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Botanical study, comparison of antifungal activity leaves of *Aspilia africana* (Pers.) CD Adams var. *africana* and *Acanthospermum hispidum* DC. on the *in vitro* growth of *Cryptococcus neoformans* and study of their toxicity on human cells HFF

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ABSTRACT: An ethnobotanical survey conducted in the Autonomous District of Abidjan (Côte d'Ivoire), showed that *Aspilia africana* and *Acanthospermum hispidum* are two species mentioned recurrent way in several therapeutic prescriptions.
To facilitate the recognition of these two species and to identify them on the ground, this study was conducted and allowed to do their botanical description followed by an exploration of their antifungal activity.
Those two Asteraceae are known to be crops rainy weeds. *A. hispidum* is an erected annual herb with several ramifications. This plant is remarkable for its hispide nature and especially by its very spiny achenes. As for *A. africana*, it is a hardy and untidy herb, to stem steep, with rigid stalk and several ramifications and rough on touch. It is dinstinguished for its bright yellow florets and its quadrangular achene covered with hard and fine hairs. Both plants have antifungal activity *in vitro* against *Cryptococcus neoformans*. Among the tested extracts, the FE70 % extracts (FE70 $\%_{Aa}$ and FE70 $\%_{Ah}$) showed very good activities. The FE70 $\%_{Ah}$ extract is the most active with CMF = 12500 µg/mL and an IC_{50} = 1280 µg/mL. At this concentration, *A. hispidum* is not toxic to human cells HFF unlike *A. africana* which shows a very high toxicity.

KEYWORDS: *Aspilia africana*, *Acanthospermum hispidum*, *Cryptococcus neoformans*, antifungal activity, human cells HFF, toxicity.

1 INTRODUCTION

With the advent of HIV/AIDS, there is an excessive progression of many diseases called opportunistic. Of these infections, candidiasis, dermatophytes, pneumocystoses, aspergillosis and cryptococcosis are fungal infections in very strong upsurges [1]. These infections have nowadays become a real public health problem. Despite considerable progress made in last years in the treatment of invasive fungal infections, thanks to the therapeutic arsenal, these diseases are increasingly difficult to eradicate [2], [3].

In addition, Renal toxicity presented by some modern drugs of references, proved inefficiency of other drugs responding to these diseases and the inaccessibility of these drugs in our population due to their high costs, are for developing country a real concern [3].

In response to this concern like worldwide researchers, our laboratories were oriented towards new therapeutic research, based on plants that would be effective against fungal infections, cheaper and accessible to all. So after ethnobotanical survey, plant species *Aspilia africana* and *Acanthospermum hispidum*, two medicinal Asteraceae, were

selected to evaluate their antifungal activity. Otherwise, note that many pharmacological, phytochemical and toxicological researches had been conducted by researchers to verify and justify the traditional use of these two plants. Thus *A. hispidum* has been scientifically studied for its antimicrobial properties ([4], [5], [6]), abortive and teratogenic ([7], [8]), antimalarial ([9], [10], [11]), immunostimulatory [12], antitrypanosoma, antileishmania, antitrichomonal ([11], [13], [14]). It was reported that *A. hispidum* contains certain chemical compounds such as lactones and sesquiterpenes ([15], [16]). Glycosides and flavonoids have been reported to be inside the aerial part of the plant [17]. It is the same for *A. africana* which has been studied for its antimicrobial activities ([18]), anti-ulcer ([19]), anti- inflammatory ([20]), parasitic ([21], [22], [23]), reproductive ([24]). The chemical test of this plant ([18], [25]) and its toxicologic one ([26], [27]) had been done. Besides to this study, our research team take great interest in antifungal properties and the selected biological target is *Cryptococcus neoformans*. It is an encapsulate and mucilaginous yeast. It is an opportunist germ, very virulent in case of waning immunity, especially in patients with HIV/AIDS ([1]). It can lead to skin, systemic and neuromeningeal infections and cryptococcal septicemia. *C. neoformans* is also known for his resistance of modern drugs (amphotericin B, terbinafine, fluconazole,...) [28]. These reasons justify the choice of this fungal germ to evaluate the extracts antifungal potency of these two plants.

This article has two objectives :

- make a complete botanical description of these two plants to facilitate their identification on the ground;
- make an antifungal exploration extracts of the leaves of both weeds and assess their toxicity.

