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RESEARCH ARTICLE

ASSESSMENT OF AQUEOUS AND ETHANOLIC EXTRACTS ANTIFUNGAL ACTIVITY FROM STEM BARKS OF ENANTIA POLYCARPA (ANNONACEAE) ON CANDIDA ALBICANS, ASPERGILLUS FLAVUS, ASPERGILLUS NIGER AND CRYPTOCOCCUS NEOFORMANS.

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Abstract

The increase of mycosis and the development of resistance to usual antifungal stimulated the search for new antifungal coming from medicinal plants. This work's aim was to evaluate the antifungal activity of aqueous and ethanolic extracts of *Enantia polycarpa* stem's barks on the *in vitro* growth of *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger* and *Aspergillus flavus*. Aqueous and 70% ethanolic extracts were tested on the fungi. The evaluation has been done by incorporating extracts in agar Sabouraud according the double dilution slant method. After 48 hours of incubation at 30^0C. The germs have been sensitive to the two extracts according to a relation dose response relationship.

70% ethanolic extract (FMC=3,125 mg/mL on *Candida albicans*, FMC = 6,25 mg/mL on *Cryptococcus neoformans* and *Aspergillus flavus* and FMC = 12,50 mg/mL on *Aspergillus niger*) had the best activity. *Candida albicans* (FMC=3,125 mg/mL and IC50 = 1,80 mg/mL) was most sensitive.

Thus, solvent hydro-alcoholic (70% ethanolic) concentrates better active ingredients of *Enantia polycarpa*.

Introduction:-

Since VIH infection advent at 1980, the humanity is coped to one the most important sanitary crises. The VIH infection is now considered as the 6th reason of mortality in the world and the 2nd in sub-Saharan Africa. VIH/SIDA epidemic's progression in this region made it the most touched zone with 25 million people living with VIH (PLVIH), either 70,82% of the population are positive VIH (**ONUSIDA, 2013**). Since the beginning of the pandemic, about 75 million people have been infected by the VIH/SIDA. In spite of the efforts provided in the handling of people living with VIH (PLVIH) through antiretroviral, Opportunist infection follow-up is less remain considered involving their evolution and vital prognosis compromises of the patients (**Lahuerta and Hoffman, 2013; Ouattara, 2009**). VIH infection advent involved a fungal infection recrudescence with *Cryptococcus*, *Candida* and *Aspergillus* infection on the first rank (**Ouchara *and al.*, 2010**).

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The neurominingeal cryptococcosis is the second most frequent opportunist infection and the most dangerous systemic mycosis during the VIH infection (**Luma and Temfackn, 2013; Millogo *and al*., 2004**). Whereas the impact of the illness decreased in the western countries with the triple therapy, the neurominingeal cryptococcosis remained a reason principal of meningitis in sub-Saharan Africa.

According to **Shivaswamy and Neelambike** (**2014**), 60 to 80% of patients positive VIH develop a candidosis with a death rate of 10 to 20%. The candidosis, the neurominingeal cryptococcosis and the aspergillosis are frequent opportunist infections during the AIDS compromising the handling of the people living with VIH (PLVIH).

The antifungal multiresistance to the germs responsible for these infections like *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus flavus*, *Aspergillus niger* etc. impose the scientific community to develop new therapeutic approaches from the natural substance of plant origin. Besides, the cost raised of the antifungal, the long period of treatment and the poverty are many factors that force the population to move toward traditional medicine by healing plants. These plants countain compounds bioactifs which can serve to develop new drugs (**Kporou *and al*., 2008b**) and many persons in the world use in first intention the traditional medicine for their needs of health. The plants exploration could constitute a promising way of development of new sources medicinal efficient antifungal and at a lower cost (**Ackah *and al*., 2008b; Yapi *and al*., 2010**). Surveys ethnobotanical and ethnopharmacological permitted to identify healing plants commonly used by the communities in their cares of primary health (**Ambé *and al*., 2015; Piba *and al*., 2015**).

Among these plants *Enantia polycarpa* a plant species reputed in traditional environment for its antimicrobic, antiparasitic, anti-inflammatory properties.

The present work is a contribution to the valorization of Ivory Coast healing plants.

