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Antifungal Activity of *Terminalia mantaly* on the *in vitro* Growth of *Cryptococcus neoformans*

Ackah Jacques Auguste Alfred Bognan[1], Yaye Yapi Guillaume[1*], Yapi Houphouet Felix[1], Kra Adou Koffi Mathieu[1] and Djaman Allico Joseph[1,2]

[1]Laboratoire de Pharmacodynamie-Biochimique, UFR Biosciences Université Felix Houphouët Boigny*, 22 BP 582 Abidjan 22 (Côte d'Ivoire).*
[2]Département de Biochimie Médicale et Fondamentale, Institut Pasteur de Côte d'Ivoire, 01 BP 490 Abidjan 01(Côte d'Ivoire).

Authors' contributions

This work was carried out in collaboration between all authors. Author AJAAB designed the study, wrote the first and final draft of the manuscript, and wrote the protocols. Author YYG executed a scientific study, and checked the results. Authors YHF, KAKM and DAJ checked the results and managed literature and scientific searches of the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Among the HIV / AIDS infected, individuals contract fungal infections of which many die as a direct consequence of these infections. In this study, the antifungal activity of ten extracts (stem bark) from *Terminalia mantaly* H. Perrier was evaluated on the *in vitro* growth of clinical isolate of pathogenic fungi (*Cryptococcus neoformans*). Agar double dilution method in slope tubes was adopted to determine anticryptococcal activity. All extracts exhibited antifungal activity in dose-response relationship. The residue extracts T_{4-2} obtained after degreasing the hydro-alcoholic extract T_0 (MFC = 24.37µg/Ml; IC50 = 5.87µg/mL) is the most active extract. Moreover, for a given concentration it is said that there are not significant differences between the different tests for each extract ($P< 0.05$). Therefore, using the hydro-alcoholic solvent (70% ethanol) followed by removal of oil is the best way to obtain an optimally concentrated

**Corresponding author: Email: yayeyapi@yahoo.fr;*

active ingredient from *Terminalia mantaly*. The present study justifies the traditional use of this plant for the treatment of fungal infections.

Keywords: Antifungal activity; cryptococcosis; opportunists; Terminalia mantaly.

1. INTRODUCTION

These last years, infectious knew a strong outbreak connected to several factors among which one of the most marking is the advent of the HIV/AIDS in the human viral pathology [16,17].

This difficult pandemic does not only have more negative impacts on the sanitary evolution in most the African countries but also involved and amplified the distribution of certain number of pathologies among which mycoses [15]. Among these opportunist mycoses, cryptococcosis takes up a disturbing place. With the global emergence of AIDS, the incidence of cryptococcosis is increasing and now represents a major life-threatening fungal infection in these patients. The most serious infections usually develop in patients with defective cell-mediated immunity. Indeed, Cryptococcus may cause pulmonary diseases and central nervous system (CNS) fungal infection (life-threatening meningitis or meningoencephalitis) potentially mortal to the HIV/AIDS patients (developed from 80 to 90% this infection), to the patients undergoing organ transplantation and corticosteroid treatment [3,8,16]. Today and in spite of the existence of numerous antifungal and the therapeutic combinations, cryptococcosis is part of the three most dangerous fungal infections in hospitable environment [22,23].

Furthermore, in the face of lack of equipment, the insufficiency of medical tool and especially the lack of efficient medicines and the increasing of the poverty, the population mainly the African ones turned to traditional medicines to relieve their ills [4,12]. But the excessive use of these healing plants can have serious consequences even dramatic on this population. Then, it turns out to be necessary to secure this traditional medicine. So, an ethnobotanic study made in Côte d'Ivoire allowed to list several healing plants with turned out to be reliable biological activities. Among the selected plants represent plants with anti-infective activities of which *Terminalia mantaly* H. Perrier (combretacea) used in the traditional environment to satisfy several troubles among which the fungal infections [21]. In fact, in traditional medicine this specie of plant treats arterial hypertension, gastro enteritis, *postpartum* care as well as mycosis infections [2,3,17,19].

