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Vaginal Yeasts and the Antifungal Action of Red Propolis Extract

ORIGINAL

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Abstract

Introduction: Vulvovaginitis is an inflammatory process that affects the vulva, vagina and cervix, most of the times of infectious cause. Vulvovaginal candidiasis is the second most frequent cause in Brazil, in Europe ranks first. *Candida* spp can be found in the vagina up to 20% of asymptomatic women.

Objective: Understanding the occurrence of vaginal yeasts and evaluating the antifungal action of a red propolis extract.

Method: This is a clinical study *in vitro* with vaginal secretion samples collected from 197 patients treated at the Health Center Frei Damiao in Patos-PB, Brazil. The species were identified using a chromogenic medium CHROMagar *Candida*®. At the end, the antifungal activity of the red propolis extract in four different concentrations was observed: 25%, 50%, 75% and 100%, by test disc Agar diffusion. The study was approved by the Research Ethics Committee of the Federal University of Campina Grande, CAAE 35203614.8.0000.5575.

Results: Of the samples analyzed *other species* identified by the medium used were the most prevalent corresponding to 59.8%, followed by *C. albicans*, with 21.7%. The antifungal activity was observed in 81.25% of the tested samples. According to the average and standard deviation of the inhibition zone of the four concentrations, the one that presented the smallest variation was the concentration at 50%, followed by concentration to 75%.

Conclusions: The results of this research showed that there was antifungal action of red propolis extract, since occurred inhibition in 81.25% of the analyzed samples. However did not show clearance regarding this action, since positivity tendency was not growing by increasing or decreasing the concentration of propolis extract.

Keywords
Propolis; *Candida*; Vulvovaginitis; Products with antimicrobial action.

Introduction

The vulvovaginal candidiasis (VVC) is an infection characterized by white, odorless vaginal morbid discharge, associated with vulvar and vaginal lesions, itching and burning. It affects more often pregnant patients, diabetic and who used antibiotics. Diagnosis is based on signs and symptoms and laboratory tests [1].

The therapy for this infection is restricted, limited to the polyene and azole. Nystatin has been used for decades; however therapeutic failures were observed [2-3]. Of the azole fluconazole is one of the most widely used; however, has shown the occurrence of strains with decreased sensitivity, and other resistant *in vivo* and *in vitro*, to certain antifungal agents, side effects and high recurrence rates [1,4].

In view of this, the natural products have been traditionally used in the treatment of diseases, because they are sources of many actives, and these are the basis for the vast majority of anti-infective therapies in current clinical in use [5].

Thus, propolis is a natural product, of a resinous aspect, variable chemical composition, collected from various plant species by honey bees that add salivary secretions; being considered an organotherapic, which means product obtained from glands, organs, tissues and animal secretions [6].

Propolis is a complex mixture composed of balsamic resin material, collected by honey bees from flowers, plant exudates or other parts of the plant tissue plus wax, pollen and products of their metabolism, as the enzyme salivary β-glycosidase, responsible for the hydrolysis of flavonoid glycosides in aglucones increasing its pharmacological action [7].

In Brazil, biological properties and different chemical compositions are described for samples collected in different regions of the country. This variation can be explained by the Brazilian biodiversity [6]. The Brazilian propolis was identified and classified into 12 main groups, according to geographic region of origin and the vegetation from which it was extracted the chemical composition [8].

Recently, a new group of Atlantic Forest in the States of Alagoas, Paraiba, Pernambuco, Bahia and Sergipe was ranked as the 13th type of Brazilian propolis, called red propolis. It had its botanical origin identified as *Dalbergia ecastophyllum (L) Taub*, known popularly as Bugiu-of-Tail, a species of legume with different physical and chemical characteristics of other samples from 12 groups, and has a potent biological action [9-10]. This biological potential of this natural product is due to a synergism that occurs between its many components [11]. In addition, the Brazilian propolis is known for its antifungal activity [12].