The general objective is to value in vitro aqueous and ethanolic extracts antifungal activity from stem barks of *Enantia polycarpa* (Annonaceae). To reach this general objective, some following specific objectives have been defined:

1. Produce aqueous and ethanolic extracts from stem barks of *Enantia polycarpa* (Annonaceae)
2. Determine the antifungal parameters: the Inhibitory Minimal Concentration (IMC) that is the smallest concentration from which no colony is visible to the naked eye, the Fungicidal Minimal Concentration (FMC) that gives 99, 99% of inhibition compared to the tube witness of control growth (100%) and the Concentration for 50% of inhibition (CI_{50}) that is determined graphically and corresponds to 50% of inhibition compared to the tube witness of control growth.

Material and Method:-

Plant material:-

It is constituted of the powder of stem barks of *Enantia polycarpa* Engl. & Diels. (Annonaceae). The plant material has been harvested by a herbalist and a herbarium has been achieved for its identification at the Floristic National Center (FNC) of the university Felix Houphouët Boigny of Cocody. It has been identified at the specimen n°11561.
After the identification, *Enantia polycarpa* barks have been harvested, washed, cut and dried during one week under cover to the sun. After the drying, the barks have been ground finely in an electric grinder. The gotten powder served at the preparation of extracts for the assessment *in vitro* of antifungal activities.

Microbial material:-

The antifungal activities assessment has been made on *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans* and *Cryptococcus neoformans*. These germs were provided by the mycology laboratory from Medical Sciences of Felix Houphouët Boigny's university (Abidjan, Ivory Coast). These germs have been isolated at patients in consultation at the service of the infectious illnesses of from Treichville's university hospital.

Methods:-

Aqueous (Xaq) and 70% ethanolic (Xet) extracts preparation:-

This preparation has been achieved according to the method described by authors **Ouattara**, (**2004**); **Ackah *and al*.**, (**2008a**). To get aqueous and 70% ethanolic total extracts, *Enantia polycarpa's* powder has been extracted using 100g of the powder in 1L distilled water in a blender of mark Life's Superb (LS-317) or 1L of 70% ethanol during

three cycles of three minutes each at the ambient temperature. The gotten homogenate is first wrung in a white cloth square, filtered five times then successively on the absorbent cotton wool and on the filter paper Whatman 3mm. Extraction solvents water and 70% ethanol were evaporated respectively with a steam room controlled at 60°C during 72 hours and 48 hours. The gotten dry evaporate has been codified Xaq for the aqueous total extract and Xet for the 70% ethanolic extract.

Antifungal activity's assessment:-

Antifungal tests were carried out on culture medium Sabouraud (BioRAD /Réf: 64494; Batch: 6J2218). Vegetable extract incorporation to agar was made according double dilution method of tilted tubes. 12 test tubes were used including 10 test tubes containing vegetable extract and 2 pilot tubes. Among these two tubes, one without vegetable extract was used as witness of germs control growth while the other without germs and extract was used as witness of culture medium sterility control. Extract concentrations range in the tubes go from 800 to 1.56 µg/mL with geometrical connection of reason ½. All the tubes were pressure-sealed (121°C during 15 min), then tilted with small base at room temperature to allow their cooling and solidification of the agar. Germs culture on agar slant previously prepared was made by sowing of 1000 cells of each stock of *Candida albicans*, *Aspergillus flavus*, *Aspergillus niger* and *Cryptococcus neoformans* (**Ackah, 2004; Kporou *and al.*, 2008b**). Cultures were carried out and incubated with 30°C during 48 hours. After this time of incubation, germs were counted with pen of germs meter (CEINCEWARE number 23382) and growth in the 10 experimental tubes was evaluated expressed as survival percentage, calculated compared to 100% of pilot tube survival of growth control (**Ackah, 2004; Kporou *and al.*, 2008b**). The processing of these data made it possible not only to determine the fungicidal minimal concentrations (FMC), but also to plot the curves of activity of the extracts graphically determine the concentrations for 50% of Inhibition (IC_{50}).

Antifungal parameters (IMC, FMC and IC_{50}):-

The IMC (Inhibitory Minimal Concentration) is the smallest concentration of extract from which there is no growth visible to the naked eye in the tube test.

