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FREE RADICALS SCAVENGING PROFILES AND ANTIFUNGAL CAPACITY OF ETHYL ACETATE FRACTION (EAF) OF STEM BARK FROM *BOSWELLI DALZIELII* HUTCH (BURSERACEAE)

Dr. Konaté Kiessoun[1,2,5]*, Ouattara Nabèrè[3], Metuor Dabiré Amana[4], Alain Souza[5] and Mamoudou H. Dicko[1]

[1]Laboratory of Food Biochemistry, Enzymology, Biotechnology and Bioinformatic, University of Ouagadougou, 03 P.O. Box: 848, Ouagadougou 03, Burkina Faso.

[2]University of Dedougou, Burkina Faso Training and Research Unit in Applied and Technological Sciences, P.O. Box 176, Dedougou, Burkina Faso.

[3]Laboratory of Biochemistry and Applied Chemistry, University of Ouagadougou, 09 P.O. Box 848, Ouagadougou 09, Burkina Faso.

[4]Laboratory of Biology and Genetic Molecular, 01 P.O. Box 1091, Ouagadougou 01, Burkina Faso, 03 BP 7021, Ouagadougou 03, Burkina Faso.

[5]Laboratory of Animal Physiology, Electrophysiology and Pharmacology, Faculty of Sciences, University of Science and Technology of Masuku, Franceville, Gabon.

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***Corresponding Author**
Dr. Konaté Kiessoun
Laboratory of Food Biochemistry, Enzymology, Biotechnology and Bioinformatic, University of Ouagadougou, 03 P.O. Box: 848, Ouagadougou 03, Burkina Faso.

ABSTRACT

Background: The present study was designed to evaluate the *in vitro*antioxidant and the antifungal activity of EAF of stem bark from *Boswelli dalzielii* Hutch (Burseraceae) against *Candida albicans, Candida parapsilosis, Candida tropicalis* and *Candida krusei* strains and to determine its mode of action and synergistic effect when combined with the synthetic antifungal nystatin. **Methods:** The antioxidant activity of EAF of stem bark from *Boswellidalzielii* Hutch has been evaluated using 1,1-diphenyl-2-picrylhydrazyl inhibitory activity and chelation of iron (II) ions.The antifungal activity was investigated using the microplate dilution method and the Fractional Inhibitory Concentration Index (FICI) of EAF of stem bark was evaluated. **Results:** EAF of stem bark exhibit maximum radical scavenging activity. As for the antifungal activity, result varied

according to microorganism. The results obtained in this antifungal activity were interesting and indicated a synergistic effect between of EAF of stem bark from *Boswellidalzielii* and the antifungal references such as Nystatin. The results of this study showed that of EAF of stem bark from *Boswellidalzielii*in combination with antifungal reference (Nystatin) exhibited antimicrobial effects against *candida* strains tested. **Conclusion:** Therefore, this study shows a fungicidal effect on *Candida* species and a synergistic effect when combined with nystatin and the results supported the utilization of this Burseraceae in treatment of *candida* infections.

KEYWORDS: Free radicals scavenging, Antifungal, Ethyl Acetate Fraction, stem bark, *Boswellidalzielii* Hutch.

BACKGROUND

Free radicals are continuously generated and metabolized as the result of metabolic processes in the body and interact with the environmental stimuli. Under normal physiological conditions wide range of antioxidant defense mechanism protect the body against free radicals.[1] The production of free radicals is a steady-state event in normal cells and is now recognized that uncontrolled production of these reactive species is the primary cause of numerous disease conditions and involve microbial infections.

In recent years a large number of plants have been investigated for their antimicrobialproperties as an integrative system of medicine for protection and management againstpathogens. Use of plant extracts having antimicrobial potential can be of great significance totreat human pathogenic diseases.[2] In African, according WHO estimations, over 80% of the populations in Africa use even traditional medicine to meet their health care needs.The indiscriminate use of commercial antimicrobial drugs for curing infectious diseases haslead to the development of multiple drug resistance in human pathogenic microorganisms. Inaddition, a variety of side effects like allergic reactions, hypersensitivity and immunesuppression are occasionally associated with antibiotics. Recent trends for the use of natural remedies as antimicrobialhave increased their use in food, cosmetic and pharmaceutical products which have beenscreened *in vitro* and indicated antimicrobial and other diverse properties.This is related to toxicity of chemicals, high cost of chemical drugs, removal and/or inadequate health facilities especially in rural areas, which limit a suitable care of public health problems. Furthermore, the control of bacterial and fungal becomes complex because of theemergence of resistant

