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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

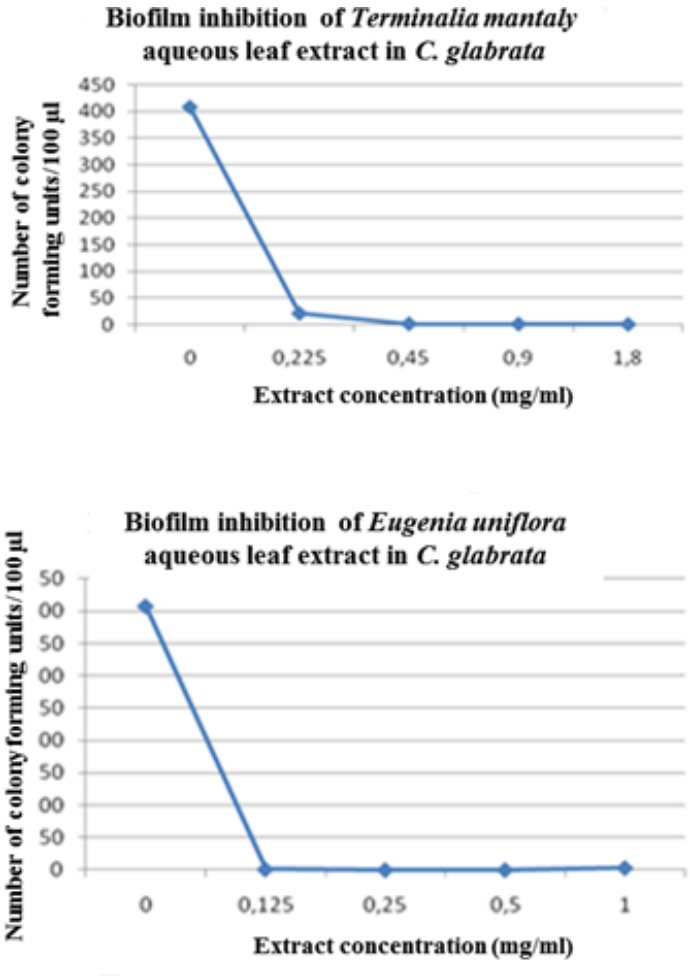

Figure 1. Biofilm inhibition concentration of *E. uniflora* and *T. mantaly* aqueous leaf extract in *C. glabrata.*

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

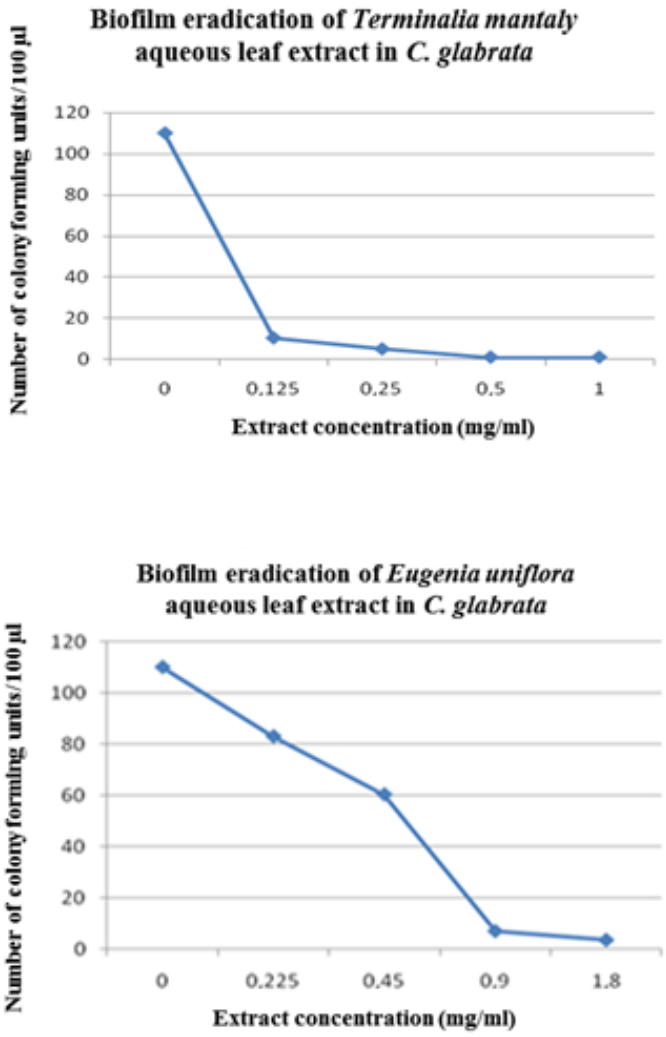

Figure 2. Biofilm eradication concentration of *E. uniflora* and *T. mantaly* aqueous leaf extract in *C. glabrata.*

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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