Besides, this plant has given satisfying results about other germs: *Candida albicans*, *Aspergillus fumigatus* [24,25]. Through these encouraging results we wanted to know if the extracts of the bark of this specie had also inhibitive activities on *Cryptococcus neoformans* especially as the Cryptococcus became the third fungi infection after aspergillosis and candidosis. Moreover, in the world cryptococcosis is one of the main causes of morbidity and mortality of HIV/AIDS patients [6,20].

Hence, in line with the current trend of finding naturally occurring anticryptoccosis activity, this study was designed to evaluate anticryptoccosis activity of the extracts of *Terminalia mantaly* on the *in vitro* growth of *Cryptococcus neoformans*, the major germ of Cryptococcus which causes illness in human.

2. MATERIALS AND METHODS

2.1 Fungus Used

For these tests, the isolate *Cryptococcus neoformans* (nº 3190 / PLR of the 06-04-2000) was used. PRL is the Spine Liquid Sample. It was supplied by the laboratory of mycology of Medical Sciences department of the University of Felix Houphouët Boigny (Côte d'Ivoire). This germ was isolated from patients in the infectious diseases department of the University teaching hospital of Treichville-Abidjan (Côte d'Ivoire).

2.2 Preparation of Extracts

The pieces of the barks harvested, cut and dried at the shade were crushed. The powder obtained was coded TEKAM1. The crude extracts (aqueous and hydro-alcoholic) were prepared as follows:

Hundred grams of TEKAM1 was extracted in blender with one liter (1L) of distilled water or ethanol-water (70 % of pure ethanol and 30 % of distilled water) respectively. After crushing, the homogenate obtained in each case was centrifuged in a square of fabric and then filtered on absorbent cotton and on wathman paper 3mm respectively. Afterward, aqueous filtrate was concentrated under a vacuum at 60°C and the extract obtained constitutes the crude aqueous extract T_{Aq}. The hydro-alcoholic filtrate was evaporated to dryness and we obtain the crude extract T_0.

Besides from the T_0 extract, 8 other extracts were prepared. Indeed, 3 portions of 10g are subjected to a liquid / liquid partition in 400 mL of 3 different mixtures of solvents (hexane-water, ethyl acetate-water and dichloromethane-water; v/v: 50/50). After decantation the various phases were separated and were concentrated under a vacuum. The following extracts were obtained: the hexane phase (plant oil) (T_{1-1}), the aqueous phase of the partition hexane-water (T_{1-2}), the acetate phase (T_{2-1}), the aqueous phase of the partition ethyl acetate-water (T_{2-2}), the dichloromethane phase (T_{3-1}) and the aqueous phase of the partition dichloromethane-water (T_{3-2}). Moreover, another portion of 10g of the extract T_0 was degreased in 350 mL of hexane at soxhlet. We obtain T_{4-1} (the hexane phase of the degreased extracts, plant oil) and T_{4-2} (the degreased extract). The extracts obtained are directly dissolved by homogenization in the agar. It's exempt from solvent as after the extraction, the solvent is evaporated whether in a dry or in under a vacuum. Therefore the extract cannot still contain solvent. After, all the obtained extracts were tested for biological assays.

2.3 Culture Media

Sabouraud Agar (Bio-Rad / ref: 64449; Batch: 8B2212) was used in this tests. This medium was prepared according to the instructions of the manufacturer's protocol. The inclusion of the plant extracts in the agar was made according to the method of the double dilution agar slopes [1]. Ten test tubes were used per series among which 8 containing the plant extract. The concentrations of these tubes ranged from 780 to 6.09 µg/mL binding by a geometrical reason of ½. The 2 other tubes are used as control tubes in which one was without a plant extract used to monitor the growth of germs, and the other germ-free tube and without plant extract was used as sterility controls to the culture medium.