2 MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 VEGETAL MATERIAL

The plant material is made from the leaves of *Aspilia africana* (Pers.) C.D. Adams var *africana* and of *Acanthospermum hispidum* DC., two Asteraceae collected in the autonomous district of Abidjan in August 2014. Their identification was performed at the National Floristic Centre (CNF) from the University Félix Houphouët-Boigny Abidjan-Cocody where samples are preserved.

2.1.2 CULTURE MEDIUM

Sabouraud medium (HIMEDIA/Ref : M1067-500G Lot 0000215703) was used for the culture of fungal germ.

2.1.3 FUNGAL GERM

The microbial strain is a fungal germ (*Cryptococcus neoformans*) provided by the Mycology Laboratory at Institut Pasteur (IPCI) (Abidjan, Côte d'Ivoire). It was isolated from cephalorachidian liquid in a patient from the department of infectious diseases at Hospital University Center of Treichville (Abidjan, Côte d'Ivoire).

2.1.4 CELLULES HFF

The cells HFF (Human Foreskin Fibroblasts) used for this work were provided by the Adaptation and Pathogenesis of Microorganisms Laboratory (LAPM) at Grenoble (France).

2.2 METHODS

2.2.1 BOTANICAL DESCRIPTION OF TWO PLANTS

The botanical description take into account : the general appearance of the plant ; the size, shape, and arrangement of leaves ; the type and arrangement of the curds on the stalk, the section of the stalk ; the type, shape and appearance of the fruit.

2.2.2 PREPARATION OF PLANT EXTRACTS

The leaves of these two species were dried separately in the Laboratory for two weeks and reduced to a fine powder using an electric grinder type IKA Labortechnik (MFC type).

Preparation of aqueous total extracts (ETA) : the preparation of these extracts was performed using the method described by [29] which consists in macerating 100 g of plant powder of each species in 1L of sterile distilled water using a blender Blinder type 7 SEVEN STAR. The homogenates were filtered over hydrophilic cotton and then on filter paper Whatman 3 mm. The aqueous filtrate thus obtained are evaporated in an oven type Med Center Venticell at 50°C to obtain powders that constitute the aqueous extracts (ETA_{Aa} for *Aspilia africana* and ETA_{Ah} for *Acanthospermum hispidum*).

Preparation of ethanolic fractions 70 % (FE70 %) and aqueous residual fractions (FRA) : these fractions were obtained separately by dissolving 5 g of each ETA in 100 mL of a ethanol 70 % solution and then homogenized. After decantation and filtration of the alcoholic fraction on hydrophilic cotton and on filter paper Whatman 3 mm, the filtrate collected is evaporated in an oven at 50 °C. The powder obtained constitutes the FE70 % extract (FE70 $\%_{Aa}$ or FE70 $\%_{Ah}$ according to the specy). Likewise the aqueous residual deposit was collected and evaporated in an oven at 50 °C. The powder obtained constitutes the FRA extract and is called FRA_{Aa} or FRA_{Ah} according to ETA.

2.2.3 ANTIFUNGAL TESTS

Preparation of medium culture and extracts incorporation : The culture medium was prepared according to the manufacturer's instructions while taking into account the quantity taken and the incorporation of different extracts prepared medium was made in tubes by the method of double dilution which leads to the obtaining of different concentration from 50 to 0.097 mg/mL according to geometrical connection of 1/2 reason. For each extract, a series of 12 test tubes was constituted with 10 experimental tubes and 2 control tubes of which one without constituting the plant extract of germs growth control and the over without extract and germ, used as a culture medium for sterility control light.

Preparation of the *inoculum* and sowing of the tubes : The inoculum preparation is made by homogenization of a young colony (48 h) well isolated from *C. neoformans* in 10 mL of sterile distilled water to give a suspension of 10^0 (10^6 cells/mL). From this suspension, 1 mL was taken and mixed in 9 mL of sterile distilled water to form the suspension 10^{-1} corresponding to 10^5 cells/mL. The 10 experimental tubes and the growth control tube was inoculated with 10 µL of the suspension 10^{-1}.

Sterilization : The 12 tubes of each series were autoclaved (PBI STEMATIC III) at 121 °C for 15 min and then all the tubes were incubated at 30 °C for 72 hours and then tilted with a small stick to the room temperature to permit cooling and solidification of the agar.