Moreover, the interest of researchers from around the world has been awakened to use propolis, especially, its therapeutic properties: antimicrobial, anti-inflammatory, immunomodulatory, antioxidant, antitumor and antiviral [13].

The ability of propolis to inhibit the growth of microorganisms is the pharmacological activity more popularly known and scientifically proven [14]. Considering that the Brazilian propolis has been the subject of intensive research in recent decades, this research aimed to identify, in women attending in a gynecology clinic of the countryside of Paraiba (Brazil), vaginal yeasts and the antifungal activity of red propolis extract.

Material and Method

This is a clinical study *in vitro* with vaginal secretion samples collected from 197 patients treated at Health Center Frei Damião, in the municipality of Patos-PB (Brazil), in the period from July to November 2014. The patients agreed to participate in the research, by clarifying and signing of the Informed Consent. In compliance with Resolution 466/12, the research was approved by the Research Ethics Committee of the Federal University of Campina Grande, CAAE 35203614.8.0000.5575 protocol.

Gynecological examination was performed to speculate, for the purpose of collecting vaginal discharge using swuab (culture swuab transport system - COPAN). Samples of vaginal secretions were obtained from collections recommended previously [15-16]. Analyses were performed in the Microbiology Laboratory Technological Vocation Center, Federal University of Campina Grande, campus of Pombal, Paraíba. The material was seeded onto plates containing Sabouraud Agar (Sabouraud Dextrose Agar - Difco). The plates were incubated at 37°C for 72 hours. After this period the reading was performed and checked the growth of *Candida* colonies characteristics. The identification takes place through the study of macroscopic and biochemical aspects Chrom Agar *Candida*® (Probac Brazil), a means of chromogen culture that allows identifying presumptive action of yeasts, because it contains several enzymatic substrates hydrolyzed by the corresponding hexoaminidases allow to identify action yeast according to the colony pigmentation displayed in a time of 24 to 48 hours [17]. The medium used indicates green colonies, for *C. albicans*; Rugose Rose for *C. krusei*; metallic blue, for *C. tropicalis*; white and violet, for *other species*. The reading of the plates and the interpretation of results were carried out by observing the morphology and pigmentation of the colonies [18].

The red propolis samples were obtained from a beekeeper from the coast of Paraíba of *Apis mellifera* bees arising from the *Dalbergi frutescens* (Bugiu-of-Tail), in which the statement was made to 30% of ethyl alcohol PA [10]. The antifungal activity of the propolis extract was analyzed in four different concentrations: 100%, 75%, 50% and 25% (dilution ethyl alcohol 70%) and was determined by the Agar diffusion test, in which samples of *Candida* were grown on Sabouraud agar and incubated at 37°C/48h. Then, filter paper discs were soaked used with 10µL extract at the concentrations and deposited on the surface of Sabouraud agar containing the colonies of *Candida* sp. The plates were incubated at 37°C/48h. After it the measurement was made with a caliper, of the halos of inhibition of *Candida* growth. From the results, it was considered active product against the species of the microorganisms under study which produced halos that above 10 mm in diameter [19].

Results

Vaginal secretion: species of *Candida* identified

The results of vaginal culture of 197 participants showed that 46.2% (n = 91) were positive for some species of *Candida*. Noting that, in a sample, there were identified two species and the patient was asymptomatic. The data in **Table 1** show the species identified in samples whose culture was positive. By

Table 1. Species of *Candida* identified in the samples of culture of vaginal secretion (*n* = 91).

Species of *Candida*	*n*	%
Other species*	55	59.8
C. albicans	20	21.7
*C. tropicalis**	15	16.3
C. krusei	2	2.2

Source: Research data. Patos-PB, 2014.
* In a sample there were identified two species (*C. tropicalis* and Other species)

those findings, prevailed for *other species* unidentified culture medium with 59.8% (n = 55), followed by the species *C. albicans*, with 21.7% (n = 20) and of *C. tropicalis* with 16.3% (n = 15).

