

Evaluation and Optimization of Antifungal Activity of Active Components of Extracts of *Eucalyptus sp*, on the *In vitro* Growth of *Candida tropicalis*

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ABSTRACT

Infectious diseases continue to be an important public health problem. Among them, candidiasis an opportunist fungal infection especially in patients living with HIV / AIDS is on the increase. Our research team tested different plant extracts (butanolic, hexane, ethyl acetate, chloroform) of crude extract hydro-ethanolic obtained from *Eucalyptus sp* on the *in vitro* growth of *Candida tropicalis*.

Among the 10 extracts tested, X_{1,2} has the highest inhibitory activity (MFC = 125 µg/mL; IC₅₀ = 09.91 µg/mL). The method of preparation of the extract (X_{1,2}) by partitioning of hydro-ethanolic extract in different solvent mixtures is the best way to obtain the best active principle concentration.

Keywords: Hydro-ethanolic extract, *Candida tropicalis*, *Eucalyptus*.

INTRODUCTION

In developing countries, infectious diseases constitute major public health problem due to their frequency and severity¹. Indeed, they are the cause of more than 17 million deaths yearly worldwide of which more than half comes from Africa alone².

This situation has worsened especially with the advent of HIV pandemia that is very difficult to eradicate and which generates various opportunistic infections like bacterial, viral diseases and mycoses due to immune deficiency³. Of these infections, fungal infections have taken a

considerable proportions^{4,5} and those caused by *Candida* are the most common in human especially in patients living with HIV / AIDS⁶⁻⁸.

Despite the damage caused by harmful microorganisms, the scientific world has developed many anti-infective treatments. These remedies have helped reduce the incidence of infectious diseases especially in developed countries⁹. But these remedies remain inaccessible to people in poor areas due to the expensive cost of drugs. This precarious situation associated with the emergence of multidrug-resistant strains, leads the financially handicapped population to use the pharmacopoeia for their treatment needs¹⁰⁻¹².

To help this population cope with this situation, our research team is assessing the scientific basis and the effectiveness of these therapeutic plants and isolate the active principles for the development of more effective and cheaper new drugs. Ethnobotanical surveys have resulted in the selection of several plants widely used by traditional healers for their anti infectious properties.

The objective of our study therefore is to improve the anticandidosic activity of hydro-ethanolic extracts from *Eucalyptus sp* on the *in vitro* growth of *Candida tropicalis*¹³.

MATERIAL AND METHODES

Material

Biologic material

The material used was some powder obtained from the bark of *Eucalyptus sp* coded EUCA. This plant has been identified by the National Floristic Center of University of Félix Houphouët-Boigny (Côte d'Ivoire, Abidjan-Cocody).

Tested Germs

The germ used in this study is *Candida tropicalis*. It was provided by the Mycology and Parasitology laboratory of the Pasteur Institute in Abidjan (Côte d'Ivoire). This microorganism was isolated from a patient with recurrent vaginal discharge.

Culture medium

Sabouraud agar medium was used for germs culture (Bio-RAD/Réf: 64449, Lot: 8B2212) buffered to acid pH (5.7). A suitable and commonly used culture medium for growing fungal germs.

Methods

Preparation of extracts

Pieces of bark of *Eucalyptus sp* was cut, collected and dried under shade, then finely ground using an electric grinder. From the obtained powder, crude hydro-ethanolic extract was prepared. Thus: 100 g of EUCA were extracted by homogenization in blender with one liter (1 L) a solvent mixture of ethanol-water 70/30, v/v. After six cycles of crushing, the homogenate obtained was first filtered in a square of fabric and then filtered twice on absorbent cotton and once on Wattman filter paper 3 mm successively. The filtrate obtained was evaporated to dryness. We have obtained hydro-ethanolic extract coded X₀.

Then, 4 portions of 10 g each of X₀ are subjected to a liquid/liquid partition in 300 mL of 4 different mixtures solvents (hexane-water, ethyl acetate-water, Butanol-water and chloroform-water; v/v, 50/50). After settling, the various phases were separated and concentrated under vacuum. The following extracts were obtained:

X_{1,1}: the hexane phase, X_{1,2}: the aqueous phase from the hexane-water partition, X_{2,1}: the ethyl acetate phase, X_{2,2}: the aqueous phase from the ethyl acetate-water partition, X_{3,1}: the butanol phase, X_{3,2}: the aqueous phase from the butanol-water

partition, X_{4,1}: the chloroform phase and X_{4,2} the aqueous phase from the chloroform-water partition.

Preparation of culture medium

Sabouraud agar medium was prepared according to the manufacturer's instructions. Thus 42 g of the medium powder were homogenized in 1000 mL of distilled water (for the purpose of manipulation, we had collected the corresponding amount). This mixture was stirred and heated on a heating magnetic stirrer.