bacteria and fungi to many conventional antibiotics. Yet bacterial infections and candidiasis are counted among the most dangerous and opportunistic diseases to vulnerable people such as children, elderly andimmune compromised individuals.In the last three decades, pathogenic resistant fungiparticularly *candida* strains, have caused major healthproblems throughout the world in women although thepharmacological industries produced quantities of antibiotics.Currently, disseminated invasive candidiasis has an estimated mortality rate of 40%, even with the use of antifungal drugs.[3] This fact justifiesthe development of new therapies for use in clinicalpractice.Among these new therapies, natural products are well candidates; they are considered sources of bioactive moleculeswith potential therapeutic applications in medicine.[4,5] Many studies have been conducted onthe antifungal activity of natural products against*Candida* species involved in fungal infections of the oralcavity.[6,7] The search for plantswith antifungal activity has gained increasing importancein recent years due to the development of resistance. The fact that microorganismsnowadays tend to develop resistance towards drugs, coupled to the undesirable side effects of certain antibioticsoffer considerable potential for the development ofnew effective antimicrobial agents, medicinalplants are a prolific source. Amongst the medicinal plants investigatedin our research team, the family of Burseraceaeislargely represented and some of these plants as *Boswellidalzielii* Hutch. A decoction of this Burseraceae is locallyused in the treatment of coughs, rheumatic and abdominalpain, and diarrhea while the leaf decoction is used inthe treatment of fever and to prevent miscarriage.[8] In this context, the present study investigated antifungal activity of bioactive fraction of stem bark from *Boswellidalzielii* Hutch against *C. albicans* strains withrespect to growth inhibition, microbial death, and synergistic effect in combination with the bioactive fraction antifungal and nystatin.

MATERIAL AND METHODS

Plants material

The vegetable materials (Fresh stem bark) of*Boswellidalzielii*Hutch (Burseraceae)were collected in August 2014 in Dedougou, 230 Km West of Ouagadougou, capital of Burkina Faso. This plant was botanically identified by Dr. TraoréLassina from the plants Biology Department of the University of Koudougou.

Bacterial strains

The studies microorganisms included reference strains of *Candida albicans* ATCC 9002, *Candida albicans* ATCC 2091, *Candida parapsilosis* ATCC 22019, *Candida tropicalis*

ATCC 750, *Candida Krusei* ATCC 6258.Fungal strains were maintained on agar slant at 4°C and sub-cultured on a fresh appropriate agar plates 24h prior to any antifungal activity. Sabouraud Glucose Agar was used for the activation of fungi. The MuellerHinton Broth (MHB) was used for the MIC and MFCdeterminations.

Extraction and fractionation

Fifty grams of powdered plant material was extracted with 80% aqueous acetone (500 mL) in 1/10 ratio (w/v) for 24 h undermechanic agitation (SM 25 shaker, Edmund BÜHLER, Germany)at room temperature. After filtration, acetone was removed underreduced pressure in a rotary evaporator (BÜCHI, Rotavopor R-200, Switzeland) at approximately 40 °C. The aqueous extracts weresubjected to sequential liquid-liquid extraction with n-hexane, dichloromethane, ethyl acetate and n-butanol. Each fraction wasthen collected and concentrated to dryness under reduced pressure toobtain n-hexane fraction (n-HF), dichloromethane fraction (DCMF), ethyl acetate fraction (EAF) and n-butanol fraction. The differentfractions were freeze-dried by Telstar Cryodos 50 freeze-dryer. Thefraction residues were packed in waterproof plastic flasks and storedat 4 °C until use.Afterquantitative tests, it revealed that ethyl acetate fraction (EAF) contents possess the best fraction than the other. Therefore, antifungal and antioxidant properties should be evaluated with this fraction.

Antioxidant profiles

DPPH radical-scavenging activity

The scavenging effect for DPPH free radical was monitored as described in [9] with minor modification. Briefly, 1.0 mL of 0.16 mM DPPH methanolic solution was added to 1.0 mL of either methanolic solution of extract (sample) or methanol (control). The mixtures were vortexed and then left to stand at room temperature in the dark. After 30 min absorbance was read at 517 nm. Radical-scavenging activity (RSA) for DPPH free radical was calculated using the following equation

$$RSA= (A_{Control} - A_{Sample})/A_{Control} \times 100$$

Where $A_{Control}$is the absorbance of the methanol control and $A_{Sample}$is the absorbance of the flavonoids. Synthetic antioxidant, BHA, was used as positive control. DPPH radical-scavenging activity was calculated as the concentration that scavenges 50% of DPPH free radical and thus has RSA = 50% (EC_{50}).