Colonies counting : After the incubation time, the colonies of *C. neoformans* were counted by direct counting with a colonies counter pen type Geiger. The growth in experimental tubes was evaluated as a percentage of survival, calculated at 100 % survival in the control growth control tube. The calculation of the percentage of survival was done according to the following formula :

$$S = (n/N) \times 100$$

(S = Survival of *C. neoformans* percentage ; n = number of colonies in the control tube ; N = Number of colonies in the test tube).

Required antifungal parameters : data processing has determined the following parameters antifungals :

-CMI (Minimum Inhibitory Concentration) : this is the concentration of extract in the tube for which there was no growth visible to the naked eye ;

-IC_{50} (Concentration for fifty percent inhibition) : is the concentration which gives 50 % inhibition. It is graphically determined from the sensitivity curve plot of each extract of *C. neoformans*;

-Fungicidal (**CMF** ou **CFS**) : after 72 hours of incubation, the surface of the agar contained in test tubes having resisted the growth of the fungal isolate was taken slightly then inoculate on a new agar and incubated for 72 h at room temperature. Two cases are possible :

- presence of colonies of *C. neoformans*, the extract is said fungistatic. Thus, it is determined the **CFS** (Concentration Fungistatic) ;
- absence of colonies of *C. neoformans*, the extract is said fungicide. This observation identified the **CMF** (Minimum Concentration Fungicide) which gave 99.99 % inhibition compared to control growth control tube.

2.2.4 TOXICITY TESTS

The HFF cells used for toxicity tests, were cultured at 37 °C, 5 % CO_2 in D10 medium (Dulbecco Minimum Essential Medium, Gibco, supplemented with 10 % fetal calf serum, 1 % glutamine ; 50 $U.mL^{-1}$ penicillin and 50 $\mu g.\mu L^{-1}$ streptomycin).

To measure the toxicity of ethanol extracts (FE70 $\%_{Aa}$ and FE70 $\%_{Ah}$), the cells HFF (Human Foreskin Fibroblasts) were sowed in 96-well plates (*CellStar*) at a rate of 3000 to 5000 cells per well in 100 µL of medium D10. These cells are maintained in culture for 24 hours (dividing cells) or 96 hours (confluent cells). Subsequently they were exposed for 24 hours at different concentrations (0-1000 µg/mL) in the plant extract solubilized in PBS buffer. This was done in three parts. Viability was determined using the 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium (MTT). In each well, the MTT is added to a concentration of 500 µg/mL and incubated for 3 h at 37°C. The formazan crystals are solubilized in dimethylsulfoxide (DMSO) 10 mM. The Measurement of the optical density at 544 nm was made using a Safir spectrophotometer (Tecan). This absorbance measurement was used to determine the relative amount of living and metabolically active cells. The results were expressed as percentage of viability compared to the control without plant extract (control) [30].

Viability rate = ($Abs_{544\ nm}$ extract/ $Abs_{544\ nm}$ control) × 100

3 RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 BOTANICAL DESCRIPTION

The two plant species studied are two qualified weed. They are common to the Guineo-Congolese region and the Sudano-Zambian region. The general appearance of the two plants is presented in Figure 1 and in Table 1 summarizes the morphological details and comparative description of the different organs.

Acanthospermum hispidum is called Saraka-weini in Malinke (Northern ethnic group of Côte d'Ivoire) and Gnéakeyébêko in Bete (West-center ethnic group of Côte d'Ivoire). This is an herb that grows in the fields, pastures, roadsides and piece of waste ground. As for *Aspilia africana*, called Zeu-nanh in Akye (Southern ethnic group of Côte d'Ivoire) and Soumadibrou in Malinke (Northern ethnic group of Côte d'Ivoire). It grows in cultivated fields. It is also found in fallow, especially in the forest area.

Aspilia africana (Pers.) C.D. Adams var africana

Acanthospermum hispidum DC.