The FMC has been determined after sowing in other agar tubes hatched at 30°C during 48h the tubes tests presenting no growth of colonies visible to the naked eye from the tube of the IMC. The microbial growth in these different sowed tubes has been compared with dilution 10^0 to 10^{-4} in search of the growth letting 0,01% of survivors (dilution 10^{-4}). The concentration of the tube in which the number of colonies is identical with the dilution 10^{-4} corresponds to the Fungicidal Minimal Concentration (FMC). The Antifungal parameters (IMC and FMC) will permit to specify if the extracts are fungistatic or fungicidal.

The data processing permitted to draw activity curves of the extracts. These draw activities permit to determine the Concentration for 50% of inhibition (IC_{50}) (**Kporou *and al.*, 2008b; Djeneb *and al.*, 2016**).

Results:-

Activity of Xaq and Xet extracts on *C. albicans* and *Cryptococcus neoformans in vitro* growth:-

After 48 hours of incubation at 30°C, we noted compared to the witness of control growth of the germs, a progressive reduction of the number of *Candida albicans* and *Cryptococcus neoformans* colonies as the concentrations of the extracts increased in the experimental tubes. The gotten applied information are translated under shape of curves summarized by the figures 1 & 2. In a general way, all curves present a decreasing pace with valuable slopes variable according to the extracts. The decreasing shape of the activities curves showed that the 2 extracts have acted according to a relation amount-effect.

On *C. albicans*, the ethanolic extract activity curve has a relatively strong slope that the aqueous extract. The antifungal parameter values have been determined for the aqueous extract at IMC=50 mg/mL, FMC = 100 mg/mL and IC_{50} = 12,55 mg/mL whereas for the ethanolic extract at IMC = 3,125 mg/mL, FMC = 3,125 mg/mL, and IC_{50} = 1,80 mg/mL.

On *C. neoformans*, the ethanolic extract activity curve has a relatively strong slope that the aqueous extract. The antifungal parameter values have been determined for the aqueous extract at IMC=200 mg/mL, FMC = 400 mg/mL and IC_{50}= 55 mg/mL whereas for the ethanolic extract at IMC = 3,125 mg/mL, FMC = 6,25 mg/mL, and IC_{50} = 2,40 mg/mL.

Activity of Xaq and Xet extracts on A. flavus and A. niger:-

After 48 hours of incubation à 30°C, we noted compared to the witness of the germs control growth, a progressive reduction of the number of *A. flavus* and *A. niger* colonies as the extract concentrations increased in the experimental tubes. The gotten applied information are translated under shape of curves summarized by the figures 3 & 4. In a general way, all activity curves presented a decreasing pace with valuable slopes variable according to the extract.

On *A. flavus*, the ethanolic extract activity curve has a relatively strong slope that the aqueous extract. The antifungal parameter values have been determined for the aqueous extract at IMC = 400 mg/mL, FMC = 800 mg/mL and IC_{50} = 62,5 mg/mL whereas for the ethanolic extract these antifungal parameter values have been valued at IMC = 6,25 mg/mL, FMC = 6,25 mg/mL, and IC_{50} = 1,15 mg/mL.

On *A. niger*, the ethanolic extract activity curve has a relatively strong slope that the aqueous extract. The antifungal parameter values have been determined for the aqueous extract at IMC = 200 mg/mL, FMC = 400 IC_{50} mg/mL et = 19,25 mg/mL whereas for the ethanolic extract, these antifungal parameter values have been valued IMC = 6,25 mg/mL, FMC = 12,5 mg/mL, and IC_{50} = 4,30 mg/mL. The different antifungal parameter values were given in Table I.

