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SUPERFICIAL ANTICANDIDOSIC ACTIVITY OF LEAVES OF COMBRETUM RACEMOSUM P. BEAUV. (COMBRETACEAE) EXTRACTS

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ABSTRACT

This preliminary work consists in evaluating the superficial anticandidic activity of the leaves of Combretum racemosum P. Beauv in humans. To this end, we first demonstrated bioactive compounds of five (5) crude extracts (aqueous and hydro-organic 70%) from C. racemosum. Then, and finally, we have separately evaluated the superficial anticandidic activity of these extracts on Candida albicans, a species of genus Candida, mostly encountered in superficial human candidiasis. C. albicans was found to be sensitive to the five extracts tested. This partly justifies the use of C. racemosum in traditional medical practice against superficial microbial diseases. For these extracts, the Inhibitory Minimum Concentrations (MIC) recorded ranged from 7.8125 mg/mL to 62.550 mg/mL; While Fungicide Minimum Concentrations (CMF) range from 31.25 ± 0.00 mg/mL to 62.550 mg/mL. Concentrations for Fifty Percent Inhibition (IC_{50}) range from 0.930 mg/mL to 3.861 mg/mL. The hydroethanolic macerated extract 70% (Eeth 70%) is the most active fraction on C. albicans (CMF = 7.8125 ± 0.00 mg/mL, IC_{50} = 0.941 ± 0.009 mg/mL) compared to the total of the five crude extracts tested. The preliminary phytochemical screening of the five crude extracts revealed the presence of alkaloids, saponins, terpenoids steroids and tannins (catechics and gallics) at various levels of concentration in our study. Eeth 70%, the most active extract in our work, contains in medium concentrations the same active ingredients cited above. Finally, we note that the hydroethanolic macerated extract 70% is the most active fractions in this work. It may, however, subject to toxicological studies, be used in the treatment of theskin, the cutaneous appendages and the mucous membranes in humans.

Keywords: Combretum racemosum, phytochemical screening, superficial anticandidic

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1. INTRODUCTION

The skin, the cutaneous appendages and the mucous membranes constitute the first natural defenses of the living organism against external aggressions of any kind, in particular the microbial invasions[1]. They contain, in fact, physical, chemical and biological parameters, in particular microbial parameters, essential for the survival of the living body. Consequently, they require regular and rigorous hygiene, and in case of external microbial

aggressions, adequate and immediate medicinal treatments.

The genus Candida is a group of commensal yeast species of humans and other animals, opportunistic pathogens, keratinophilic and having an affinity for keratinized tissue to cause superficial and invasive candidiasis.

At the global level, for example in Central Africa, species of the genus Candida are at the forefront of opportunistic leprosy in healthy people and those living with HIV, and are the cause of pathological manifestations of AIDS [2]. Moreover, in recent years, the incidence of previously unrepresented candidiasis species is responsible for the increased frequency of yeast infections [3]. As an indication, Candida albicans is today, the most pathogenic and wide spread Candida [4]. However, although it is the most widespread species, these other species defined as non-albicans have, in recent years, a higher prevalence related to ecology [5].

Moreover, in Côte d'Ivoire in particular, a 2008 study in Abidjan on C. albicans, the most pathogenic and most frequently isolated Candida, showed that C. albicans accounted for 72.6% of the original strains vaginal disorders [6].

The superficial candidiasis, the object of this study, is opportunistic infections due to yeasts of the genus Candida, which affect the epidermis of the skin, the cutaneous appendages and the mucous membranes [7]. They represent the most frequent and natural candidiasis in the intestine, folds and buttocks, especially in young children [8]. They are cosmopolitan, recurrent and recurring superficial fungal infections that are difficult to cure in some cases, do not threaten life, but cause physical discomfort for those affected and may be factors that contribute to serious diseases including HIV infections [9]. They often lead to deaths in immune compromised individuals because of recurrence and difficulties in curing somecases. Overall, the rate of the global population with superficial candidiasis ranges from 70% to 80%; babies, children and immune compromised persons are the first victims [10, 11]. This frequency has increased steadily in recent years, with five to six recurrences a year. Moreover, the risk of complications of these infections in immune compromised subjects, for example indigestive candidiasis patients, is enormous and the mortality rate varies from 40 to 60% [12].