Figure 1 : General appearance of the leaves and flower stalks of Acanthospermum hispidum and Aspilia africana

Table I : Description of the two plants

Plant species / Described parts	*Acanthospermum hispidum* DC.	*Aspilia africana* (Pers.) C.D. Adams var *africana*
General appearance of the plant	Hispide to dichotomous branching, annual plant Stems with angular section, 30-60 cm high	Hardy herb with ramification Stem with regular section, 60 cm to 1.5 m high
Section of the stem	Quadrangular	circular
Size of leaves	Length: 6 to 8 cm; width: 2 to 4 cm	Length : 6 to 15 cm ; width : 3 to 7 cm
Leaf shape	Oval	Oval-lanceolate
Leaf arrangement	Opposite (sessile)	Opposite
Types of inflorescences	Solitary capitules, inserted in each branch, greenish color	Terminals and solitary capitules Color of the jewels: bright yellow
Type of fruit	Akenes	Akenes
Fruit size	Length : 6 to 8 mm ; width : 3 to 4 mm	Length about 5 cm width : 0,5 to 2 mm
Appearance fruit	Prickly	Quadrangular and covered with hard and thin hairs

3.1.2 YIELD EXTRACTIONS

Maceration with distilled water of *A. hispidum* gives a blackish powder of 17 g (ETA_{Ah}) a yield of 17 % for 100 g of drug. As to the partition, for 5 g of ETA_{Ah} dissolved in 100 mL of ethanolic 70 %, there was obtained 2.61 g of FE70 $\%_{Ah}$ that is to say a yield of 52.2 % and 2.14 g of FRA_{Ah} that is to say a yield of 42.8 %.

As for *A. africana*, maceration with distilled water gave a blackish powder of 11 g (ETA_{Aa}) is a yield of 11 % for 100 g of drug. As for splitting for 5 g of ETA_{Aa} dissolved in 100 mL of 70 % ethanol solution, gives 1.44 g of FE70 $\%_{Aa}$ that is to a yield of 28.8 % and 3.10 g of FRA_{Aa} that is to say a yield of 62 %.

3.1.3 ANTIFUNGAL TESTS

After 72 hours of incubation at 30°C, we observed compared to control, a gradual decrease in number of colonies of *Cryptococcus neoformans* as and as the concentration of plant extracts increased in experimental tubes. This reduction in the number of fungus colony was more remarkable for the ethanolic extracts 70% (Figure 2). Clear and effective inhibitions were obtained at different concentrations for 70% ethanolic extracts and for ETA_{Aa} extract. On the other hand for ETA_{Ah} and aqueous extract residual (FRA_{Aa} and FRA_{Ah}) it not observe any significant antifungal activity on *C. neoformans* in the concentration range established.

Moreover, among the two ethanol extracts (FE70 $\%_{Aa}$ and FE70$\%_{Ah}$) FE70 $\%_{Ah}$ showed higher antifungal activity on *C. neoformans* compared with FE70$\%_{Aa}$ (CMF = 12.5 mg/mL for FE70$\%_{Ah}$, CMF = 25 mg/mL for FE70 $\%_{Aa}$).

Generally all the extracts had curves showing a decreasing pace with more or less steep slopes. The extracts FE70 $\%_{Ah}$, FE70 $\%_{Aa}$, FRA_{Aa} and ETA_{Aa} have curves with relatively steep slopes, especially that of FE70 $\%_{Aa}$. The lowest slopes are observed with that of ETA_{Ah} and particularly the FRA_{Ah} extract.

The experimental data reflected in the form of sensitivity curves are summarized in Figure 2 and the values of the parameters antifungals (CMI, CMF and IC_{50}) of the various extracts are reported in Table 2.