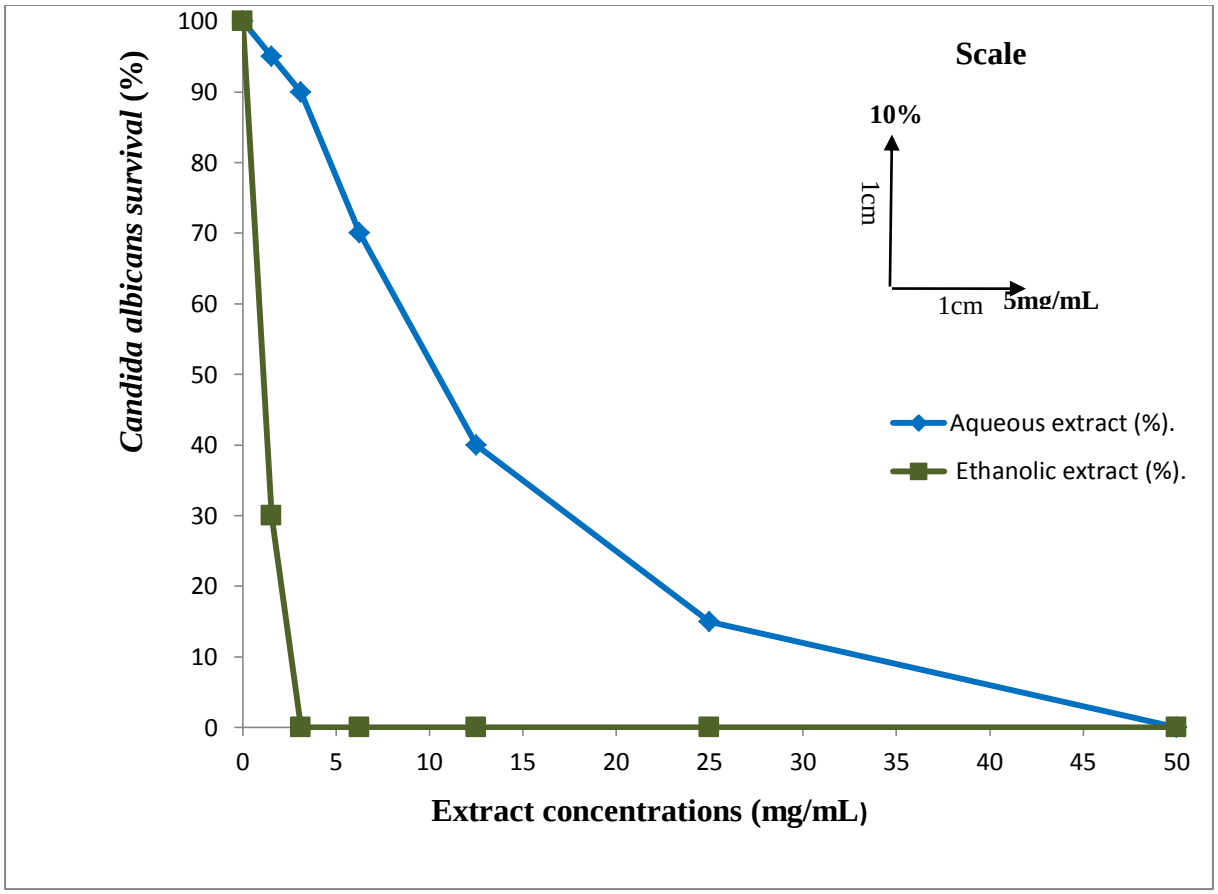

Figure 1:- Activity curves of Xaq and Xet extracts from *E. polycarpa* on *C. albicans.*