It was noticed by the findings that the percentage of symptomatic women 85% (n = 17) was higher in the kind of *C. albicans*, compared to 53.52% (n = 38) of species other than *albicans*. (Data not shown in table).

In **table 2**, divided the groups of species of *Candida* in *C. albicans* and non-*albicans*, there are presented according to age and characteristics of the menstrual cycle of the women interviewed. It appears that the percentage of *C. albicans* species 37.5% was higher in younger women, compared to the lowest percentage of this kind 4.7% in women of higher age, demonstrating statistical dependence between variables (p = 0.001) since by increasing the age of women it was reduced the percentage of species *C. albicans* and increased of the non-*albicans*. Moreover, the variables of species of *Candida* and menstrual cycle characteristics did not show statistically dependence (p = 0.122), although there was a tendency to increase the percentage of *C. albicans* in women with menstrual cycles present. (**Table 2**)

Antifungal action of red propolis extract

Of the 91 vaginal secretion samples with the presence of *Candida,* only 48 samples formed fungal growth with the number of colonies sufficient to conduct the inhibition test with extracts of propolis, according to the method used for this purpose.

When testing the antifungal action of red propolis extract on 48 samples referred to, it was noticed that in 81.25% (n = 39) the result was positive, forming halo of inhibition higher than 10 mm. However, the positive antifungal action prevailed in 95% (n = 19/20) on C. *albicans*, followed by 87.5% in C. *tropicalis* (n = 7/8) (**Table 3**).

Facing the different extract concentrations of red propolis, added to samples with identification of *Candida* species, it was possible to identify variations of 12 to 100 mm in positivity of antifungal action, considering that this action is only proven in the presence of halo of inhibition greater than 10mm. Thus, **Table 4** shows that, according to the average and standard deviation of the inhibition halo of the four concentrations, presented the lowest change was the concentration at 50%, followed by concentration to 75%.

Table 2. Distribution of species of *Candida* according to age and characteristics of the menstrual cycle of the interviewed.

Variables		Species of *Candida*				*p*
		C. albicans (*n* = 20)		Non *C. albicans* (*n* = 71)		
		n	%	*n*	%	
Age (in years)	14 - 38 (*n* = 48)	18	37.5	30	62.5	<0.001*
	39 - 79 (*n* = 43)	2	4.7	41	95.3	
Menstruation**	Present (*n* = 75)	19	25.3	56	74.7	0.122*
	Absent (*n* = 14)	1	7.1	13	92.9	

Source: Research data. Patos-PB, 2014.
p - Chi-square Test (X^2) or * Fisher Exact Test; Statistical significance if $p < 0.05$.
**Excluded two hysterectomized.

Table 3. Distribution of species of *Candida* identified in the culture of vaginal secretion, according to the antifungal action of red propolis extract (n=48).

Species of *Candida*	Positive	
	n	%
C. albicans (*n* = 20)	19	95.0
C. tropicalis (*n* = 08)	7	87.5
Other species (*n* = 18)	12	66.7
C. krusei (*n* = 2)	1	50.0

Source: Research data. Patos-PB, 2014.

The data shown in Table 4 indicate that the lowest and highest number of cases of positive antifungal action, respectively, were found at a concentration of 100% (*n* = 17) and 25% (*n* = 35). This association was not observed in the average positive aspects of mergers, as the lowest average/standard deviation difference was found in the concentration to 50%. Thus, considering the greater appearance of inhibition zones, there are indications that the lowest concentration of propolis extract has a higher antifungal action.

It is also noticed that although there was total inhibition (100mm) in a sample tested at a concentration of 100%, the concentration with the highest quantity of positivity in the samples tested was 25% (87.2% *n* = 35/39), with inhibition zones ranging from 12 to 80mm; and the positivity of 20, 30, 40 and 50mm were observed at all concentrations. (**Table 4**)

Discussion

Fungal vulvovaginitis is caused predominantly by the genus *Candida*, being *C. albicans* the most prevalent. But actually there has been an increase in species non-*albicans*, in some populations. The greatest concern lies in the fact that these other species, in general, tend to be more resistant to antifungal agents [20-21].

