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Antifungal Activities of *Erythrina senegalensis* Leaves Partitioned Extracts on the Germs Responsible for Opportunistic Cryptococcosis of HIV/AIDS

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Abstract | **Original Research Article**

Introduction: The mortality rate remains very high (45.1%) among HIV-infected subjects with cryptococcosis in developing countries due to the inaccessibility and toxicity of conventional antifungals. Thus, the alternative search for new, more effective and less expensive antifungal molecules required this study on *Erythrina Senegalensis*, a plant used against mycoses in ivorian traditional medicine. ***Methodology:*** The double dilution method in liquid medium and the diffusion method in solid medium were used for the antifungal tests of partitions of the of *Erythrina senegalensis* leaves hydroalcoholic extract, in comparison with conventional antifungals. ***Results:*** Among the partitions of *Erythrina senegalensis* leaves hydroalcoholic extract obtained and tested, the partition with hexane was the most active with an MIC of 3.12 mg/ml, an IC50 of 1.55 mg/ml. Having obtained an inhibition diameter of 15 mm, this hexane partition containing flavonoids, sterols and terpenes has antifungal activity on *Cryptococcus neoformans* in the same way as amphotericin B and Fluconazole. ***Conclusion:*** Thanks to its fungistatic action, the *Erythrina senegalensis* leaves hexane partition has a fungistatic action on HIV/AIDS opportunistic cryptococcosis.

Keywords: *Erythrina senegalensis*, hydroalcoolic, HIV/AIDS, cryptococcosis, flavonoids.

INTRODUCTION

Cryptococcoses are mycoses caused by *Cryptococcus neoformans*, encapsulated yeast-type fungi which mainly infect people with weakened immune systems [1]. Having become the main factor favoring these candidiasis, HIV infection has modified the epidemiology of these opportunistic diseases. In sub-Saharan Africa, cryptococcosis, which remains the major cause of opportunistic mycoses, ranks fourth in deaths due to infectious diseases [2, 3]. This very high mortality rate among people infected with HIV in developing countries is due to the inaccessibility of conventional antifungals to populations given their very high cost. Also, some studies have shown the renal and hematological toxicity of Amphotericin B [4]. To resolve this problem of managing cryptococcosis in HIV-infected subjects, alternative research into new, more effective and less expensive molecules in our floristic heritage is necessary. Hence this interest in *Erythrina senegalensis*, a plant used in ivorian traditional medicine against cryptococcosis. Called "Kinjê" by the AKAN ethnic group of Côte d'Ivoire, *Erythrina senegalensis* is a thorny shrub 6 to 7 m high which is very widespread in West Africa [5, 6].

The present study, which is part of the search for a more effective and suitable antifungal phytomolecule, is a contribution to the fight against cryptococcosis in HIV subjects infected. It will consist of determining the antifungal activity of partitions derived from *Erythrina senegalensis* leaves hydroalcoholic extract, on *Cryptococcus neoformans* in comparison with conventional antifungals regularly prescribed to HIV patients infected. It will also involve carrying out a phytochemical screening in order to identify the chemical groups responsible for these antifungal activities.

MATERIAL

Plant material

Plant material consists of *Erythrina senegalensis* (Fabaceae) leaves located in NAWA region of Côte d'Ivoire southwest whose geographical coordinates are: 5°47′08″ North - 6°36 ′29″ West,

Altitude: 134 meters. This plant was authenticated at the national floristic center of Cocody Félix Houphouët Boigny University in Côte d'Ivoire (Figure 1).

Figure 1: Photographie de feuilles de *Erythrina senegalensis* (Photo Coulibaly, 2016)

Biological material

The fungal pathogen used in this study is *Cryptococcus neoformans* isolate MY18-20739/18299. This strain was isolated from samples from HIV/AIDS infected patients in the mycology laboratory of Côte d'ivoire Pasteur Institute. It was grown in a culture medium consisting of Sabouraud Chloramphenicol agar.