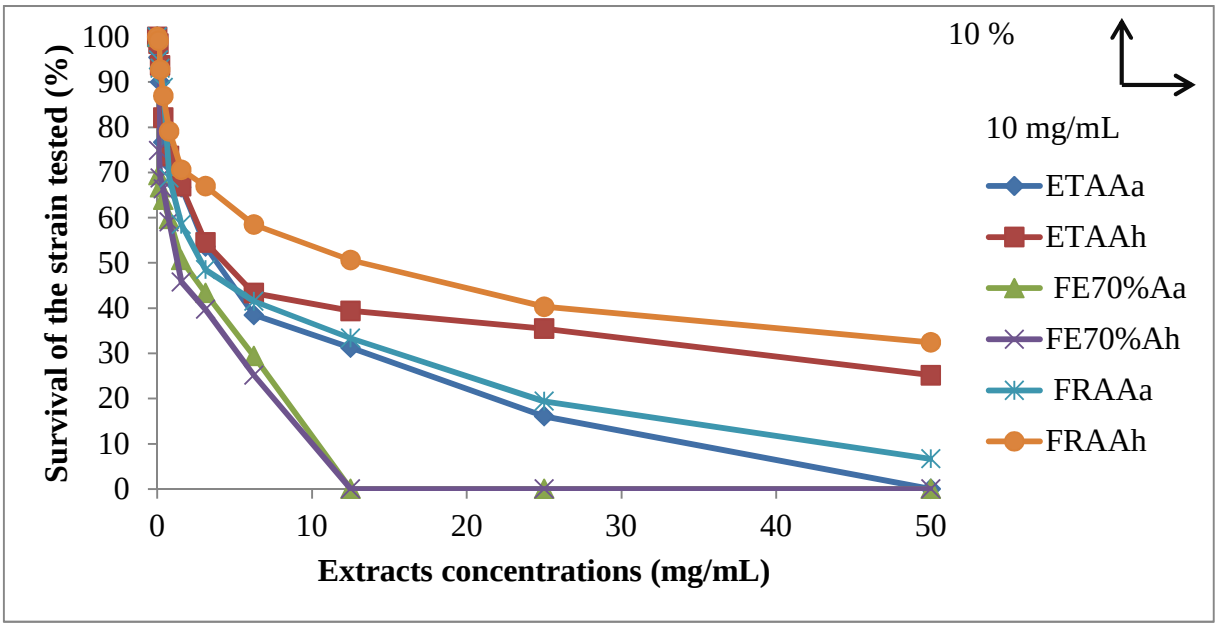

Figure 2 : Sensitivity of Cryptococcus neoformans to the extracts of Acanthospermum hispidum and Aspilia africana

Table 2 : Values of antifungal parameters of six Acanthospermum hispidum and Aspilia africana extracts at 72 hours of incubation at 30 °C.

Plant extract	Antifungal parameters			Fungicidal
	CMI (mg/mL)	IC_{50} (mg/mL)	CMF (mg/mL)	
ETA_{Aa}	50	2.80	50	Fungicide
ETA_{Ah}	>50	4.40	>50	Fungistatic
FE70 $\%_{Aa}$	12,5	1.78	25	Fungistatic
FE70 $\%_{Ah}$	12,5	1.28	12.5	Fungicide
FRA_{Aa}	>50	2.90	>50	Fungistatic
FRA_{Ah}	>50	10.50	>50	Fungistatic

3.1.4 TOXICITY TESTS

The results of ethanolic extracts toxicity tests 70 % (FE70 $\%_{Ah}$ and FE70 $\%_{Aa}$) on HFF cells are respectively summarized in Figure 3 and Figure 4.

These results reveal that the FE70$\%_{Ah}$ extract shows little toxic effect on human cells studied in relation to the FE70 $\%_{Aa}$ extract and this compared to controls. We observe for FE70$\%_{Ah}$ extract, a decrease of the drop of HFF cells viability rate when the concentration of the plant extract increases (from 125 µg / mL to 1000 µg / mL). Thus for confluent cells, lower viability rate is between 14 % (125 µg/mL) and 7 % (1000 µg/mL). As for dividing cells, this decrease is between 38 % (125 µg/mL) and 19% (1000 µg/mL).

However, for FE70$\%_{Aa}$ extract, the lower viability rate of HFF cells is developing with increasing of the extract concentration. This is observed as well for the confluent cells for those in division. For confluent cells, this decline in viability rate is higher and ranges from 26 % (125 µg/mL) and 74 % (1000 µg/mL). As regards the dividing cells, this decline varies between 30 % (125 µg/mL) and 59 % (1000 µg/mL).