It is estimated that about 75% of adult women have at least one episode of fungal VVC in their life. Of these, 40 to 50% experience further outbreaks and 5% become applicants. On the other hand, studies indicate that 20-25% of asymptomatic healthy women and have positive cultures of vaginal discharge for yeast and that among the colonized patients, about 50% present VVC at some point in their life [20, 22].

About 80 to 90% of cases of VVC are due to species *C. albicans* and 10 to 20% of the species non-*albicans* (*C. tropicalis, C. glabrata, C. krusei, C. parapsilosis, C. pseudotropicalis, C. lusitaniae*) [23]. However, studies show that, in some populations, the frequency of isolation of yeasts non-C. *albicans* has increased [20].

Other authors have also observed the predominance of non-*albicans* species. In a study with 4.228 Italian women met in gynecology services found a higher prevalence of non-*albicans*, 57.2% [24]. Similar in another study involving 44 women with candidiasis history, they found 40.91% of positive cultures for *Candida* spp and of these, identified 52.63% of *non- C.albicans* [25].

Table 4. Results of antifungal action of red propolis extract in concentrations to 100%, 75%, 50% and 25% (n=39).

Results (mm)	Concentrations of propolis extract			
	100% (43.6% *n* = 17)	75% (53.8% *n* = 21)	50% (51.3% *n* = 20)	25% (89.7% *n* = 35)
Average (SD)	40.00±22.492	30.00±13.684	27.7±11.973	30.00±18.991
Minimum-Maximum	20-100	15-60	20-50	12-80
12 mm	-	-	-	2.9% (= 1)
15mm	-	14.3% (= 3)	-	8.6% (= 3)
20 mm	41.2% (= 7)	28.6% (= 6)	45.0% (= 9)	20.0% (= 7)
21mm	-	-	-	2.9% (= 1)
23mm	-	-	-	2.9% (= 1)
25mm	-	-	5.0% (= 1)	2.9% (= 1)
28mm	-	-	-	2.9% (= 1)
30 mm	5.9% (= 1)	19.0% (= 4)	20.0% (= 4)	14.3% (= 5)
35mm	-	-	-	2.9% (= 1)
40 mm	5.9% (= 1)	19.0% (= 4)	10.0% (= 2)	14.3% (= 5)
50 mm	23.5% (= 4)	14.3% (= 3)	20.0% (= 4)	11.4% (= 4)
60 mm	17.6% (= 3)	4.8% (= 1)	-	5.7% (= 2)
80mm	-	-	-	8.6% (= 3)
100mm	5.9% (= 1)	-	-	-

Source: Research data. Patos-PB, 2014.

Regarding the *Candida* species found in the sample investigated (Table 1), it is known that up to 40% of women may have one or more species, as constituent of the vaginal flora without symptoms [26]. Other researchers believe that most vulvovaginitis is caused by a single species of *Candida*. However two or more species may be involved simultaneously [27].

Following the increasing trend of *Candida non-albicans* species, this study showed a predominance of these, corresponding to 78.3% of the species (Table 1). The reasons for this increase in non-*albicans* species, over the years, are the inappropriate use of topical and systemic antifungal once more select species resistant to antifungal agents most widely used. Studies have linked the emergence of *C. albicans* species not to the use of fluconazole [28- 29].

In turn, researchers [30] reported that the erroneous, inappropriate or incomplete use of antifungals would enable the elimination of *C. albicans* most sensitive species, selecting the most resistant non-*albicans*. Furthermore, it is reported that various antifungal agents such as clotrimazole, ketoconazole, miconazole, nystatin, butoconazole, are not active *in vitro* or *in vivo* against non-*albicans* species

[30-31]. However, other factors can contribute: the geographical location, the characteristics of the studied populations and other inherent in the diagnosis [20,27]. Since non-*albicans* species show a significant association with absence of symptoms [32].

