components: saponins, tannins, flavonoids, alkaloids, polysaccharides, anthraquinones, organic acids and reducing sugars, all according to the protocols established by Mouco et al. (2003).

Statistical analyses

The data were analyzed by the statistical system R 2.5 (R Development Core Team, 2008) via generalized linear models (Crawley, 2007). For the analysis of extract inhibitory activities, the presence or absence of activity was used as response variable. In this case, a model with binomial error distribution and logit link was used. To examine the responses of tested strains to the pure extracts and to the positive control, a normal error distribution model was used. Interactions between the plant extracts were analyzed taking into consideration inhibition zone diameters, in which the presence of agonism, antagonism or lack of interaction follows a quadratic trend.

RESULTS

Antibacterial activities of the metabolites crude from plants

According to the results of the antibacterial test, extracts from *Piper guineense* and *Myracrodruon urundeuva* plants resulted in growth inhibition of *S. aureus* and *P. aeruginosa* with the two solvents used (ethanol and methanol) with no apparent difference between them. The *Anacardium humile* extract, although less effective, showed inhibition against the same bacterial strains. None of the plants evaluated showed antibacterial activity against *E. coli*. The negative control did not show antibacterial activity against any bacteria tested (Table 2). The results of the agar diffusion antibacterial test were expressed by measuring the diameters of the inhibition zones of the tested plant extracts and the positive control. There was an increase in the inhibitory responses of the extracts as their concentrations increased (Table 3). The negative control did not show antimicrobial activity against the microorganisms tested, and the positive control showed inhibition as expected. Figure 1 shows the relationship between the presence/absence of antibacterial activity and the different concentrations of plant extracts. The *M. urundeuva* extract began its antibacterial action at a lower concentration (30 µg/L), followed by the *A. humile* and *P. guineense* extracts in disc diffusion assays.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

All results for MIC and MBC are shown in Table 4. The MICs for the *P. guineense, M. urundeuva* and *A. humile* extracts were all 4.1 µg/L for *S. aureus*. For the *P. guineense* and *A. humile* extracts tested against *P. aeruginosa*, the MICs were both 8.2 µg/L, and for the *M. urundeuva* extract, it was 4.1 µg/L. As shown from the MICs and MBCs, the extracts had effective antibacterial activities compared to the control; the samples obtained had MBCs up to 16 times lower than the initial values of the crude extracts tested. According to the classification proposed by Aligiannis et al. (2001), MIC values ≤ 100 mg/ml are considered strongly inhibitory when testing fractionated plant material. In this specific case, MICs of ≤ 10 mg/ml for crude, unpurified extracts were considered promising.

Interactions between plant extracts

The results of the assessment of the interactions between plant extracts are illustrated in Figure 2. All combinations resulted in synergistic interactions, which the results of the combined action were greater than the sums of the effects of each isolated compound. According the analysis of variance of the plant extracts as a function of the bacterial strain of *S. aureus* tested (Table 5), there was no significant difference between them, demonstrating a similar behavior to the extracts tested. In the analysis of deviance for the interaction between the plants extracts studied, all interactions were significant.

Phytochemical analyses

All extracts were positive for the presence of tannins and flavonoids and negative for alkaloids. The detection of tannins with ferric chloride indicated the presence of

Table 5. Analysis of Deviance from results by disc diffusion protocol between the extracts plants, S. aureus ATCC 25923 and the combination of extracts*.

Plant extract	GL	Residual GL	Deviance	Residual deviance	p
P. guineense	15	512	288	14422	0.8063
M. urundeuva	15	512	340.4	15522.4	0.735
A. humile	15	512	237	13409	0.8732
Chloramphenicol (30 µg)[1]	15	512	1568.8	5667.2	<0.0001
P. guineense + *M. urundeuva*	1	563	420.6	7406.5	<0.0001
P. guineense + *A. humile*	1	563	466.5	6112.0	<0.0001
M. urundeuva + *A. humile*	1	563	317.4	6010.7	<0.0001

*Tests performed in triplicates. [1]Positive control.