Chelating activity (ChA)

The chelation of iron (II) ions was studied as described by[10]. An aliquot of the fraction in methanol (1.3 mL) was added to 100 μL of 2 mM FeCl2. After 5 min, the reaction was initiated by adding 200 μL of 5 mMferrozine. Following 10 min incubation at room temperature, the absorbance at 562 nm was recorded. For preparation of control, 1.3 mL of methanol was used instead of polyphenols solution. EDTA was used as a chelating standard. The Fe2+ chelating activity (ChA) was calculated using the equation below:

$$\mathbf{ANT = (A_{Control} - A_{Sample})/A_{Control}\ x100}$$

Where $A_{Control}$ is the absorbance of the negative control (solution to which no flavonoid was added) and A_{Sample} is the absorbance of the extract solution. Chelating activity was expressed as $ChEC_{50}$, the concentration that chelates 50% of Fe^{2+} ions and thus has ChA = 50%.

***In vitro* antifungal activity**

Preparation of inocula

The fungal strains grown on nutrient agar (MullerHinton broth) at 35°C for 72 h were suspended in a salinesolution (0.9%, w/v) NaCl and adjusted to a turbidityof 0.5 Mac Farland standard (5×10^5 CFU/ml).[11]

Preparation of fraction substances

The stock solutions of ethyl acetate fraction (EAF) were dissolved in 10%dimethylsulfoxide (DMSO) in water[11,12] at a final concentrationof 800 μg/ml. The stock solutions were sterilizedby filtration through 0.22 μm sterilizing Milliporeexpress filter.

Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration (MIC) was determinedby the microdilution method in culture broth as recommendedby.[11,13] with low modifications. 12 serialtwo-fold dilutions of EAF solutions or conventionalantibiotic were prepared as described before, to obtainfinal concentration ranges of 800–0.78125μg/ml and50–0.0488 μg/ml for EAF and reference substances respectively.The last wells (n°12) served as sterilitycontrols (contained broth only) or negative control(broth + inoculums). The 96-well micro-plates (NUNC, Danemark) containing 100 μL of Mueller Hinton (MH) broth were used. For each fungi strain, three columns ofeleven wells to the micro-plate were used. Each well hasgetting: the culture medium + EAF solution or Nystatin or the combination of fraction solutionwith Nystatin +

inoculum standardizedat 5×10^5 CFU/ml (10 µl of inocula) and INT (50 µl; 0.2 mg/ml for 30 min). The plates were sealed with parafilm, then agitated with a plate shaker to mix their contentsand incubated at 35°C for 48 h. All tests wereperformed in triplicate and the fungi activity wasexpressed as the mean of inhibitions produced. Viablemicroorganisms reduced the yellow dye to a pink colour.The MIC was defined as the lowest concentration of EAFsubstance at which no colony was observed after incubation.So, the MIC was defined as the lowest concentrationwhere no change was observed, indicating nogrowth of microorganism.

Minimum fungicidal concentration (MFC)

Minimum fungicidal concentration (MFC) was determinedby the microdilution method in culture broth asrecommended by,[11,13] with low modifications. Minimumfungicidal concentration (MFC) was determinedby adding 50 µl aliquots of the clear wells to 150 µl offreshly prepared broth medium and incubating at 35°Cfor 48 h. The MFC was regarded as the lowest concentrationof test sample which did not produced a colour.All tests were performed in triplicates.

Evaluation offungicidal and fungistatic capacity

The MFC/MIC ratio was calculated to determinewhether EAF has a fungistatic (MFC/MIC $\geq$ 4) orfungicidal activity (MFC/MIC $<$4).[14]

Evaluation of the fractional inhibitory concentration index (FICI)