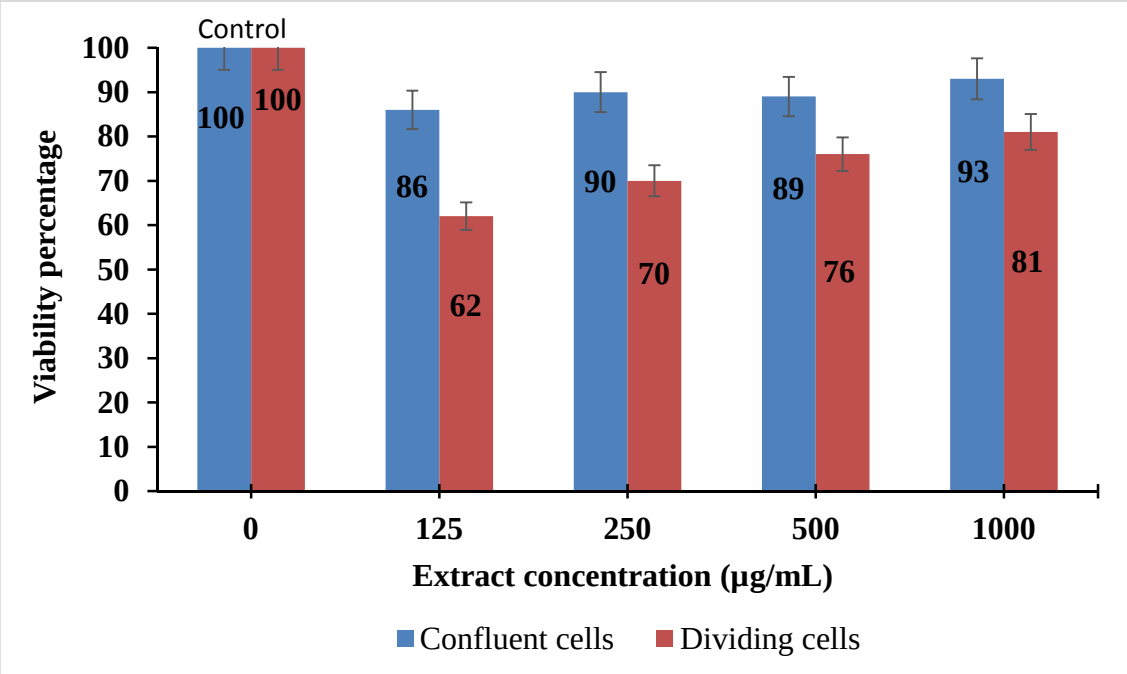

Figure 3 : Viability rate on HFF cells of FE70%$_{Ah}$

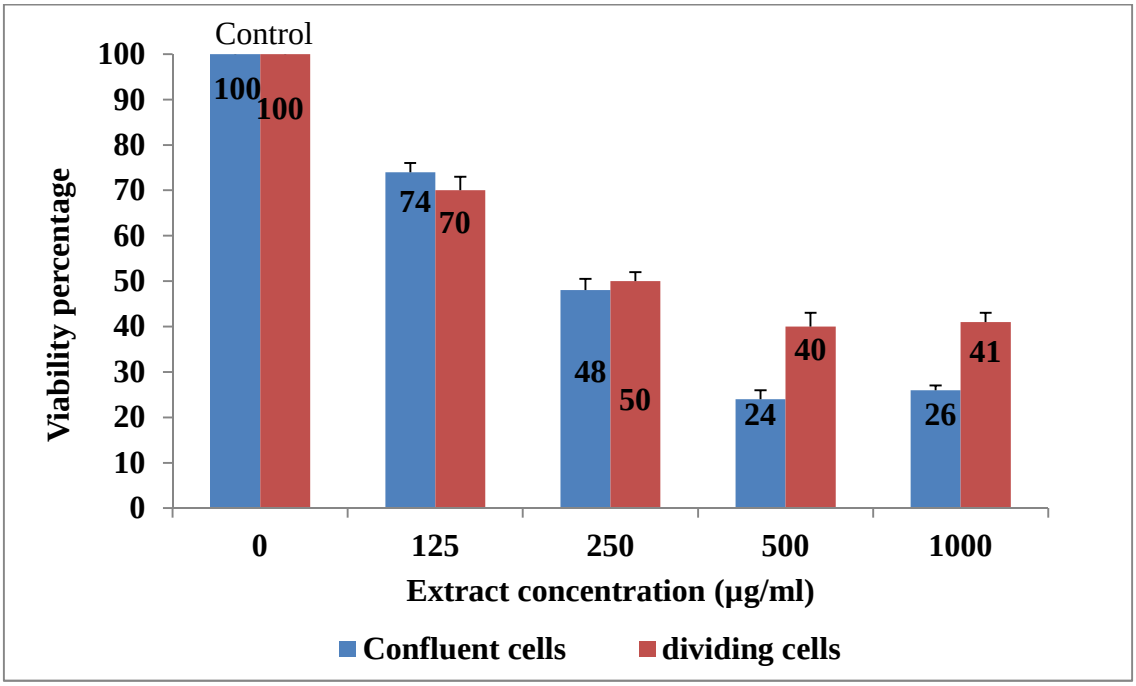

Figure 4 : Viability rate on HFF cells of FE70%$_{Aa}$

3.2 DISCUSSION

Botanically, *Anthospermum hispidum* and *Aspilia africana* are two Asteraceae presenting all the characteristics of this family. They are two ruderary and characteristic species of cattle way zones and two weed plots grazed after harvest. According to [31], two species known to be rainy weed of crops and plantations.