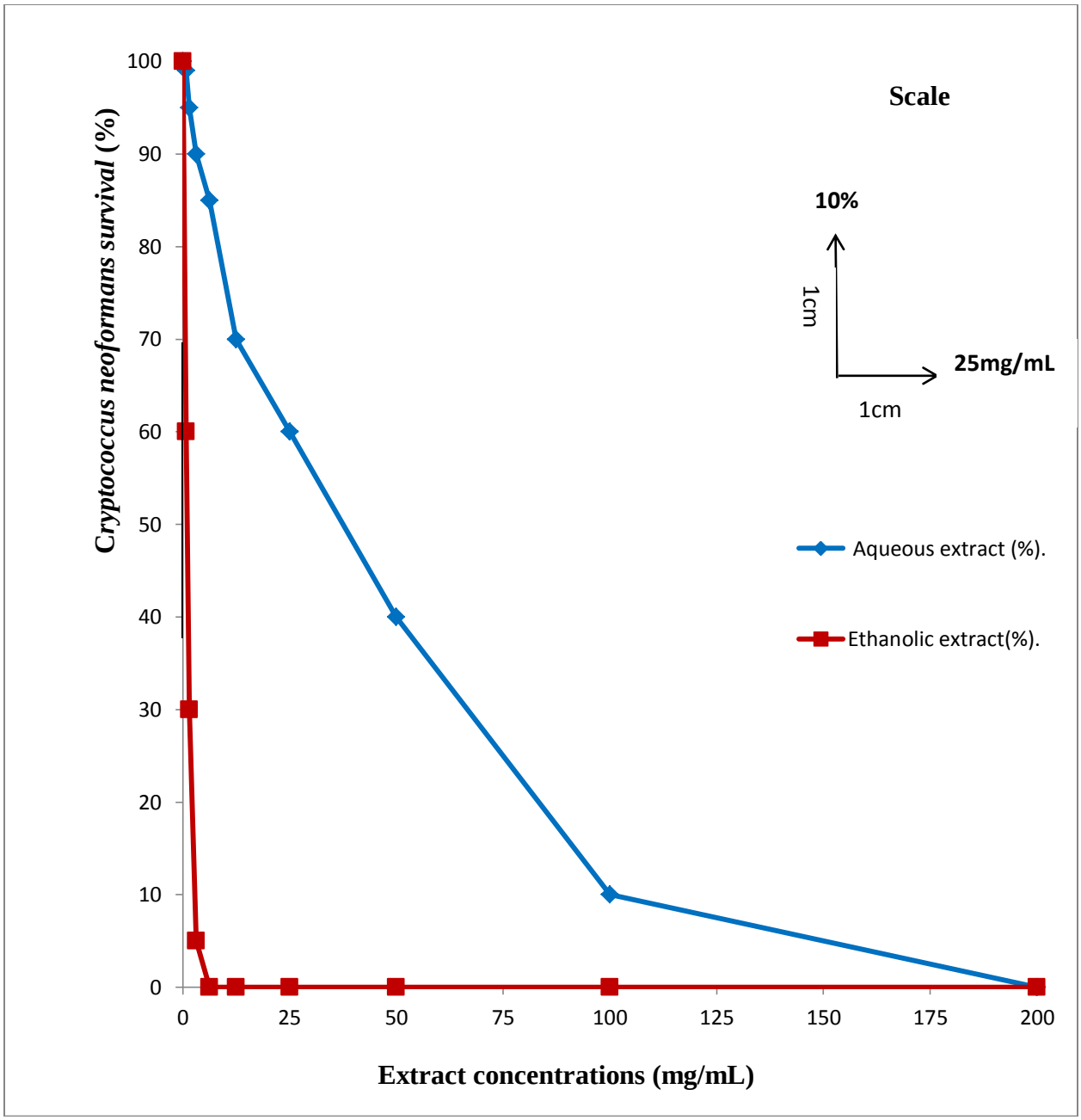

Figure 2:-Activity curves of Xaq and Xet extracts from *E. polycarpa* on *C. neoformans*