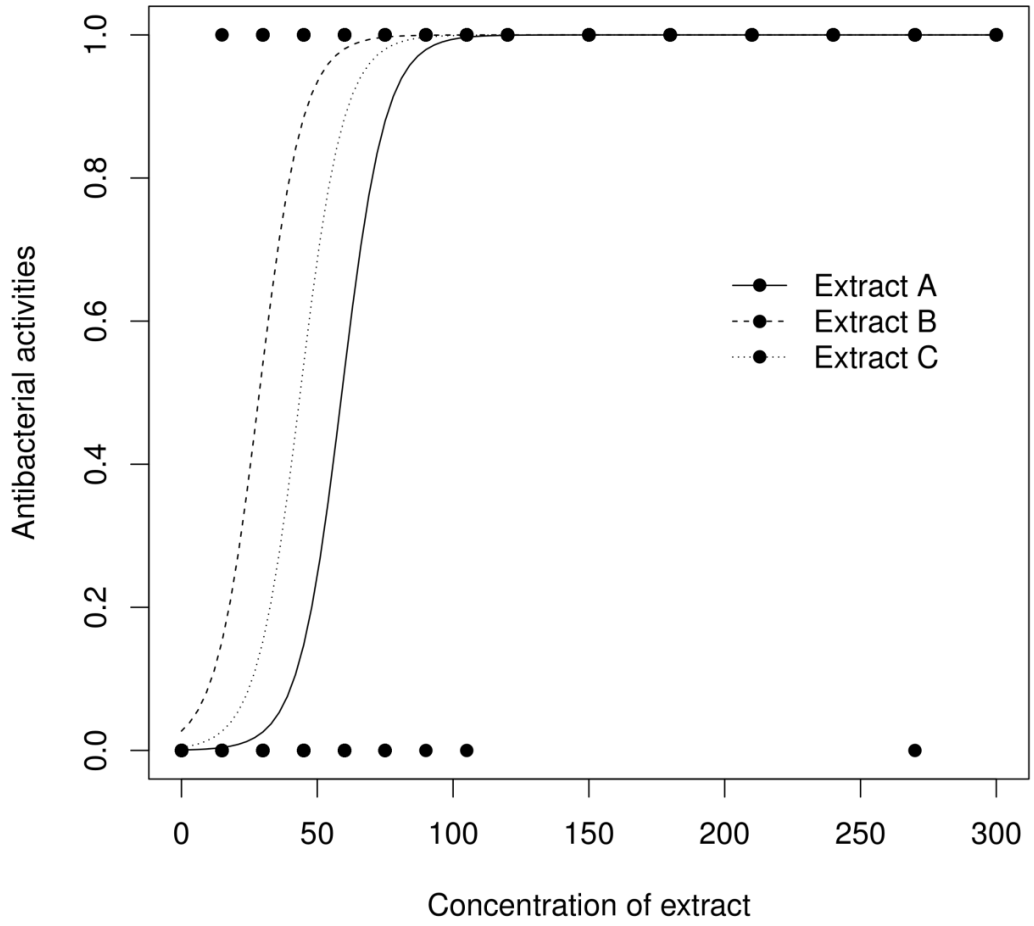

Figure 1. Relationship between bacterial activity and plants extract concentration, where A, B and C represent *Psidium guineense*, *Myracrodruon urundeuva* and *Anacardium humile*, respectively by disc diffusion protocol against *S. aureus* ATCC 25923.

hydrolyzable tannins in *M. urundeuva* and condensed tannins in *A. humile.* These results were confirmed by the reactions with lead acetate and glacial acetic acid. The detection of flavonoids with Shinoda reagent indicated the presence of flavonoids in *A. humile* and flavones in *M. urundeuva.* These results were confirmed by the reactions with ferric chloride. No foaming saponins, polysaccharides, organic acids, anthraquinones or reducing sugars were detected in the plant species evaluated.