METHODS

1. Preparation of the total hydroalcoholic extract [7]

One hundred (100) grams of *Erythrina senegalensis* leaf powder were dissolved in 500 ml of a 70% ethanol solution then homogenized for 24 hours at room temperature, using the IKA-MAG magnetic stirrer. After decantation, the supernatant was collected, filtered twice through hydrophilic cotton then through 3 mm diameter Wathman paper. The filtrate obtained was evaporated using a BUCHI rotary evaporator. The resulting paste was dried in an oven at 60°C until a dry powder was obtained which constituted the total hydroalcoholic extract.

2. Partitioning of the total hydroalcoholic extract [8, 10].

The hydroalcoholic extract obtained was introduced into a separatory funnel and exhausted three times with 150 mL of hexane. After decantation, the hexane phase was recovered, dried over magnesium sulfate then filtered using 3 mm diameter WATTMAN paper. The hexane was removed using the BUCHI rotary evaporator and the resulting hexane partition (Fhex) powder was stored until use in a dark jar in the EXPRESSCOOL refrigerator at -4°C. The same operation was successively carried out with dichloromethane (Fdcm) and ethyl acetate (Face). At the end, the aqueous partition was evaporated then dried in a MEMMERT type oven at 60°C.

3. Preparation of the fungal inoculum

Using a platinum loop, a *Cryptococcus neoformans* young colony was collected which was homogenized in 10 ml of sterile distilled water in order to obtain the mother suspension (10^0) concentrated at 10^6 cells/ml. From the suspension (10^0), a second suspension (10^{-1}) is prepared by diluting 1/10 of the first in order to obtain a suspension of 10^5 cells/ml which constituted the fungal inoculum.

4. Carrying out evaluation tests

The evaluation tests make it possible to determine the antifungal parameters using the double dilution method in an inclined tube [7, 9].

For each of the partitions of the ETHA extract from *Erythrina senegalensis* leaves, a series of 12 test tubes was prepared. It included 10 test tubes and 2 control tubes, one of which without plant partition constituting the growth control of the fungal pathogen and the other without plant partition and without fungal pathogen serving as the agar medium sterility control. Thus, 2g of plant partition were homogenized in 20 ml of liquid Sabouraud chloramphenicol agar at 40°C in T1 tube in order to obtain a concentration of 100 mg/ml in this tube. Half of this T1 tube was transferred to a T2 tube containing 10 ml of agar in order to obtain a concentration of 50 mg/ml in this T2 tube. This operation is repeated successively for the other tubes until T10 tube which obtained the lowest concentration of 0.195 mg/ml. The 10 test tubes therefore made it possible to obtain a range of decreasing concentrations of plant partitions

ranging from 100 mg/ml to 0.195 mg/ml according to a geometric connection of ratio 1/2. After sterilization in an autoclave at 121°C for 15 minutes, the test tubes obtained were tilted at room temperature to allow cooling and solidification of the agar containing the partitions of *Erythrina senegalensis* leaves ETHA extract. . The prepared fungal inoculum (10^5 cells/ml) is used to inoculate the 12 prepared test tubes except the sterility control control. The test tubes were then incubated at 37°C for 48 hours and then the colonies present in each tube were counted by direct counting (the tests were repeated 3 times).

The growth of the germ in each test tube is expressed as a percentage of survival (S) and calculated according to the following formula:

S= n / N x 100

Where,

S= % survival
n= number of colonies in the test tube
N= number of colonies in the control tube

The processing of these experimental data makes it possible to determine the antifungal parameters which are the MIC, IC50 and MFC:

- MIC or minimum inhibitory concentration is the minimum concentration for which there is no growth visible to the naked eye.
- IC50 or 50% inhibitory concentration is the concentration which corresponds to 50% inhibition. It is determined graphically from the sensitivity curve.
- MFC or minimum fungicide concentration is the lowest concentration above which there is no resumption of fungal growth. It is determined from a subculture carried out after 48 hours of incubation on new agar from tubes in which no growth was observed.