Then, all tubes of each series were removed by the use of forceps sterilized by flaming at 121°C for 15 minutes and were inclined to room temperature of the laboratory to cooling and solidification of the agar [14].

2.4 Antifungal Assays

A fresh colony of *Cryptococcus neoformans* (48 hours of incubation) was homogenized in 10 mL of sterilized distilled water. The suspension 10^0 obtained was concentrated to 10^6 cells/mL. Afterward, a second suspension (10^{-1}) has been prepared from the suspension 10^0 by dilution ($1/10^{th}$). It's charged to 10^5 cells /mL. To check the charge of the inoculum, secondary dilutions of the inoculum was performed (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}). All these suspensions were seeded on agar in Petri dishes incubated at the same time as the test cultures. After 48 hours of incubation, the colony count of 10^{-5} dilution, based on the sampled volume (10µL) and the dilution factor, give an inoculum charge, which is consistent with normal standard for culture. In this verification, the number of colonies counted in suspensions (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}) are taken into consideration. Besides for each tests tube except the sterility control tube, the culture of the germ was made by sowing of 10 µL of the suspension 10^{-1}. This corresponded to 1,000 cells seeded.

After incubation at 30°C for 48 hours, the colonies were numbered with a pen colony counter (serial number 23382 Scinceware de Bel-Art). Furthermore, the growth in the experimental tubes was assessed as a percentage of survival calculated compared to 100 % survival in the control tube growth. The treatment of experimental data was used to determine:

- The Minimum Inhibitory Concentration (MIC): it is the smallest extract concentration in the tube where there is no growth.
- The Minimum Fungicidal Concentration (MFC): it is the smallest extract concentration in the tube which gave 99.99 % inhibition compared to the control tube growth or conversely it is the tube which leaves 0.01 % survival. It's obtained after transplanting the agar of the tube corresponds to the MIC.
- The Concentration for 50 % Inhibition (IC_{50}): it is the concentration which gave 50 % inhibition. This parameter was determined graphically.

These parameters were used to evaluate the antifungal activity of different extracts.

2.5 Statistical Analysis

The analysis of variance (ANOVA) at $p< 0.05$ means and standard deviations were carried out to compare the levels of extracts. The principal components analysis (PCA) was performed using the software Graph Pad Prism 5.

3. RESULTS

After 48 hours of incubation at 30ºC, it was observed compared with control tube a progressive decrease in the number of colonies gradually as the concentrations of the plant extracts increased in the experimental tubes. This is observed for all series of each extract. These results are average of 6 experiments of each extract.

So, effective inhibitions were observed at different concentrations level according to the extracts (Table 1). The experimental data from different partitions extracts were drawn to

sensitivity curves as presented in Fig. 1. In general, all sensitivity curves showed a decreasing pace with slopes more or less strong according to the extract. They intersect x-axis at different levels according to the extracts (Fig. 1).

The value of the MIC is equal to the one of the MFC. In fact, the gelose of the MIC is seeded on a barren gelose made for that. After the incubation period, we still notice that there are not visible colonies on the agar gelose.The values of the MFC and the IC_{50} for all the extracts are shown in Table 1. Therefore the statistic values from extracts are confining to Table 2.

The control tube used to monitor the growth of germs with concentration 0µg/mL compared to the extract is elevated at 100% and this value is represented for each extract on the Fig. 1 by the value 100% of survival.