Microbiologically, analysis of the results shows that only FE70 % and ETA_{Aa} extracts are active on *Cryptococcus neoformans*. This fungal isolate is sensitive to the extracts in a dose-response relationship.

The results reveal that the value of the FE70%$_{Aa}$ extract CMF is 25 mg/mL and that of ETA_{Aa} extract is 50 mg/mL. The comparison of the activities of both extracts showed that FE70 %$_{Aa}$ extract is 2 times more active than the ETA_{Aa} extract who served in its preparation. Similarly, the results also reveal that the value of the FE70 %$_{Ah}$ extract CMF is 12.5 mg/mL and that of ETA_{Ah} extract is > 50 mg/mL. The comparison of the activities of these two others extracts shows that FE70 %$_{Ah}$ extract is 4 times more active than the ETA_{Ah} extract. Thus, the comparison of the activities of three extracts (FE70 %$_{Aa}$ ETA_{Aa} and FE70 %$_{Ah}$) show that the FE70 %$_{Ah}$ extract is 2 times more active than the FE70 %$_{Aa}$ extract and 4 times more active than the ETA_{Aa} extract.

Overall, the results show that FE70 % extracts from the partition ethanol/water, significantly improve the effectiveness of ETA extracts that were used as a basis for their preparation.

The performance comparison of the two FE70 % extracts shows that the FE70 %$_{Ah}$ extract (CMF = 12.5 mg/mL) is 2 times more efficient than the FE70 %$_{Aa}$ extract (CMF = 25 mg/mL). The performance comparison of the two FE70 % and ETA_{Aa} with the Eaq extracts (aqueous total extract) Eeth (ethanol 70% extract) and EacH_2O (acétatique H_2O extract) of leaves of *Morinda morindoides* (Baker) Milne-Redh. ([32]) on this same fungal germ shows that FE70 %$_{Ah}$ extract is 24 times more active than the Eaq and EacH_2O extracts, 2 times more active than the Eeth extract. Similarly, the works of [33] on the same germ, show that the FE70 % extracts and ETA_{Aa} have better performance on *C. neoformans* that *Cymbpogon nardus* L. and *Cymbopogon schoenanthus* L.

In terms of toxicity, analysis of the results reveals that the FE70 %$_{Ah}$ extract presents little toxic effect on confluent cells. Moreover, the toxicity effect decreases with further increase in the concentration of the extract. This can be caused by the presence in the extract molecules stimulating cell division. According [7], *A. hispidum* taken orally is not toxic for mice. Which confirms these results.

As for FE70 %$_{Aa}$ extract, analysis of toxicity results show that the extract has a high toxic effects on human cells HFF. Bisides, this toxic effect further increases with extract concentration increase. Indeed, the results of the works of [26] show that the use of *A. africana* causes damages on the uterine tissue of rats. This is in agreement with the results obtained.

4 CONCLUSION

This study allowed us to show that all the extracts of *Anthospermum hispidum* and *Aspilia africana* have antifungal activity more or less pronounced on the *in vitro* growth of *Cryptococcus neoformans*. The FE70 % extracts were the most active. Ethanol was the solvent that would allow a better concentration of the active principles. This study showed that the extraction method used, would be a way that would concentrate the active principles and improve the activity of ETA, traditionally used against cryptococcosis.

Besides, this study reveals that the use of *A. hispidum* for therapy would be safe but that of *A. africana* would be carefully. At last, we can say that the use in traditional environment of these two plants as antifungal is warranted but with a very moderate use of *A. africana*. Further analysis by phytochemical screaning followed by chromatography on CM and column, we allow us to isolate the different molecules in the FE70 % extracts in order to specify the nature of the active molecules.

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