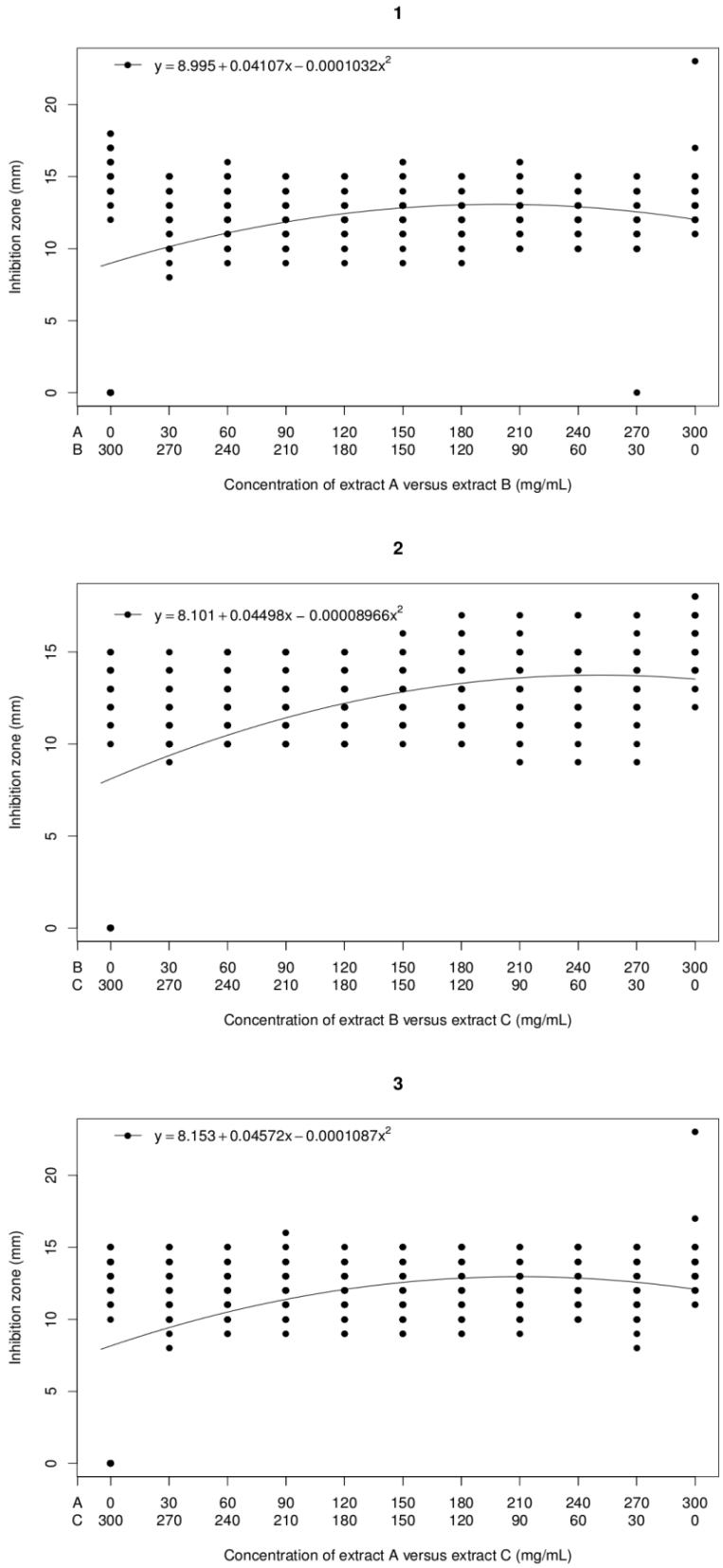

Figure 2. Interactions between plant extracts (A: *Psidium guineense*, B: *Myracrodruon urundeuva* and C: *Anacardium humile*) by disc diffusion protocol against standard strain *S. aureus* ATCC 25923. Tests performed in triplicates.