5. Carrying out efficiency tests

The antifungal activity of the partitions was confirmed by the diffusion method in agar medium using discs soaked in plant partitions or reference antifungals described by Traore *et al*., (2012) [10]. The principle of this method is based on the diffusion from a disk of the active antifungal substance into the agar containing the fungal germ with creation of a concentration gradient.

Sterilized discs of 6 mm diameter cut from Wathman paper are placed in the partitions of *Erythrina senegalensis* leaves ETHA extract for 1 hour. The prepared fungal inoculum (10^5 cells/ml) is spread on the surface of the Sabouraud-chloramphenicol agar poured into a sterilized Petri dish then dried at 37°C for 5 min. In each inoculated petri dish, the discs thus soaked with plant partitions are placed next to discs soaked with Amphotericin B and Fluconazole and 5-Fluorocytosine, which are left to incubate at 37°C for 48 hours. Around each disk containing a partition or a reference antifungal, inhibition diameters are observed and measured (the test was repeated 3 times for each partition).

6. Phytochemical screening

Phytochemical screening was carried out with the aim of detecting the major chemical groups contained in *Erythrina Senegalensis* leaves total extracts.

RESULTS

1. Antifungal activity of partitions derived from *Erythrina senegalensis* leaves hydroalcoholic extract

After 48 hours of incubation at 37°C, the growth of *Cryptococcus neoformans* colonies in the test tubes, expressed as a percentage of survival (S), is translated in the form of sensitivity curves presented in Figure 2.

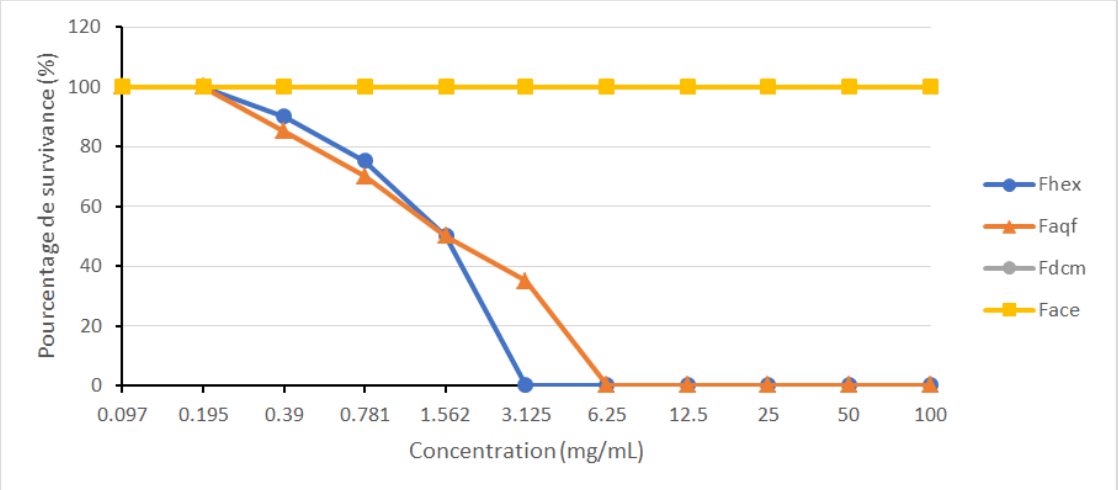

Fig 2: Sensitivity curves of partitions from the hydroalcoholic extract of *Erythrina senegalensis* leaves against *Cryptococcus neoformans*

The MIC of the Fhex and Faqf partitions of *Erythrina senegalensis* are respectively 3.12 mg/mL and 6.25 mg/mL on *Candida albicans* while the Fdcm and Face partitions of the same plant do not present any antifungal activity on *Cryptococcus neoformans* which maintains 100% survival.

MFC was investigated experimentally and none of the new concentrations applied was able to prevent the reappearance of colonies after 48 hours of reincubation. The Fhex and Faqf partitions of *Erythrina senegalensis* therefore have a fungistatic effect on *Cryptococcus neoformans* strains.