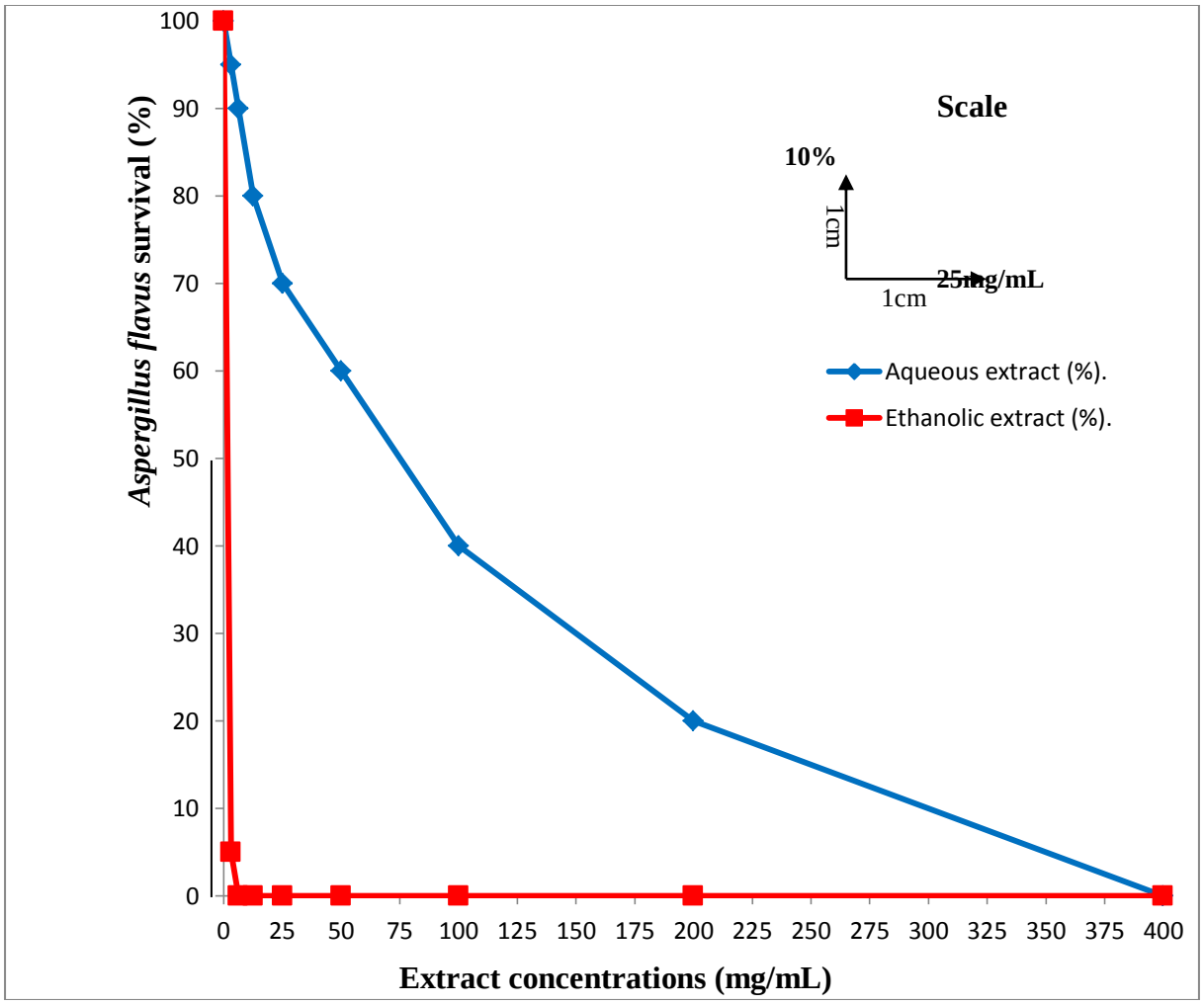

Figure 3:-Activity curves of Xaq and Xet extracts from *E. polycarpa* on *A. flavus*