The Muller Hinton agar dilution method was used toevaluate the Fractional Inhibitory Concentration Index (FICI) of EAF and the tested anti-microbial standard asreported earlier [15, 16]. Eleven (11) serial two-fold dilutionsof EAF solutions were prepared as described before, toobtain final concentration range of 800 to 0.78125µg/ml.A series of two-fold serial dilutions of Nystatin was also prepared in the same conditions as EAF. Inthis way, antifungal standard dilutions were mixed with theappropriate concentration of EAF solution thus obtaining aseries of the combinations of conventional antifungaland EAF solution. The concentrations prepared correspondedto 1-1/1024 of MIC values. The 96-well microplate (NUNC, Danemark) containing 100 µL of MuellerHinton (MH) broth were used. For each fungal strain, three columns of eleven wells to the micro-plate were used.Each well has getting: the culture medium + combinationof EAF solution with Nystatin + inoculum standardized at 5×10^5 CFU/ml (10 µl of inocula) and INT (50 µl; 0.2 mg/ml for 30 min). The plates were covered andincubated at 35°C for 48 h. All tests were performed intriplicate and

the fungicidal activity was expressed as themean of inhibitions produced. Viable microorganismsreduced the yellow dye to a pink colour. The analysis ofthe combination of EAF solution and antifungal reference (Nystatin) was obtained by calculatingthe Fractional Inhibitory Concentration Index (FICI) as follows:

FICI= (MICa of EAF in combination/MICa alone) + (MICb of the standard antifungal agent in combination/MICb alone), where MICa (Minimal Inhibitory Concentrationof EAF) and MICb (Minimal Inhibitory Concentrationof Nystatin). The FICI was interpreted asfollows: (1) a synergistic effect when FICI =0.5; (2) an additiveor indifferent effect when FICI >0.5 and < 1; (3) an antagonisticeffect when >1.[11]

Statistical analysis

The data wereexpressed as Mean±Standard deviation (SD) of threedeterminations. Statistical analysis (ANOVA with a statisticalsignificance level set at $p<0.05$ and linear regression)was carried out with XLSTAT 7.1.

RESULTATS

Antioxidant of DPPH radical-scavenging activity

The basis of DPPH assay is the discoloration of DPPH• solution in presence of an antioxidant. In its radical form, DPPH absorbs with maximum at 517 nm, but upon reduction with an antioxidant. In this study, EAF of stem barks from *Boswellidalzielii*demonstrated notable antiradical activities albeit lower than the activity of BHA (P<0.001). Results are consigned in the (Figure 1).

Chelating activity (ChA)

The chelating ability of the fraction toward ferrous ions was investigated (Figure 1) in presence of ferrozin, Fe2+ ion chelator, which upon binding of the metal ionabsorbs with maximum at 562 nm. In the investigated EAF of stem barks from *Boswellidalzielii*demonstrated significant chelating ability in the present research, although lower than the ability of EDTA (P<0.0001).

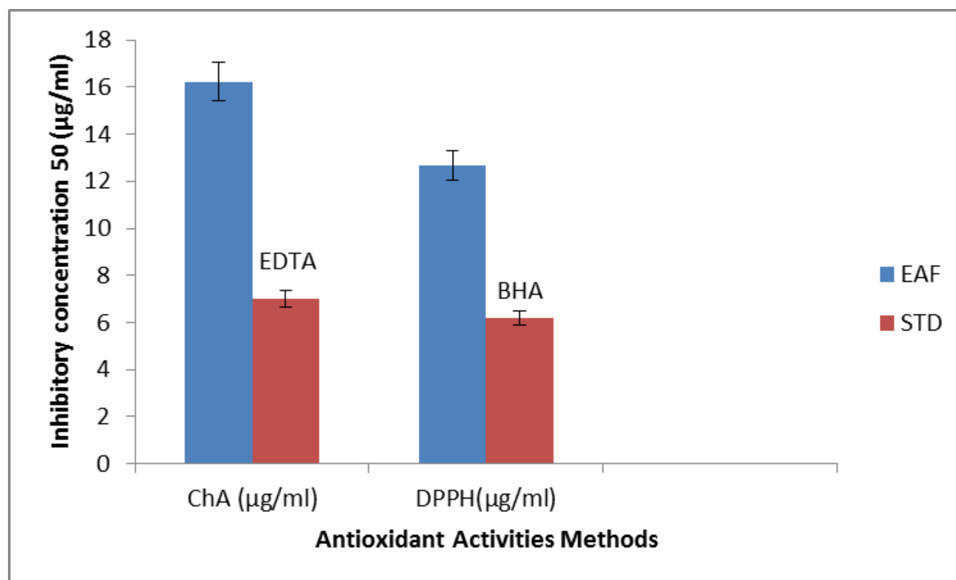

Figure 1: Antioxidant activity in DPPH radical scavenging-activity (EC_{50}), and metal chelating activity ($ChEC_{50}$) of EAF of stem barks from *Boswellidalzielii*and Standards (STD).