Table 1. Antifungal parameters of the different extracts of *Terminalia mantaly*

		Antifungal parameters	
	Extracts	**MFC (µg/mL)**	**IC_{50} (µg/mL)**
Crude extracts	T_{Aq}	195	35
	T_0	97.5	16.5
T_0 Partitions	T_{1-1}	390	150
	T_{1-2}	48.75	9.5
	T_{2-1}	195	62.5
	T_{2-2}	24.37	10
	T_{3-1}	780	345
	T_{3-2}	48.75	10
	T_{4-1}	780	165
	T_{4-2}	24.37	5.87

Table 2. Statistical data of the different extracts of *Terminalia mantaly*

Concentrations	6.09	12.18	24.37	48.75	97.5	195	390	780	F
T_{Aq}	89.50 a	78.40 a	62.00 a	34.50 a	20.00 a	0 a			318.4***
	±0.577 b	±0.333b	±0.333 b	±0.577 b	±0.333 b				0.9862ns
T_0	80.00 a	62.33 a	30.17 a	15.00 a	0				283.1***
	±0.577 b	±0.333 b	±0.288 b	±0.288 b					0.9986ns
T_{1-1}	96.00 a	93.33 a	86.00 a	71.00 a	63.33 a	42.00 a	0		897.6***
	±0.577 b	±0.333 b	±0.577 b	±0.577 b	±0.333 b	±0.577 b			0.9956ns
T_{1-2}	77.33 a	37.5 a	10.33 a	0					725***
	±0.333 b	±0.288 b	±0.333 b						0.9937ns
T_{2-1}	92.87 a	90.50 a	88.67 a	55.33 a	38.50 a	0			639.7***
	±0.133 b	±0.288 b	±0.333 b	±0.333 b	±0.288 b				0.9939ns
T_{2-2}	71.17 a	49.90 a	0						623.8***
	±0.166 b	±0.100 b							0.9993ns
T_{3-1}	95.17 a	93.17 a	85.17 a	80.17 a	78.17 a	67.17 a	45.17 a	0	171.8***
	±0.166 b	±0.166 b	±0.166 b	±0.166 b	±0.166 b	±0.166 b	±0.166 b		0.9973ns
T_{3-2}	61.5 a	42.17 a	20.33 a	0					115.5***
	±0.288 b	±0.166 b	±0333 b						0.9954ns
T_{4-1}	97.17 a	92.83 a	89.33 a	75.17 a	62.17 a	44.17 a	31.33 a	0	303.3***
	±0.166 b	±0.166 b	±0.333 b	±0.166 b	±0.166 b	±0.166 b	±0.166 b		0.9827ns
T_{4-2}	48.17 a	26.83 a	0						195.1***
	±0.166 b	±0.166 b							0.9766ns

ns: not significant **The indicated values represented the average of six tests (n = 6); a: mean; b: standard deviation * in every extract the values affected by different letters were significantly different in p < 0. 05.*