On the other hand, conventional modern medicines such as azoles, allylamines, equinocandins and polyenes are effective against fungal infections and are currently available on the market [13]. However, they are limited in number; costly, difficult to access by the poor and do not always correspond to the effective treatments for these diseases [14]. However, they consist of heavy drug treatments, not without undesirable side effects and constitute on the market, the main therapies used [15; 16]. Another major problem with these modern medicines is he resistance that certain pathogenic organisms put in place against these drugs, which, moreover, have quite high toxicity a few times [17; 18].

All these factors listed above make it necessary to develop new, effective, low-toxicity medicines that are readily available to populations from all walks of life. This research would be based on plants, because they are an important source of an immense variety of bioactive molecules[19]. Moreover, these phytomolecules have multiple interests used in industry, food, cosmetology and dermopharmacy. Among these molecules are alkaloids, coumarins, quinones, phenolic compounds, tannins, lignins, flavonoids and terpenes [20; 21].

On the international markets, demand for natural herbal medicines is also high. Today, according to the WHO, this demand is estimated at about 81% and represents for humanity, primary health care[22]. Combretum racemosum P. Beauv. could meet this demand. Combretum racemosum P. Beauv. is a very common shrub of the genus Combretum and widely used in African traditional medicine for its antimicrobial activities[23; 24]. Several scientific studies have been carried out on species of the family Combretaceae of which it forms part [25]. However, so far no special report has been made on C. racemosum against superficial candidiasis.

This work consisted in evaluating the superficial anticandidic activity of five crude extracts (aqueous and hydro-organic 70%) of C. racemosum in humans. In addition, a preliminary triphyochemical study of the various extracts was also carried out.

2. MATERIALS AND METHODS

2-1. Plant material

The plant material used consists of fresh leaves of C. racemosum. This plant is found in East Africa, in the forestry recruits of the Guineo-Congolese region, in Senegal and Cameroon. It is also found in southern Nigeria where, in view of its therapeutic utility, it bears the name "Ebi-odo" or "Rose de Noel" [26; 27]. In Côte d'Ivoire, C. racemosum occurs in the southern forest zone; It is known under the vernacular name of betso (in the Attié language in the Department of Anyama) and calo wôrô (in the Bambara language). It is a large climbing vine or a sprouting shrub up to 25-30 m long.

The leaves, ovate or elliptic, acuminate and rounded at the base, average 10 cm in length and 5 cm in width. They include about 8 to 10 lateral ribs. The inflorescences, of the pancicles of short speciform races, are remarkable for the white bractal leaves, the terminal or axillary clusters of yellow flowers. The flowers have 5 free sepals and 5 petals. They have 6 to 7 fertile stamens, very long anthracenes and poricides. The fruits, with four wings, are white- greenish or pinkish in the fresh state.

These leaves of C. racemosum are collected in June 2015 in the Department of Anyama (Côte d'Ivoire) and authenticated by Professor AKE Assi at the National Floristic Center of the University Félix Houphouët Boigny of Abidjan where samples are deposited Herbarium of the Plant under No. 19649, on 17 July 1985. These sheets were used to make powders and then the five crude extracts (aqueous and hydro-organic 70%).

2-2. Candidic isolates used

The candidosic isolate used is Candida albicans (13-304). Candida albicans is a commensal filamentous yeast of the skin and mucous membranes which, under special conditions, cause acute, subacute or chronic candidiasis of the skin, mucous membranes and more rarely visceral and generalized [28]. Among the yeasts of the genus Candida, it is the most widespread but also the most pathogenic species of superficial candidiasis. It infects newborns, immune suppressed individuals, diabetics and surgical patients, as well as healthy people. For example, 75% of women have had vaginal candidiasis in C. albicans and 5% have chronic candidiasis [29].

The choice of C. albicans is made because of its majority (60%) involvement in superficial and invasive candidiasis.

Our strain of C. albicans (13-304) is supplied by the Laboratory of Mycology of the Institute Pasteur of Ivory Coast (IPCI). It is derived from the vaginal sampling of AIDS patients from infectious diseases.

2-3. Plant treatments and sampling

After identification, the leaves of C. racemosum are first thoroughly sorted and freed from foreign bodies. They are then washed with distilled water, cut and dried out of the sun and in the open air. After drying, they are sprayed using a Culatti type Mfc electric grinder. Finally, the fine powder obtained is stored in sterile, clean and dry bottles. The whole is kept away from moisture and at ambient temperature of the laboratory to serve the preparation of the five crude extracts (aqueous and hydro-organic 70%).