Several authors associated the presence of symptoms with the isolation of *C. albicans* and other species, to the absence of symptoms [32]. A study with 223 samples identified *Candida* spp in 31% of symptomatic women and in 8% of asymptomatic women; and non-*albicans* species corresponded to 33% of asymptomatic group and 13% of symptomatic [33].

Regarding the association between the presence of *Candida* and the age of the participants recently survey did not observe this relationship, since the average age of cultures positive for *Candida* was 37 years old, ranging from 14 to 65 years old [34]. Other research found that the higher frequency of patients with positive cultures was between 14 and 46, and showed no association between the identified species of yeast and the age of the patients [35].

Regarding the menstrual cycle it was observed that when regular, was significantly associated with vulvovaginal candidiasis; and explained that it is possible that estrogen peaks facilitate fungal invasion of the vaginal mucosa [36].

The presence of regular menstrual cycles have been identified as relevant, since the acidity subsequent to hormone peaks of FSH, LH, estradiol and progesterone may contribute to the invasion of the vaginal mucosa [33]. Moreover, it is possible to relate the high levels of estrogen and a positive culture for *Candida*. It is believed that, due to higher estrogen levels, the incidence of symptoms is higher in women of reproductive age [35].

About antifungal action of propolis extract, verified in this study, other researchers also found such action on different *Candida* species in sensitivity following order: *C. albicans* > *C. tropicalis* > *C. krusei* > *C. guilliermondii* [37].

Several studies have demonstrated the action of propolis on yeast, especially on *C. albicans* [12,38]. Despite the differences in propolis composition studies at different times and in different regions demonstrated their antifungal activity [39-40].

Comparing the activities of propolis and fluconazole against *Candida* spp isolated from the mouths of HIV-positive patients, researchers found that propolis extract was able to inhibit the yeast with a minimum inhibitory concentration (MIC) less than fluconazole [41]. Propolis also showed antifungal activity against dermatophytes *C. neoformans* and *onychomycosis* and exhibited a synergistic effect with macrophages against *Paracoccidioides brasiliensis*.

Some research found that the ethanol extract of propolis has fungistatic activity at a concentration of 0.55 mg/ml [42]. Other studies have demonstrated fungicidal activity at concentrations of 3 to 7 mg/ml, being more susceptible species *C. albicans* [43].

This difference is due to different strains analyzed because researchers gave the antifungal potential of propolis in asymptomatic and symptomatic patients, compared to Nystatin, which obtained positive results before the vulvovaginal candidiasis [44].

Moreover, it can still be related to the isolation site and also the virulence of the agent in question, since studies underscore the yeast's ability to adhere to infect and cause disease by its potential virulence and pathogenicity, determined by virulence factors expressed genetically, when subjected to certain situations [45].

An in vivo study in Paraná noted that the propolis extract has shown activity similar to Nystatin, with inhibition profile of the vaginal infection by different *Candida* species [46]. The antifungal and antibacterial activities of propolis are attributed mainly to pinocembrine flavonona, the galargina flavonol and phenylethyl ester of caffeic acid, substances whose mechanism of action is probably in inhibiting bacterial RNA polymerase [47]. The compounds flavonoids, caffeic acid, cinnamic acid and benzoic acid,

probably acting on the wall or cell membrane of microorganisms, causing structural damage [13].

Conclusion

Despite the antifungal action of propolis extract, with inhibition in 81.25% of the samples analyzed, it appears that, although the most effective concentration was 25% for all other concentrations also observed antifungal action, especially considering that there were different strains in different patients, with varied responses due to particularities.

The positive trend of antifungal action of red propolis extract was not increased by increasing or decreasing the concentration of propolis extract. Therefore, such studies are relevant because of the need for new therapeutic alternatives, especially low-cost, more efficient and safe for the treatment of vulvovaginal candidiasis, considering the few treatment options available and the observed resistance of *Candida* to some usual drugs.

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