2. Efficacy of active partitions of *Erythrina senegalensis* on strains of *Cryptococcus neoformans*

The evaluation of the effectiveness of the partitions resulting from *Erythrina senegalensis* leaves hydroalcoholic extract made it possible to obtain in the petri dishes, inhibition zones diameters of *Cryptococcus neoformans growth*, expressed by the histogram of Figure 3.

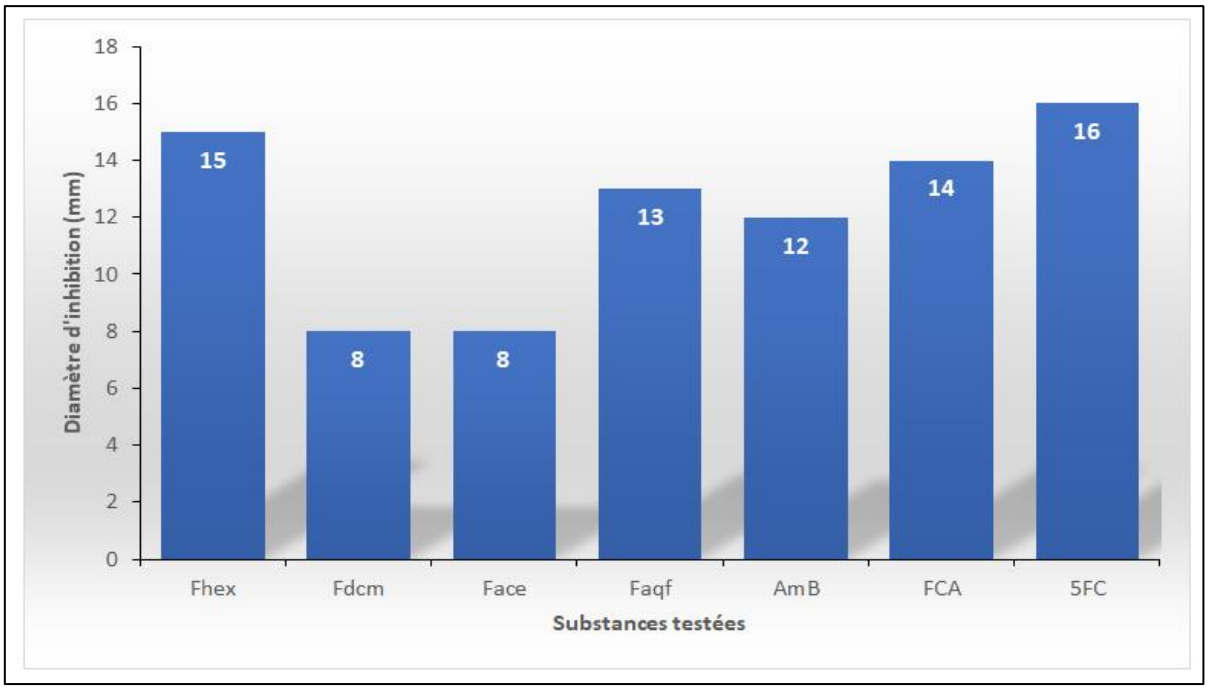

Fig 3: Effectiveness of partitions derived from the hydroalcoholic extract of *Erythrina senegalensis* and reference antifungals on *Cryptococcus neoformans*

The inhibition diameter of Fhex, Faqf partitions and antifungals AmB, FCA and 5FC which are respectively 15 mm, 13 mm, 12 mm, 14 mm and 16 mm, are greater than the sensitivity threshold of 10 mm unlike Fdcm partitions and Face which have an inhibition diameter of 8 mm.

3. Phytocompounds of *Erythrina senegalensis* leaves hydroalcoholic extract

The phytochemical screening made it possible to detect in the partitions of *Erythrina senegalensis* leaves hydroalcoholic extract, the different families of secondary metabolites present. The results obtained are presented in Table I.