Incorporation of plant extracts

The incorporation of various plant extracts into Sabouraud agar was done following the method of double dilution in slope tubes¹⁴⁻¹⁶. For each plant extract, each series consists of 10 test tubes, 8 of these tests tubes contained plant extracts, and the other 2 tubes are considered control tubes one without plant extract and germs-free served as control of the sterility of the culture medium and the other without plant extract used as a control for the growth of germs. For the 8 test tubes concentrations ranging from 2,000 to 15.62 µg/mL binding by geometrical reason of 1/2.

After incorporation of the extracts, all 10 tubes of each extract were removed by the use of forceps sterilized by flaming at 121 °C for 15 minutes and then inclined to room of the laboratory to cooling and solidification of the agar^{16,17}.

Antimicrobial test

A young colony of *Candida tropicalis* taken with a handle was homogenized in 10 mL of sterilized distilled water. This gives the suspension (10⁹) concentrated to 10⁶ cells/mL. From this suspension, a second suspension (10⁻¹) was prepared by dilution of the first solution by

1/10th. This brings the concentration to 10⁵ cells/mL.

For each of the test tubes of each series of 9 extracts (except the control tube of sterility of the culture medium) the germs culture was done on the agar slant previously prepared culture medium by seeding 10 mL of the 10⁻¹ suspension in transverse striations until exhaustion. This corresponds to 1,000 cells. The cultures thus produced were incubated at 30 °C for 48 hours.

After incubation, the colonies of *Candida tropicalis* were counted with a pen colony (N^o 23382Scinceware Bel-Art serial). Growth in eight (8) experimental tubes of each series was evaluated as a percentage of survival calculated relative to 100 % survival in the control tube growth¹⁵⁻¹⁷.

The analysis of experimental data was used to determine the following antifungal parameters:

- The Minimum Inhibitory Concentration (MIC) is the lowest concentration for which there is no growth visible to the naked eyes.
- The Minimum Fungicide Concentration (MFC) is the minimum concentration of extract in the tube which gave 99.99 % inhibition compared to the control tube growth control tube or the concentration which allows a survival of 0.01 % relative to the growth control tube. It is determined by a sterility test of the tube corresponding to the MIC by inoculating a sample taken from the agar surface of the tube into a new agar.
- The concentration for 50% inhibition (IC₅₀) is the concentration that gave 50 % inhibition estimated relative to the number of counted colonies in the control tube growth control tube. This parameter is determined graphically.

RESULTS AND DISCUSSION

After 48 hours of incubation at 30 °C we observed compared to the control tube, a gradual decrease in the number of colonies gradually as the concentration of plant extract in the test tube increases. This was observed for all series of 9 extracts. These results are statistical averages of 6 experiments for each extract.

Moreover, clear and effective inhibitions were obtained at different concentrations depending on the extracts. Experimental data represented as activities curves are presented in Figure 1 for the crude extracts (X_{Aq} and X_0)¹³ and Figure 2 for extracts partitioned.

In general, all activity curves have a decreasing pace with slopes greater or lesser degree depending on extracts. Regarding the crude extracts, the activity curve of the X_0 extract has a stronger slope than X_{Aq} extract (Figure 1)¹³.

In Figure 2, the lowest slope was obtained with X_{1-1} , average slopes were obtained with X_{2-1} , X_{2-2} and X_{4-1} , X_{3-1} extract. The steeper slopes were obtained with X_{1-2} , X_{3-2} and X_{4-2} extracts, in particular that of the X_{1-2} extract. The values of the MFC and IC_{50} for all the extracts are shown in Table I.

The analysis of results obtained show that the extracts are active on the isolated fungal studied in a dose response relationship. Indeed, we observed a progressive decrease in the number of colonies as the concentration of extracts in the experimental tubes increases.

The comparison of crude extracts based on the values of MFC shows that the X_0 extract (MFC = 250 µg/mL) has a better activity than the X_{Aq} (MFC = 500 µg/mL) extract. It is 2 times more active¹³.

Comparing our results to those of Kporou *et al.*, 2009¹⁸ reveals that the hydro-ethanolic extract of EUCA are significantly more active than the MISCA- X_0 (MFC =

6250 µg/mL). It is 200 times on the same isolate. In addition, this improved performance was also observed by comparing the activity of hydro-ethanolic extract of EUCA to activity of hydro-ethanolic extract of *Salvia tigrina* 50 % tested by Dulger and Hacıoglu in 2008¹⁹ on *Candida tropicalis*. Indeed these authors obtained with leaves and roots' bark of this plant, the respective values of MFC 25000 µg/ml and 12500 µg/ml on *Candida tropicalis*. The X_0 extract of EUCA is 100 and 50 times more active.