DISCUSSION

To elucidate the pharmacological activities of chemical components present in plant species is the objective of several studies. A preliminary phytochemical analysis can identify the relevant metabolite groups that may be related to the identified biological activities, thus guiding the research to obtain an effective and safe herbal medicine, in addition to identifying possible toxic active ingredients (Cragg and Newman, 2013). Previous phytochemical researches with the plant species in this study confirm the results found in the phytochemical analysis like antibacterial activity (Al-Mariri and Mazen, 2014; Rodrigues et al., 2014; Tankeo et al., 2016). Ferreira (2005) while studying the anti-ulcerogenic activity of *A. humile*, detected the presence of tannins and flavonoids. Correia et al. (2006) reported that substances with antibacterial and antifungal activities were present in species from the Anacardiaceae family. Lignins and tannins were isolated from *M. urundeuva* by Morais et al. (1999) and Queiroz et al. (2002). Vergas et al. (2007) and Rodrigues et al. (2014) reported the presence of tannins in leaves from plants of the genus *Psidium.*

The results obtained in this study corroborate with use of plants as antimicrobials in popular therapy. Pathogenic bacteria such as *S. aureus* and *P. aeruginosa* frequently shows resistance to the antibiotics used against them (Oliveira et al., 2007), however, they were inhibited by the three ethanol and methanol extracts tested. The *M. urundeuva* crude extract showed the largest inhibition zone against the microorganisms tested, and its inhibition started at a lower concentration (30 µg/L), followed by the *A. humile* and *P. guineense* extracts (60 µg/L). The antibacterial activity detected in these crude extracts may be related to the identified compounds (tannins and flavonoids). Studies performed with extracts from *Psidium* species plants showed antibacterial activities against Gram-positive and Gram-negative bacteria as well as antifungal action (Oliveira et al., 2007; Nair and Chanda, 2007; Carvalho et al., 2008; Rodrigues et al., 2014). González et al. (2005) attributed the antibacterial activity observed in *P. guineense* extracts to secondary metabolites such as tannins and flavonoids. Soares et al. (2006) reported the antibacterial activity of *M. urundeuva* Allemao (Aroeira) against *S. aureus*. Antibacterial activities were also reported in studies with plants of the genus *Anacardium* (Melo et al., 2006). A better understanding of the antimicrobial activities of plants aids in the selection of new substances for this purpose (Gonçalves et al., 2005; Dias et al., 2012; Cragg and Newman, 2013). Given that bacteria are resistant to multiple antimicrobials compounds become a problem in the treatment of infections, being clear that there is a need to find new substances with these properties to be used in the treatment against these pathogenic micro-organisms.

The antibacterial potentials of substances produced naturally in several plant species must be explored, and the relevant components or active fractions must be identified (Ríos and Recio, 2005; Zago et al., 2009; Dias et al., 2012; Cragg and Newman, 2013). Research with medicinal plants involves investigating traditional and popular medicine (ethnobotany); isolating, purifying and characterizing active ingredients (organic chemistry and phytochemistry); investigating pharmacological extracts and isolating chemical compounds (pharmacology); chemically transforming active ingredients (synthetic organic chemistry); studying the relationships between structure, activity and the mechanisms of action of the active ingredients (medicinal and pharmacological chemistry); and finally, formulating herbal medicines. Integrating these areas into the study of medicinal plants leads to a promising and effective path for the discovery of new medications.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Anti-HIV-1 activity in human primary cells and Anti-HIV-1 RT inhibitory activity of extracts from the red seaweed *Acanthophora spicifera*

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First generation drugs such as zidovudine have been extensively used in clinical practice, resulting in the development of HIV resistance to these nucleoside analogs. Several studies have demonstrated the effective anti-HIV activity of natural products derived from seaweeds, suggesting promising sources of substances for the development of novel antiviral drugs. In this paper, the antiviral effect of extracts from the red seaweed *Acanthophora spicifera* on HIV-1 replication was evaluated *in vitro*. Peripheral blood mononuclear cells obtained using the Ficoll-Hypaque gradient were used for cytotoxicity and antiviral activity testing. The dichloromethane extracts, ethyl acetate, acetone, and methanol were found to have CC_{50} values of 31±7.4, 45±11, 38±3.5, and 179±25 µg/mL, respectively. With the control, the extract prepared in ethyl acetate inhibited approximately 60% of the viral load, which is the best result among the extracts. This same extract showed an IC_{50} value of 33.17±4.84 µg/mL for the reverse transcriptase. The EtOAc extract from *A. spicifera* showed to be an efficient HIV antiviral due to its phenolic compounds, as evaluated by nuclear magnetic resonance.