2-4. Preparation of aqueous crude extracts

The macerated aqueous crude extract (codified Eaq) is obtained according to the following method [30]:

100 g of leaf powder are homogenized in 1 liter (1 L) of distilled water in a mixer at room temperature. The homogenate obtained is first drained through a square of white cloth. Then, double filtered on hydrophilic cotton (ref: 87071) and once on 3 mm wattman paper. The filtrate obtained is concentrated in an oven at 50 ° C. to give the macerated aqueous crude extract (Eaq).

The soaked aqueous crude extract (codified Edec) is obtained according to the following method [31]:

100 g of leaf powder is first dissolved in one liter (1 L) of cold distilled water in a beaker. The whole is then boiled for 15 minutes on a hot plate, and homogenized in a blender (Mixer) at room temperature. At the end of the homogenization, the homogenate obtained is first drained through a square of white cloth. Then, double filtered on hydrophilic cotton (ref.: 87071) and once on 3 mm wattman paper. The filtrate obtained is concentrated in an oven at 50° C.

The Eaq and the Edec obtained are finally stored in sterile, clean and dry bottles and then kept away from heat and humidity to be separately used for the evaluation of the bioactive compounds and the surface anticandidosic activity.

2-5. Preparation of the hydro-organic crude extracts 70%

The macerated hydroacetate extract 70% (codified Eace 70%) is prepared according to the following method [32]:

100 g of leaf powder is dissolved in one liter (1 L) of a solution of cold water and pure ethyl acetate (300 mL of cold distilled water per 700 mL of pure ethyl acetate 99.5° G.L) and then homogenized in a blender. After homogenization in a blender at room temperature, each homogenate obtained is first filtered through a square of white cloth. Then, double filtered on hydrophilic cotton (ref: 87071) and once on 3 mm wattman paper. The filtrate obtained is concentrated in an oven at 50 ° C.

The macerated hydro-ethanolic crude extract 70% (codified Eeth 70%) and macerated hydro-methanolic crude extract 70% (codified Emet 70%) are prepared according to the preceding method [32]. However, the purity of ethanol is 96° G.L while that of methanol is 99.85° G.L.

The masses of Eace 70%, Eeth 70% and Emet 70% obtained are stored in sterile, clean, dry bottles then stored away from heat and humidity to be separately used For the evaluation of the bioactive compounds and then the superficial anticandidosic activity.

2-6. Phytochemical screening of the various C. racemosum extracts

The five crude extracts of C. racemosum are distinctly subjected to a phytochemical screening for secondary metabolites contained in the leaves of the plant [33]. Each bioactive compound is demonstrated in accordance with the method of Mangambu et al. [34]. It is a set of identification reactions and colored indicators based on the reduction (alkaline or basic) of the reagent mixture by the oxidizable groups of the bioactive compounds, leading to the formation of color reduction products which are a function of the environment. For each extract, 10 screening tests per colored reactions are carried out. Solutions with indicators have a positive reaction; This indicates the presence of bioactive compounds in the extracts and therefore in the leaves of C. racemosum.

2-7. Evaluation of the superficial anticandidosic activity of the various extracts

2-7.1. Preparation of culture media

Sabouraud agar (ref: Bio-RAD, batch: C8B2212, n° 74994) buffered to pH 5.7 is used for this test. The medium of culture is prepared according to the instructions of the manufacturer's protocol.

The incorporation of extracts of C. racemosum in Sabouraud agar is carried out using the method of double dilution of agar on slopes [35; 36]. For each C. racemosum extract, each series consists of 10 test tubes. Eight (8) of these test tubes contain extract. And the other two tubes are considered as control tubes. Of these two (2) tubes, one extract was used as control for growth control of C. albicans while the other without C. albicans strain and without extract was used as a control for the sterility control of Sabouraud agar. The eight (8) test

tubes with concentrations ranging from 125 to 0.78 mg / mL are bound by a geometric reason of ½. All the tubes are autoclaved at 121° C. for 15 minutes and then inclined at ambient temperature to allow the cooling and solidification of Sabouraud agar [37]. Ketoconazole is used as a drug control.