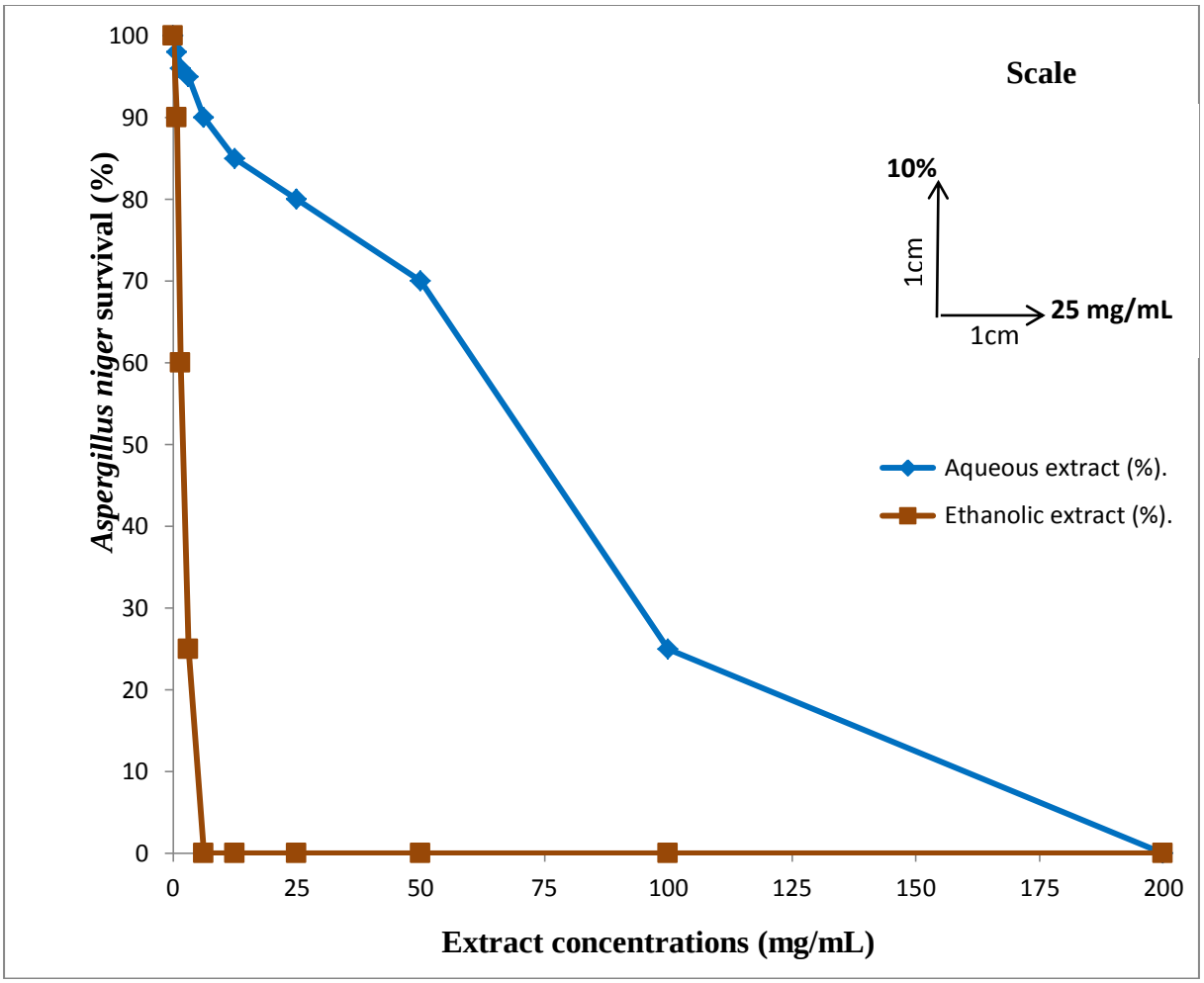

Figure 4:-Activity curves of Xaq and Xet extracts from *E. polycarpa* on *A. niger*

Table I:- Summary of the antifungal parameter values of the extracts from *E. polycarpa* at 48 hours of incubation at 30° C.

Germs	Aqueous extract			Ethanolic extract		
	IMC (mg/mL)	IC_{50} (mg/mL)	FMC (mg/mL)	IMC (mg/mL)	IC_{50} (mg/mL)	FMC (mg/mL)
Candida albicans	50	11	100	3,125	1,50	3,125
Cryptococcus neoformans	200	42	400	6,25	2,50	6,25
Aspergillus flavus	400	76	800	6,25	2,5	6,25
Aspergillus niger	200	75,1	400	6,25	4,30	12,50

Discussion:-

The antifungal test was about the yeasts mushrooms (*Candida albicans* and *Cryptococcus neoformans*) and filamentous mushrooms (*Aspergillus niger* and *Aspergillus flavus*) on the culture medium Sabouraud. The control cultures (100% of survival) presented an aspect of germs growth very dense on the different streaks witnesses. It means that this agar is a culture medium enable for the germ growth. The results analysis showed that all tested germs were sensitive to the different extracts from *Enantia polycarpa*. Indeed, there was a progressive reduction of the number of colonies as the concentrations of the various extracts from *E. polycarpa* increased in the experimental

tubes, compared to the witnesses (Figures 1, 2, 3 & 4). The two types of mushrooms are sentive to the different extracts.

The antifungal parameter values of the Table I revealed that:

1. The aqueous extract had a better activity on *C. albicans* than the other mushrooms because the IMC value is the weakest. *Aspergillus flavus* seems to be the least sensitive with IMC = 400 mg/mL most elevated.
2. The ethanolic extract is more active on *C. albicans* (IMC = 3,125 mg/mL). The others strains sensibility is identical. For this extract, the others strains had IMC and FMC identical values, while taking into account the IC_{50} value, it came out again that *A. niger* is less sensitive than ethanolic extract because the IC50 is the most elevated.