Key words: Marine natural products, red seaweed, *Acanthophora spicifera*, HIV-1, Antiviral activity, Anti-HIV-1 RT.

INTRODUCTION

Since the discovery of the human immunodeficiency virus, many drugs have been developed in an attempt to inhibit its replication. However, HIV is resistant to treatment with known drugs (Kuritzkes, 2007; Hirsch et

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al., 2008; Manhanzva et al., 2015). This is due to the high mutation rate of HIV and does not have an effective mechanism for error correction during replication. One of the strategies adopted was the combination of two or more drugs known as Highly Active Antiretroviral Therapy (HAART) (Marrazzo et al., 2014). Such treatment may reduce viral load to undetectable levels in the blood and provide long-lasting clinical benefit. However, some patients do not respond to this treatment, making the search for new molecules with anti-HIV activity an urgent need. Seaweeds are a source of many bioactive compounds. Several extracts, fractions, and natural products isolated from seaweeds have demonstrated effective anti-HIV activity (Vo and Kim, 2010), making it an interesting base from which to develop new medicines. Of the seaweeds, red seaweeds produce natural products such as acetogenins (Gutiérrez-Cepeda et al., 2011), sesquiterpenes (Chen et al., 2016), monoterpenes (Silva et al., 2015), bromophenols (Popplewell and Northcote, 2009), and sulfated polysaccharides (Coura, 2012) that can be used for anti-HIV drug development. *Acanthophora spicifera* (Rhodophyta) is an excellent model for studies of biological activity in Brazil because it has natural banks on the coast of Rio de Janeiro, is easily identified (Perrone et al., 2006), has an experimental field cultivation described in the literature (Kaliaperumal et al., 1986), and is a part of the food chain to other species, indicating a low toxicity (Cruz-Rivera and Villareal, 2005). Furthermore, fractions rich in sulfated polysaccharides from the red seaweed *Acanthophora* are an effective antiviral against HSV-1 and HSV-2 strains (Duarte et al., 2004). Therefore, the objective of this study was to evaluate the antiviral effect of extracts from *A. spicifera* regarding HIV-1 replication in human primary cells and their ability to inhibit the enzyme reverse transcriptase.

MATERIALS AND METHODS

Preparation of seaweed extracts

Specimens of the red seaweed *A. spicifera* (M.Vahl) Børgesen (Rhodomelaceae, Ceramiales, Rhodophyta) were collected in May of 2013 by snorkeling to a depth of 0.5-1 m at Orla Bardot (22° 05'03'' S; 41° 53'01'' W) in the city of Armação de Búzios, Rio de Janeiro, Brazil. The algal material was washed with local seawater and separated from sediments, epiphytes, and other associated organisms. The air-dried algal material (204 g) was submitted to an exhaustive and sequential extraction using the following solvents in increasing polarity: dichloromethane (CH_2Cl_2 – 5X 1L), ethyl acetate (EtOAc - 5X 1L), acetone (Me_2CO - 4X 1L), and methanol (MeOH – 3X 1L) at room temperature for one week.

Cell and virus

Peripheral blood mononuclear cells (PBMCs) from healthy human donors (confidential information) were obtained through density centrifugation over Ficoll-Hypaque (Sigma) as described by Yeap et al. (2007). Cells were re-suspended in a RPMI 1640 medium supplemented with 10% fetal bovine serum and stimulated with 5 $\mu g/mL^{-1}$ of phytohemagglutinin (PHA, Sigma) for three days and further maintained in culture medium containing 5 U/mL^{-1} of recombinant human interleukin-2 (Sigma). The viral strain HIV Ba-L (R5-tropic) Virus type 1 was donated by the National Institutes of Health (NIH, USA) and kept in storage at -80°C.