2-7.2. Anticandidosic test

The candidosic inoculum is prepared from culture of 48 hours of young colonies of C. albicans, previously isolated from Yeast glucose chloramphenicol (ref.: 51078, batch: 777666501) which is a microscopic fungal selective medium supplied by Biomedis. One (1) colony of C. albicans is removed and homogenized in 10 mL of sterilized distilled water to obtain a 100 germ suspension. On dilution to 1/10, 1 mL of this suspension is transferred and homogenized in 9 mL of sterilized distilled water to give a final volume of 10 mL to suspension 10^{-1}. The latter is stored for inoculation at the rate of 10 µL per tube.

The anticandidosic test concerns the 8 test tubes and the growth control (Tc) control. Thousand (1000) cells of C. albicans, equivalent to 10 µL, are seeded by transverse streaks tight until exhaustion; on Sabouraud agar medium prepared before hand [38].

For each extract, all tests are carried out by triplicates. The cultures are incubated at 30 ° C. for 48 hours. They are used to determine the superficial anticandidosic activity.

2-8. Determination of the anticandidosic activity of the various extracts

This part concerns microbiological factors: colony counts of C. albicans, determination of antifungal parameters (MIC, CMF and IC_{50}) and the growth curve of C. albicans.

2-8.1. Enumeration of Candidate Colonies

After this incubation time, the colonies of C. albicans are first counted by direct counting with a colony counting pen (serial number of Cebeware 23382, brand Bel-Art), then, and finally, growth in 10 experimental tubes on the percent survival is evaluated, compared to 100% survival in the Tc growth control pilot tube [39].

2-8.2. Determination of antifungal parameters

After counting the colonies, the value of each antifungal parameter is determined. Data processing allows for minimum values of inhibitory concentrations (MIC), fungicide concentration (CMF), graphically to determine on the activity curves of the extract 50% of the inhibition concentration values (IC_{50}) and activity report (CMF/CMI).

The MIC is here the concentration of extract of C. racemosum in the tube for which there was no visible growth with the naked eye. CMF is the concentration of extract which gives 99.99% inhibition as compared to the growth control tube. The IC_{50} is determined graphically from the sensitivity curve of C. albicans to each extract.

2.9. Statistical analysis

The values of the antifungal parameters of each extract are determined using the Graphpad software. The results are given as mean ± SE (n = 4), using Colum Statistica.

3. RESULTS AND DISCUSSION

In this work, bioactive compounds (active ingredients) of the five crude extracts (aqueous and hydro-organic 70%) of C. racemosum leaves are demonstrated by phytochemical screening. The in-vitro anticandidosic activity of these extracts is also evaluated with respect to C. albicans.

3-1. Bioactive compounds

The results of the phytochemical screening of the five crude extracts of C. racemosum leaves are shown in table 1. Analysis of these results clearly reveals the presence in the five extracts of alkaloids, flavonoids, phenolic compounds, saponins, steroids, terpenoids and tannins (catechics and gallics) in varying degrees of concentration (table 1).

The 70% (70% Eeth) hydroethanolic crude extract contains alkaloids, saponins, steroids, terpenoids and tannins (catechic and gallic) at medium concentrations (table 1).

Table 1: Bioactive compounds of the five leaf extracts of C. racemosum

Chemical constituents		Aqueous extracts		Hydro-organic extracts		
		Eaq	Edec	Eace 70%	Eeth 70%	Emet 70%
Alkaloid	B	+	+	+	++	+++
	D	+	+	+	++	+++
Flavonoids		+	++	-	-	+
Phenolic compounds		+	++	-	-	+
Free quinone		-	-	-	-	-
Saponins		+	++	+	++	+++
Steroids		+	++	+++	++	+
Terpenoids		+	++	+++	++	+
Tannins	Cat	+	++	-	++	+
	Gal	+	+	-	++	+

Eaq: Macerated aqueous crude extract; Edec: Soaked aqueous crude extract; Eace 70 %: Crude Macerated hydro-ethyl acetate crude extract 70%; Eeth70 %: Macerated hydro-ethanolic crude extract 70%; Emet 70 %: Macerated hydro- methanolic crude extract 70%; B: Bouchardat; D: Dragendorff; Cat: Catechics; Gal: Gallics; -: Lack of bioactive compounds; +: Weak of bioactive compounds; ++: Medium concentration of bioactive compounds; +++: High concentration of bioactive compounds

By triphyochemical studies confirmed on C. racemosum extracts, similar results have been obtained by Harbone, Onocha et al. (a), Okwuosa et al. and Kamou et al.[40; 41; 42; 43]. These results could confirm in part the use in traditional C. racemosum against microbial superficial infections. These compounds are already known for their antimicrobial activities [44, 45, 46].