Table I: Families of secondary metabolites contained in the partitions from hydroalcoholic extracts of *Erythrina senegalensis* leaves

Familles de métabolites secondaires	Partitions			
	Fhex	Fdcm	Face	Faqf
Stérols et Terpènes	+	-	-	+
Alcaloïdes	-	-	-	-
Polyphénols	+	+	-	-
Flavonoïdes	+	-	-	+
Anthocyanes	-	-	-	-
Tanins	-	-	-	-
Quinones	-	-	-	-

NB: (+): presence (-): absence Fhex: Partition with hexane, Fdcm: Partition with dichloromethane, Face: Partition with ethyl acetate, Faqf: Final aqueous partition

The partitions of *Erythrina senegalensis* richest in families of chemical groups are the partitions Fhex and Faqf which have sterols and terpenes, as well as flavonoids, in common. The Fhex partition also contains polyphenols. The Fdcm and Face partitions are the poorest in secondary metabolite families. Also none of the scores contain alkaloids, tannins, quinones and anthocyanins as was the case for the hydrolcoholic extract.

DISCUSSION

The objective of this work was to search in *Erythrina senegalensis* leaves extracts for antifungal substances of the same effectiveness as the reference antifungals against the germs responsible for opportunistic cryptococcosis of HIV/AIDS.

Among the partitions derived from *Erythrina senegalensis* hydroalcoholic extract, only Fhex and Faqf partitions resulted in a clear and effective inhibition of *Cryptococcus neoformans* with respective MICs of 3.125 and 6.25 mg/mL. These responses are justified by the principle of successive partitioning which consisted of using several solvents of different polarities in a precise order to extract all the extractable compounds solvent after solvent. The order of increasing polarity of the solvents being hexane, dichloromethane, ethyl acetate and water, the bioactive compounds are isolated and concentrated progressively according to their polarity, during partitioning [8, 10]. Thus the fungistatic activity of the Fhex partition of *Erythrina senegalensis* would be due to the content of this plant in lipid compounds, notably sterols and polyterpenes [11].

(a)

(b)

Efficacy tests carried out on a strain of *Cryptococcus neoformans* made it possible to compare the antifungal activity of the hexane partition of *Erythrina senegalensis* leaves with that of antifungals regularly used for the treatment of cryptococcosis in subjects VIH infected.

On the *Cryptococcus neoformans* strain, the inhibition diameters of the Fhex partition and the antifungals tested, which vary from 12 to 16 mm, are greater than the sensitivity threshold of 10 mm. This confirms that the Fhex partition and the antifungals tested have antifungal activity on *Cryptococcus neoformans* because according to Biyiti *et al*., (2004) [12] a substance is said to be active on a germ when its inhibition diameter is greater than or equal to 10 mm. Thus, we deduce that *Erythrina senegalensis* Fhex partition is effective on the *Cryptococcus neoformans* strain in the same way as Amphotericin B, Fluconazole and 5-Fluorocytosine.

The phytochemical screening revealed in the Fhex partition which is the most active of *Erythrina senegalensis* leaves hydroalcoholic extract, the common presence of flavonoids, sterols and terpenes. These results are in agreement with the work of Ligor *et al*., (2018) [13] for whom hexane is one of the most used solvents for the extraction of polyphenols (flavonoids) and polyterpenes (sterols and terpenes). According to Owoseni *et al*., (2010) [14], these families of secondary metabolites are well known for their antimicrobial activities. Indeed, thanks to their free hydroxyl groups, the flavonoids and sterols and terpenes of *Erythrina senegalensis* Fhex partition could lead to the death of fungal pathogens through the destruction of cell membranes following a total loss of homeostasis [15, 16].

We deduce that hexane partition of *Erythrina senegalensis* leaves hydroalcoholic extract has a fungistatic activity on strains of *Cryptococcus neoformans* as interesting as that of amphotericin B and fluconazole which are the antifungals commonly prescribed to AIDS patients suffering from cryptococcosis.

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Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Author Contributions

CO: Ethnobotanical surveys, antifungal tests, final writing
GKRC: Extractions and fractionations of leaf extracts
SPL: Analysis and interpretation of results
OK: Establishment of experimental protocols

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