Cytotoxicity assay

The cytotoxicity of extracts from the red seaweed *Acanthophora spicifera* was assessed by monitoring the conversion of MTT 3-(4,5-dimethylthiazol-2yl)-2,5diphenyl tetrazolium bromide (Sigma-Aldrich) to formazan as previously described (Mosmann 1983) with some modification. Peripheral blood mononuclear cells were maintained in 96-well plates containing $2x10^5$ cells per well and treated with increasing concentrations of the extracts (6.75, 12.5, 25, and 50 $\mu g/mL^{-1}$ in DMSO) for one week at 37°C under a 5% CO_2 humidified atmosphere in triplicate. The DMSO concentration in the final volume of the well was less than 0.5%. After seven days of incubation, the supernatant was removed, and the MTT (20 µL of 5 mg/mL^{-1} in medium) was added to each well. Plates were incubated for two hours at 37°C with a 5% CO_2 atmosphere. The 96 well plates were then centrifuged at 100 X g for ten minutes, the supernatant was discarded, and 100 µl of DMSO was added to each well. Finally, the optical density was measured at 545 nm on a microplate reader. The result was expressed as the 50% loss of viable cells concentration (CC_{50}). The compound concentration required to reduce the optical density of MTT in relation to not treated cells was calculated using linear regression.

Anti-HIV-1 activity in human primary cells

PBMCs were maintained in 96-well plates containing $2x10^5$ cells per well and stimulated with IL-2. PBMCs were infected with 5 ng/mL^{-1} of the HIV-1 Ba-L strain and incubated for two hours at 37°C under a 5% CO_2 humidified atmosphere. Then, the 96 well plate was centrifuged at 100 X g for ten minutes, its supernatant was removed, new medium containing 50 $\mu g/mL^{-1}$ of extracts diluted in DMSO was added, and it was incubated at 37°C with a 5% CO_2 atmosphere for seven days. After incubation, the supernatant was collected, and the production of the p24 antigen was evaluated using the immunoassay ELISA (Zeptometrix). The absorbance was measured in a spectrophotometer at 450 nm. Each value is expressed as mean±SEM in triplicate experiments. The average value of absorbance was used to determine the concentration of p24 in the samples treated by comparison to a standard curve (Trinchero et al., 2009).

Anti-HIV-1 RT inhibitory activity

E. coli strain BL21 (DE3) was used as a recipient for DNA transformations. Overnight, the *E. coli* cells transformed with the plasmid containing RTp66 and RTp51 HIV-1 genes were cultured in Luria-Bertani (LB) containing ampicillin (100 $\mu g/ mL^{-1}$) under shaking at 220 rpm at 37°C. These overnight cultures were used as the inoculum for one liter of LB medium containing 100 µg/mL of ampicillin. Cells were grown for six hours at 37°C with vigorous shaking and then induced with isopropyl-b-D-thiogalactopyranoside (IPTG) (1 mM) for two hours. Cells were harvested by centrifugation (5000 x g, 15 min), and bacterial lysates were prepared using a lysis buffer (50 mM Tris-HCl (pH 7.9 at 4°C), 60 mM NaCl, 1 mM EDTA, and lysozyme/DNAse I treatment. Clarified lysates were used for the isolation of the p51/p66 heterodimeric RT. The active RT heterodimer was purified using the MagneHis™ Protein Purification System according to the manufacturer's instructions.

Table 1. The cytotoxicity of dichloromethane (CH_2Cl_2), ethyl acetate (AcOEt), acetone (Me_2CO) and methanol (MeOH) extracts obtained in increasing polarity from red seaweed *Acanthophora spicifera*.