***In vitro* antifungal activity**

Minimum inhibitory concentration (MIC) assay and Minimum fungicidal concentration (MFC)

As for the Minimum inhibitory concentration assay(MIC) and Minimum fungicidal concentration (MFC) ofEAF and their combination with antifungal reference(Nystatin), result varied according tomicroorganism and results are summarize in Tables 1, 2, 3 and 4.

Table 1: Minimal Inhibitory Concentration (MIC) of EAF of stem barks from *Boswellidalzielii*and antifungal references (Nystatin).

Microorganisms	MIC (µg/ml) EAF of *Boswellidalzielii*	MIC (µg/ml) Nystatin
***Candida albicans* ATCC 9002**	7.29 ± 4.77^{b}	3.65±2.39
***Candidaalbicans* ATCC 2091**	6.25 ± 0.00^{a}	3.125±0.00
***Candida parapsilosis* ATCC 22019**	10.42 ± 3.61^{d}	4.17±1.80
***Candida krusei* ATCC 6258**	8.33 ± 3.61^{c}	3.65±2.39
***Candida tropicalis* ATCC 750**	11.46 ± 11.83^{e}	4.43±3.61

Values are Mean ±SD (n=3). Different letters in the same column indicate significant difference (P<0.05) for EAF

Table 2: Minimal Fungicidal Concentration (MFC) of EAF of stem bark from *Boswellidalzielii*and antifungal references (Nystatin).

Microorganisms	MFC (µg/ml) EAF of *Boswellidalzielii*	MFC (µg/ml) Nystatin
Candida albicans ATCC 9002	20.83±7.22[b]	7.30±4.77
Candida albicans ATCC 2091	12.50±0.00[a]	6.25±0.00
Candida parapsilosis ATCC 22019	50.00±0.00[c]	16.66±7.22
Candida krusei ATCC 6258	12.50±0.00[a]	6.25±0.00
Candida tropicalis ATCC 750	50.00±0.00[c]	16.66±7.22

Values are Mean ±SD (n=3). Different letters in the same column indicate significant difference (P<0.05) for EAF.

Table 3: Fungicidal (+) and Fungistatic (-) effects of EAF of stem barks from*Boswellidalzielii.*

Microorganisms	MIC (µg/ml) EAF of *Boswellidalzielii*	MFC (µg/ml) EAF of *Boswellidalzielii*	Effect
Candida albicans ATCC 9002	7.29±4.77[b]	20.83±7.22[b]	+
Candida albicans ATCC 2091	6.25±0.00[a]	12.50±0.00[a]	+
Candida parapsilosis ATCC 22019	10.42±3.61[d]	50.00±0.00[c]	-
Candida krusei ATCC 6258	8.33±3.61[c]	12.50±0.00[a]	+
Candida tropicalis ATCC 750	11.46±11.83[e]	50.00±0.00[c]	-

The results are the means of number of the colonies ± standard deviations.

+: fungicidaleffect, **-** : fungistaticeffect

The MIC values of EAF were ranged from 6.25 to 12.5 g/ml.

Table 4: Minimal Inhibitory Concentration (MIC) of combination of EAF of stem barks from*Boswellidalzielii*and antifungal references (Nystatin).

Microorganisms	MIC (µg/ml) combination of EAF+ Nystatin
Candida albicans ATCC 9002	0.520±0.23[b]
Candida albicans ATCC 2091	0.390±0.00[a]
Candida parapsilosis ATCC 22019	1.04±0.45[d]
Candida krusei ATCC 6258	0.78±0.00[c]
Candida tropicalis ATCC 750	1.82±1.19[e]

Values are Mean ±SD (n=3). Different letters in the same column indicate significant difference (P<0.05) for EAF.

Evaluation of the fractional inhibitory concentration index of fraction

For FICI, our results indicate a synergistic effect betweenEAF and the antifungal references (Nystatin) (Table 5).

Table 5: Fractional Inhibitory Concentration (FIC) and FICI of combination of EAF of stem bark from *Boswellidalzielii*with antifungal references (Nystatin).

Microorganisms	FICa	FICb	FICI	Effect
Candida albicans ATCC 9002	0.07	0.14	0.21	Synergistic
Candida albicans ATCC 2091	0.06	0.12	0.18	Synergistic
Candida parapsilosis ATCC 22019	0.09	0.25	0.34	Synergistic
Candida krusei ATCC 6258	0.09	0.21	0.30	Synergistic
Candida tropicalis ATCC 750	0.16	0.41	0.57	Additive

FICa= MIC of EAF in combination/MICa alone;

FICb = MIC of the antifungalagent in combination/ MICb alone, and FICI= FICa + FICb.

a= EAF compounds; b= Nystatin.