Considering the two extract activities on each of the mushrooms, it appears that the ethanolic extract (Xet) would be the most effective with lowest antifungal parameter values. The activity difference of Xaq and Xet could explain by the nature of the molecules contained in these extracts. Indeed, there is a capacity difference of solubilization and extraction of the solvents to the secondary metabolites. We could deduce that the antifungal compounds contained in *E. polycarpa* are more soluble in the ethanol than in the water. The 70% ethanol concentrate better the active principles.

The germs susceptibility to the extracts revealed the plant antifungal character. The decreasing shape of the activities curves showed that the 2 extracts have acted according to a relation amount-effect

Besides, it comes out that *C. albicans* was the most sensitive mushroom because the antifungal parameter values were the weakest. On the basis of activity report (FMC-Xaq / FMC-Xet), ethanolic extract was respectively 32, 32, 64 and 128 times more active than the aqueous extract on *C. albicans*, *A. niger*, *C. neoformans* and *A. flavus*. On each target strain, the antifungal parameter values (IMC and FMC) for the aqueous extract were different; so aqueous extract was fungistatic on these germs. On the other hand ethanolic extract was fungicidal on *C. albicans*, *A. flavus* and *C. neoformans* (IMC=FMC) and fungistatic on *A. niger*.

On the basis of the FMC value gotten, the activity of Xet on *C. albicans* is identical to the one gotten by authors **Kporou *and al*** in **2008a** who had tested *Mitracarpus scaber's* extract on the same germ *Candida albicans* (FMC = 3,125 mg/mL). The results of our works were better than those gotten with the MISCA-F1 extract (FMC = 150 mg / mL), MISCA-F2 (FMC = 50 mg/mL), MISCA-F3 (FMC = 25mg/mL) tested respectively by **Kporou, Ouattara** and **Ackah** in **2004** on the same germ Candida *albicans* (**Ackah, 2004; Kporou, 2004; Ouattaras, 2004**). Nevertheless this activity was distinctly better than the one gotten with the extract of Terminalia species tested by **Ambé *and al.*** (**2016**) and **Yapi *and al.*** (**2010**) with respective values (FMC = 1,56 mg/mL, FMC = 0,39mg/mL) because these values are even weaker. According to **Ambé *and al.*** (**2016**) with **Yapi *and al.*** (**2010**) works and also on the basis of the FMC reports, the activity hydroethanolic extract from *Terminalia superba* was 2 times more active than the activity ethanolic extract from *Enantia polycarpa* (**Ambé *and al.*, 2016**). Besides, the activity hydroethanolic extract from *Terminalia mantaly* was 8 times more active than the activity ethanolic extract from *Enantia polycarpa* (**Yapi *and al.*, 2010**).

For the same germ *Candida albicans* with the same plant ethanolic extract from *E. polycarpa* gotten by **Ambé *and al.*** in **2016** had an activity 2 more important times than the Xet extract. This extract activity's difference could be due to the difference of place of species harvest, or harvest period, even the temperature and drying length.

Conclusion and Perspectives:-

This survey permitted to identify the *Enantia polycarpa* antifungal potential. The *Enantia polycarpa* aqueous and ethanolic extracts of *Enantia polycarpa* had antifungal activity more or less accentuated on *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger* and *Aspergillus flavus in vitro* growth at 48 hours of incubation. The extracts acted according to a relation amount-effect and the inhibitions have been gotten with different extracts at concentrations from 100 to 800 mg/mL for the aqueous extract and 3,125 to 12,5 mg/mL for the ethanolic extract. The ethanolic extract was more active than the aqueous extract on each of the studied strains. The aqueous extract shown a fungistatic effect on the four germs whereas the ethanolic extract a fungicidal action on *C. albicans*, *A. flavus* and *C. neoformans* and a fungistatic on *A. niger*. This study revealed that 70% hydroethanolic solvent permits to concentrate better the active compounds of *E. polycarpa*.

In perspectives, we consider:

1. To improve ethanolic extract activity from *Enantia polycarpa* by bio-guided extraction coupled with column chromatographic dividing;
2. To achieve a phytochemical sorting of the most fraction chromatographic active in order to identify the bioactive compounds;
3. To isolate and to characterize bioactive molecule contained in the basis ethanolic extract;
4. and to propose a formulation an improved traditional remedies from the basis ethanolic extract.

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