Regarding the partitioning, no resistance of fungal germs was observed. Moreover, this activity varies from one extract to another. The most active extract X_{1-2} is 16 times more active than X_{1-1} , 8 times more active than X_{2-1} and X_{4-1} and 4 times more active than X_{Aq} and 2 times more active than X_0 (basic extract from which it was prepared) and X_{3-1} .

Furthermore, the activity of X_{1-2} is also better than that obtained by Kporou *et al.*, 2009¹⁸ with hexane extract X_{1-1} (MFC= 3125µg/mL) on the same fungal germs. The same for Fézan *et al.*, 2007²⁰ that obtained with the dichloromethane and methanolic extract 80 % of *Erigeron floribundus* MFC values of 500 µg/mL and > 2000 µg/mL respectively. The X_{1-2} extract is 4 times more active than dichloromethane and more than 16 times than methanolic extract 80 %.

This interesting activity of the extracts of *Eucalyptus sp* confirms the antimicrobial activity that this plant of the genus *Eucalyptus* possesses. Indeed, Oyedeji *et al.*, in 1999²¹ showed significant antibacterial activity (gram-positive and gram-negative) and fungal (*Candida albicans*) of essential oils from five varieties of *Eucalyptus* in Nigeria. Similar result was obtained by the work of Elaissi *et al.*, in 2011²² that tested the essential oil of *Eucalyptus odorata* on the *in vitro* growth of *Staphylococcus aureus*. According to Bashir

et al., 2012²³, the *Eucalyptus globulus* essential oil has an effect on *Escherichia coli* and *Staphylococcus aureus*. The works of Javad and Atefeh, 2010²⁴, they have demonstrated the antimicrobial activity of essential oils and methanolic extracts of *Eucalyptus largiflorens* and *Eucalyptus intertexta*.

CONCLUSIONS

This study elaborates the real anti-infective potential of *Eucalyptus sp.* The results of these investigations have led us to understand that the extracts of this plant have antifungal activity more or less pronounced on the *in vitro* growth of *Candida tropicalis*.

The method using in this study, gave us the most active extract to be X_{1,2} (MFC = 125 µg/ml, IC₅₀ = 09.91 µg/ml) whose anticandidotic activity is relatively high.

Therefore from our observation and results obtained we can then conclude that from a solvent mixture of ethanol-water (70/30, v / v) followed by a partition in a solvent mixture of hexane-water is the best method to employ to better concentrate the active components of EUCA. Thus, the use of this plant as antimicrobial in traditional medicine is justified.

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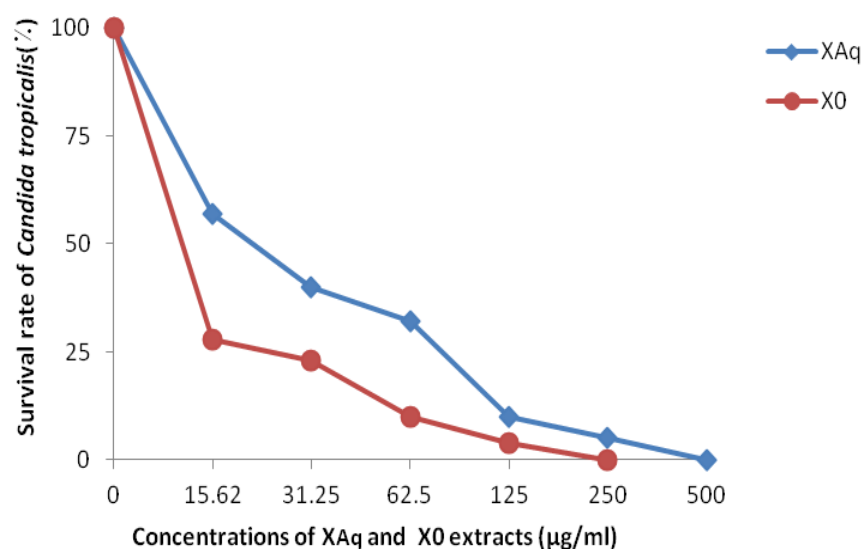
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Table 1. Values of antifungal parameter of different extracts of EUCA

Different extracts of EUCA	Antifungal parameter	
	CI ₅₀ µg/mL	MFC µg/mL
X _{Aq}	21.30	500
X ₀	11.15	250
X ₁₋₁	98.21	2000
X ₁₋₂	09.91	125
X ₂₋₁	19.52	1000
X ₂₋₂	12.35	500
X ₃₋₁	10.62	250
X ₃₋₂	11.51	125
X ₄₋₁	14.75	1000
X ₄₋₂	11.71	125

**Figure 1.** Activity curves of crude extracts X_{Aq} and X₀ on *in vitro* growth of *Candida tropicalis*¹³