The FICI was interpreted as follows

1. A synergistic effect when FICI $\leq$0.5;
2. An additive or indifferent effect when FICI >0.5 and <1;
3. Anantagonistic effect when >1.

DISCUSSION

The World Health Organization,[17] has estimated that between the years 2000 and 2020 nearly one billion people will be infected and more than 200 million will develop the disease. Therefore, it is of great interest to carry out a screening of these plants in order to validate their use in folk medicine and to reveal the active principle by isolation and characterization of their constituents.

Infectious diseases caused by fungi are still a major threat to public health, despite numerous efforts by researchers. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance.[18] Use of ethnopharmacological knowledge is one attractive way to reduce empiricism and enhance the probability of success in new drug-finding efforts.[19] Validation and selection of primary screening assays are pivotal to guarantee sound selection of extracts or molecules with relevant pharmacological action and worthy following up.[20] The number of multi-drug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing.The high prevalence and severity of infections caused by yeasts of the genus *Candida*, which can cause significant morbidity and mortality in affected patients, has encouraged investigations to elucidate new therapeutic approaches to treat candidiasis.[21] Because the oral cavity includes *Candida* spp. in its normal microbiota

and because this disease is considered an opportunistic infection, is an important site of disease development. This study used the *Candida* species usually identified in oral fungal infections. *C. albicans* is present in the oral cavities of approximately 30 to 50 % of people. It is among the most virulent species,[22] it is able to produce phospholipases and proteases that can destroy host tissues,[23] and it expresses genes that are responsible for the cellular responses involved in invasive growth, cell wall formation, adaptation to osmotic stress and resistance to currently available antifungal agents in the form of efflux pumps,[24,25] changes in the drugs' site of action,[26] and changes in the lipid composition of the fungal plasma membrane, which prevent the drugs 'inflow into the cell.[27] *C. tropicalis* and *C. krusei* are also important pathogensthat contribute to the development of the disease. Strains that are resistant to available therapeutic agents have begun to appear, and there has been an increasing number of immunocompromised people and increasingly frequent use of antifungal agents for treatmentand/or prophylaxis,[28,29] The elucidation of the action mechanisms of agentswith pharmacological potential, whether of natural orsynthetic origin, contributes to the development of rationaltherapeutic approaches, particularly in terms ofinfections caused by resistant microorganisms, whichfrequently require combinations of drugs or the use ofnew drugs when the first-choice agent is not effective.

Natural products with intrinsic antimicrobial activity or products that promote the activity of commonly used antibiotic/antifungal agents may represent new ways to combat multi-resistant microorganisms and prevent the contact of these microorganisms with synthetic products, thus reducing the risk of selecting new or improved resistance mechanisms.[30] Natural products may also be combined with traditional antimicrobials to enhance the antimicrobial activity of both.[31] According to the literature, there are scientific evidences for the use of nystatin in the treatment of fungal infections of the oral mucosa[32] and it has been indicated, with advantages over the other antifungals, such as topical use and less side effects. However, fungal resistance has been reported[33] and the association between synthetic and natural antifungal is an alternative to reducing the dose required for the effect and may reduce undesired side effects and prevent the development of resistance to antifungals.[34]

To promote greater efficiency of EAF and nystatin when used at lower concentrations, the association of these substances was proposed, and synergistic effect was observed. There are several mechanisms involved in the synergistic activity of antifungal agents such as the inhibition of different stages in the fungal intracellular pathways that are essential for cell

survival; increased penetration of one antifungal agent resulting from the action of another antifungal agent on the fungal cell membrane; the inhibition of carrier proteins; and the simultaneous inhibition of different cell targets.[35] It is noteworthy that this is the first study that reports the synergistic effect of combined EAF of *Boswellidalzielii*and nystatin and its potential use to treat superficial infections in the oral mucosa caused by *Candida* species. Therefore, the findings described here encourage the development of clinical trials to evaluate the efficacy of this combinationtreatment.

CONCLUSION: Theresults shows a fungicidal effect on *Candida* species and a synergistic effect when combined with nystatin and the study supported the utilization of this Burseraceae in treatment of *candida* infections.

CONFLICTS OF INTEREST

The author (s) declare that they have no competing interests.

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