Extract	CC_{50} (µg/mL^{-1})
Dichloromethane	31±7.4
Ethyl acetate	45±11
Acetone	38±3.5
Methanol	179±25

Data are expressed as mean±S.E. of three independent experiments. The CC_{50} of each extract was calculated using regression line.

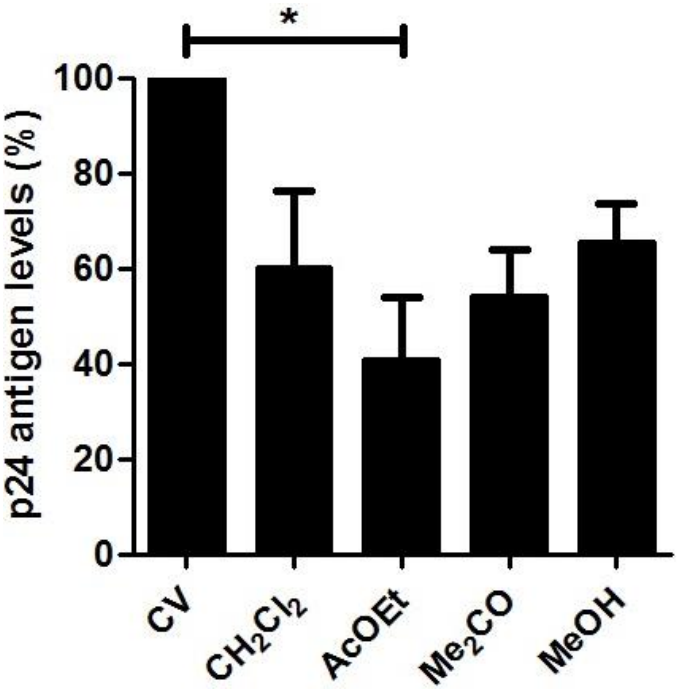

Figure 1. Histograms showing p24 levels found in the supernatant of untreated culture (Virus Control - CV) and treated with the different extracts in dichloromethane (CH_2Cl_2), ethyl acetate (AcOEt), acetone (Me_2CO) and methanol (MeOH). Each value is expressed as mean±SEM in triplicate experiments. *p<0.05, significant value as analysed by Tukey's multiple comparison test.

Table 2. Inhibitory Effects of ethyl acetate extract from Red Seaweed *Acanthophora spicifera* and Efavirenz on HIV-1 Reverse Transcriptase. Data are expressed as mean±S.E. of three independent experiments. The IC_{50} of each extract was calculated using regression line.

Sample	IC_{50}	Maximum inhibition
Ethyl acetate extract	33.17±4.84 µg/mL^{-1}	79±2.1%
Efavirenz	0.006 µM	97±3.6%

The ability to inhibit the enzyme HIV-1 reverse transcriptase was evaluated using a fluorescence RT assay kit (EnzChek® Molecular Probes) according to the manufacturer's protocol. Briefly, 20 µL of reaction mixture containing a poly(A) ribonucleotide template/oligo d(T)16 primer and dTTP was added to the wells of a microtiter plate and mixed with 5 µL of increasing concentrations of the EtOAc extract. Finally, one µL of the enzyme (15−80 ng/mL) in reaction buffer was addedand incubated at 37ºC for one hour. 2 µL of 200 mM EDTA was added to stop the reaction. Fluorescence intensity was measured using a microplate reader (Spectramax-M4 Molecular Devices) (ex. 480 nm, em. 520 nm) after the addition of 173 µL of fluorescent PicoGreen® reagent, which selectively binds to dsDNA or DNA-RNA heteroduplexes over single-stranded nucleic acids or free nucleotides. Efavirenz was used as a positive control. The IC50 values were determined using Prism5 (GraphPad Software). All assays were performed in triplicate.

Chemical profile of extract

To analyze the chemical profile, a 5 mg aliquot of EtOAc extract was removed and analyzed by proton nuclear magnetic resonance (NMR-H). The NMR spectra were obtained on a Varian Unity Plus 300 at 300 MHz. The internal reference standard was TMS, and the samples were dissolved in $CDCl_3$.