Mcgaw et al., Have also demonstrated the presence of these secondary metabolites in a hydro-ethanolic crude extract (Eeth 70%) [47].

3-2. Superficial anticandidosic activity of the different extracts

The anticandidic activity of each C. racemosum crude extract was evaluated. The values of the antifungal parameters are determined (figure 1, table 2). Analysis of the experimental results shows that, compared with growth control (TC) controls, there is a decrease in the number of C. albicans colonies in the test tubes as the concentration of each extract increases (figure 1). The curve that characterizes this decrease shows a decreasing rate (figure 1). Thus, our results show that the five (5) extracts tested are active at various levels ranging from 7.8125 ± 0.00 mg / mL to 62.5 ± 0.00 mg / mL and show anticandidic activity by inhibiting. The in-vitro growth of C. albicans in a dose-response relationship.

The sensitivity of C. albicans to each extract as well as the curve obtained make it possible to determine the different antifungal parameters of each extract, namely the Minimum Inhibitory Concentration (MIC), the Minimum Fungicidal Concentration (CMF), the Concentration for Fifty percent Inhibition (IC_{50}) as well as the activity report (CMF/CMI) (table 2).

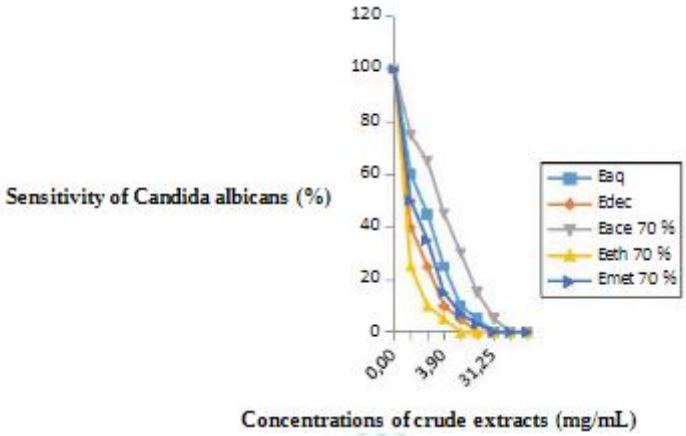

Figure 1: Sensitivity of C. albicans to C. racemosum extracts

Table 2: Superficial anticandidosic activity of C. racemosum five crude extracts tested

Strain	Crude extracts	Anticandidosic parameters (mg/mL)			Activity report (CMF/CMI)	Effect (Fungicidal)
		CMI	CMF	CI_{50}		
Candida albicans (13-304)	Eaq	31,25 ± 0,00	31,25 ± 0,00	1,903 ± 0,015	1	Fungicide
	Edec	15,625 ± 0,00	15,625 ± 0,00	0,930 ± 0,010	1	Fungicide
	Eace 70%	62,5 ± 0,00	62,5 ± 0,00	3,861 ± 0,009	1	Fungicide
	Eeth 70%	7,8125 ± 0,00	7,8125 ± 0,00	0,941 ± 0,009	1	Fungicide
	Emet 70%	31,25 ± 0.00	31,25 ± 0.00	0,946 ± 0.005	1	Fungicide
	Két	0,0076 ± 0.00	$7,65x10^{-3}$ ± 0,00	$4,653x10^{-6}$ ± $1,528x10^{-8}$	1	Fungicide

Eaq: Macerated aqueous crude extract; Edec: Soaked aqueous crude extract; Eace 70 %: Macerated hydro-ethyl acetate crude extract 70%; Eeth 70 %: Macerated hydro-ethanolic crude extract 70%; Emet 70 %: Macerated hydro-methanolic crude extract 70%;Ket: ketoconazole, MIC: Minimum Inhibitory Concentration; CMF: Minimum Concentration Fungicide; IC_{50}: Concentration for Fifty percent Inhibition