RESULTS AND DISCUSSION

The CC_{50} values of the extracts are reported in Table 1. The methanol and ethyl acetate extracts showed lower cytotoxicity in comparison with the extracts prepared in dichloromethane and acetone.

The percentages of p24 in the supernatants of each extract can be seen in Figure 1. When the culture supernatant infected with the Ba-L strain of HIV-1 was treated with 50 µg/mL^{-1} of the extract obtained by organic solvent ethyl acetate, it was possible to observe approximately 60% reduction in the p24 levels. Treatment of infected cells with ethyl acetate extract resulted in a reduction of p24 level compared to infected untreated cells. Therefore, the extract obtained with solvent ethyl acetate was used to evaluate the ability to inhibit the activity of reverse transcriptase enzyme, an important step of HIV replication cycle.

The inhibitory activity of the EtOAc extract against HIV-1 RT is shown in Table 2. The chemical profile of ethyl acetate extract from *A. spicifera* was analyzed with NMR. The ethyl acetate extract showed signs in the chemical shift region from 7 ppm, which is characteristic of aromatic compounds (doublet in 7.53 ppm, triplet in 7.36 ppm and double doublet in 7.13 ppm).

Cytotoxicity is critical in drug development (Putnam et al., 2002). Natural seaweed products have demonstrated low levels of cytotoxicity (Karadeniz et al., 2014) – even lower than commercial drugs such as AZT (Barbosa et al., 2004). The toxicity of *A. spicifera* extracts has already been studied in mice (Naqvi et al., 1980) and Vero cell line (Duarte et al., 2004). However, the present article reports for the first time the cytotoxicity of extracts from *A. spicifera* in human cells.

When the infected culture was treated with 50 µg/mL^{-1} of the extract prepared in ethyl acetate, there was approximately 60% decrease in p24 levels. A similar

study was conducted used partitions of CH_2Cl_2/MeOH, hexane, CH_2Cl_2, and CH_2Cl_2/EtOAc with brown seaweed *Dictyota menstrualis*. This group showed an inhibition in p24 levels of approximately 40% when tested at a concentration of 50 μg/mL (Pereira et al., 2004). Compared with the data found in this article, treatment using AcOEt extract from *A, spicifera* showed more efficient results.

The partition in ethyl acetate from brown seaweed *Ecklonia cava* had anti-HIV-1 activity, and this activity was confirmed by the presence of phenolic compounds. Other phenolic compounds such as flavonoids also showed effective anti-HIV activity (Casano et al., 2010; Wang et al., 2014). We evaluated the presence of phenolic compounds in the extracts obtained in increasing polarity from red seaweed *A. spicifera* by ^{1}H-NMR. Only the extract obtained with ethyl acetate showed phenolic compounds. These data corroborate the results obtained by Zeng et al. (2001), which showed the isolation of two phenolic compounds from the extract of *A. spicifera* obtained in ethyl acetate. Therefore, we believe that the anti-HIV effect of the EtOAc extract of red seaweed *A. spicifera* is due to the presence of phenolic compounds.

In a Korean study, 26 extracts from red seaweeds were tested to evaluate the inhibition of reverse transcriptase (Ahn et al., 2002). Most of the extracts were not able to inhibit more than 65% of the enzyme activity when tested at a concentration of 200 μg/mL. These data indicate that our results were interesting, since the AcOEt extract was able to inhibit 80% of enzyme activity at a lower concentration. Ahn et al. (2004) demonstrated that the inhibitory effect of brown seaweed *E. cava* was caudal by the presence of phenolic compounds. In conclusion, the AcOEt extract from red seaweed *A. spicifera* presents an efficient activity against HIV-1 virus. Finally, in accordance with the observed data, the inhibitory activity of the ethyl acetate extract in HIV reverse transcriptase-1 may be due to presence of aromatic compounds.

Conflict of Interests

The authors have not declared any conflict of interest.

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