The sensitivity of the C. albicans strain to the five extracts tested justifies in part the use in traditional C. racemosum P. Beauv. against microbial diseases. In separate studies evaluating phytochemical and antimicrobial properties of leaf extracts of C. racemosum validated, Onocha et al. (b) and Okwuosa et al., also showed the sensitivity of C. albicans to crude aqueous, ethyl acetate, methanolic and hexanic extracts of C. racemosum [48; 49]. The synergistic action of alkaloids, flavonoids, phenolic compounds, saponins, steroids, terpenoids and tannins at various degrees of concentration would be the basis of the anticandidosic activity observed in the five crude extracts in our study. Indeed, alkaloids, for example, are endowed with antimicrobial properties [50]. Flavonoids, tannins and triterpenoids are recognized as antifungal molecules [51].

Flavonoids are in particular responsible for the antiallergic, antiparasitic power and also inhibit the activation of the supplements [52; 53]. Phenolic compounds have been found to have antibacterial and antifungal activities, and are indeed natural compounds widely distributed in the plant kingdom, which are of increasing importance, in particular because of their beneficial effects on health [54]. They are also used as additives in the food, pharmaceutical and cosmetic industries [55].

The five extracts tested were fungicide on the strain of C. albicans studied. For the five extracts, the recorded MIC ranged between 7.8125 ± 0.00 mg/mL and 62.5 mg/mL for C. albicans; while CMF ranges from 7.8125 ± 0.00 mg/mL to 62.5 mg/mL. IC_{50} range from 0.930 ± 0.010 mg/mL to 3.861 ± 0.009 mg/mL.

The macerated hydro-ethanolic extract 70% (Eeth 70%) is the most active fraction compared to the others on C. albicans (CMF = 31.25 ± 0.00 mg/mL, IC_{50} = 1.910 ± 0.009 mg/mL). This activity is comparable to that of the reference drug ketoconazole tablet (CMF = 7.65x10-3 ±0.00 mg/mL, IC_{50} = 4.653x10-6 ± 1.528x10-8 mg/mL. The nature of the Eeth 70% bioactive compounds could justify the high anti-candidosic activity compared to the other extracts. The activity of Eeth 70% is indeed due to alkaloids, saponins, steroids, terpenoids and tannins.

These ethano-soluble molecules, which were moderately concentrated in this extract, were then able to express their anticandidosic potential better, hence the activity obtained.

Alkaloids, for example, generally have an important function in biological structures; they are also potent anticholinergics and are known for their high antibacterial potency [56; 57]. The activity of Eeth 70% is attributed in part to the alkaloids which not only are concentrated in Eeth 70% but also possess strong antifungal activity according to the literature. An analysis of the CMI values shows that Eeth 70% enhances anticandidosic activity than the other extracts.

We recall that the activity of a plant substance depends on several factors including the mode of extraction and the concentration of active ingredient [58; 59].

The activity report (CMF/CMI) of Eeth 70% for C. albicans is one (1). The Eeth 70% thus has a fungicidal activity against C. albicans. This extract can therefore be described as a fungicidal substance. When the activity ratio (CMF/MIC) of an antimicrobial substance is less than or equal to four (≤ 4), the latter is referred to as a fungicidal substance and if the CMF/MIC ratio is greater than four (> 4), then it is called fungistatic [60].

4. CONCLUSION AND PERSPECTIVES

This analytical study, which consisted in demonstrating bioactive compounds and evaluating the superficial anticandidosilic activity of five crude extracts of Combretum racemosum, makes it possible to conclude that all the extracts analyzed contain bioactive compounds at various degrees of concentration. The macerated hydro-ethanolic crude extract 70% (Eeth70%) contains, in medium concentrations, the same compounds mentioned above.

The C. albicans strain studied is sensitive to the five extracts tested. This sensitivity is different depending on the extract. C. albicans is more sensitive to Eeth 70%, while it is more resistant to macerated hydro-ethyl acetate crude extract 70% (Eace 70%). Eeth 70% of C. racemosum is the most active fraction on C. albicans. It concentrates the active ingredients better.

Taking into account the results obtained in this work, Eeth 70% could, after toxicological studies, be used as phytomedicine to combat skin, mucosal and skin apposition in humans.

In our future work, we will first prepare the partitioned extract of the Eeth 70% of C. racemosum, then purify its phytomolecules which we will test again on C. albicans. Finally, we will be interested in the toxicological study of Eeth 70% on laboratory animals.

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