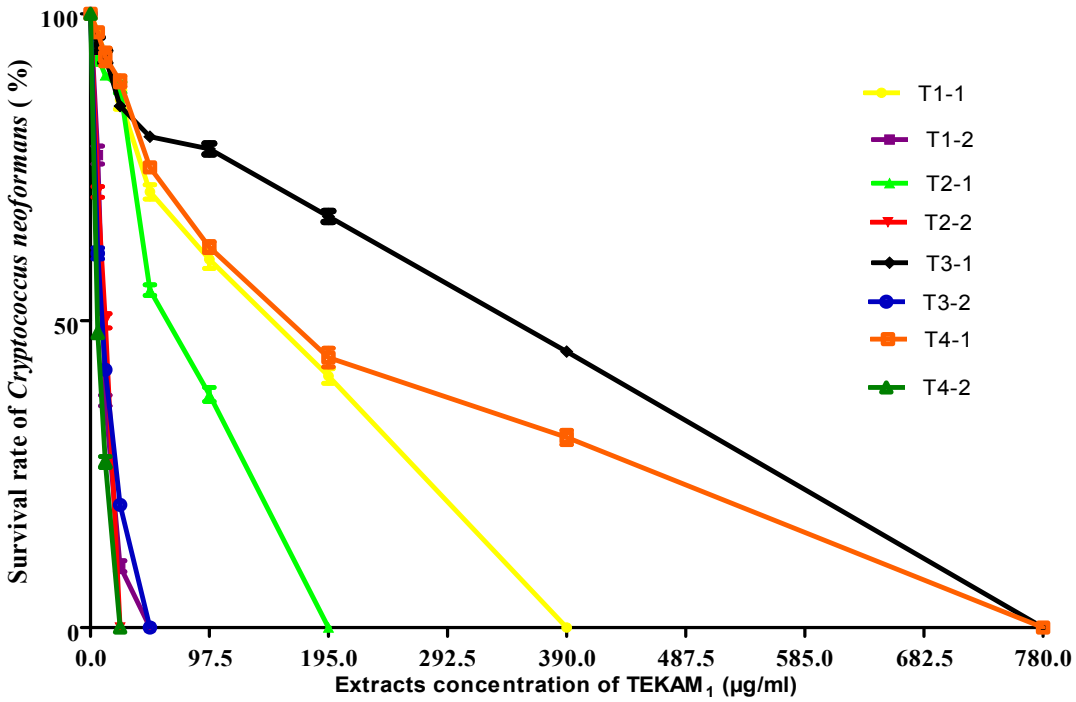

Fig. 1. Activity of T_0 partitions extracts of *Terminalia mantaly* on the *in vitro* growth of *Cryptococcus neoformans*

4. DISCUSSION

Taking for granted that for the traditional medicine treatment, a great part of the population use water as solvent, so we have checked the antifungal properties granted to the specie by testing the aqueous extract first. The very interesting results obtained, bring us to improve the performance of this extract. From then on, we used other solvents that can better concentrate active ingredients among which the ethanol, the ethyl acetate, the dichloromethane and the hexane.

The crude extracts, the sensitivity curve of the extract T_0 slope is stronger than of the extract T_{Aq}. However, the extracts $T_{1\text{-}2}$, $T_{2\text{-}2}$, $T_{3\text{-}2}$ and $T_{4\text{-}2}$ presented curves whose slopes are stronger in particular the extract $T_{4\text{-}2}$. The extract $T_{2\text{-}1}$ has curve slope as average. While, the weakest slopes are observed with $T_{1\text{-}1}$, $T_{3\text{-}1}$ and $T_{4\text{-}1}$.

Moreover, the analysis of all these results shows that the tested isolate is sensitive to drugs and the progressive decrease in the number of colonies in the experimental tubes confine the dose-response relationship of each extracts. The curves of sensitivity of each extract allow comparing their performance. Indeed, more the slope of a curve is strong more the extract is active on the germ.

It rereleases from the analysis of the crude extracts (T_{Aq} and T_0) that the extract T_0 is 2 times more active than the extract T_{Aq} on the *in vitro* growth of *Cryptococcus neoformans* (extract

T_0: MFC = 97.5 µg / mL and extract TAq: MFC = 195µg / mL) (Table 1). Besides the aqueous extract results are better than those obtained by Fyhrquist, 2007 [10] on the *in vitro* growth of *Cryptococcus neoformans*. In fact, the aqueous extracts of *Combretum molle*, *Combretum padoïdes* and *Terminalia kaiserana* gave as values for MFC: 12500µg/ mL, 6250µg/mL and 1560 µg/mL respectively. The extract TAq is still 64 times, 32 times and 8 times respectively more active than the aqueous extracts of these different species. Indeed, the crude extracts T_{Aq} and T_0 is 32 times more active than the aqueous extract (MFC = 6250µg/mL) and hydro-alcoholic extract (MFC = 3125µg/mL) of *Thonningia sanguine*a on the *in vitro* growth of *Cryptococcus neoformans* [18].

Besides, the results of 8 other extracts reveal that the sensitivity curve of extract T_{4-2} presents the strongest slope (Fig. 1) and the values of its antifungal parameters are the best (Table 1). Comparing this extract to others based on the values of MFC show that the T_{4-2} extract is still 32 times more active than extracts T_{3-1} and T_{4-1}, 16 times more active than T_{1-1}. It is also 8 times more active than the extract T_{2-1} and 2 times that extracts T_{1-2} and T_{3-2}. As for the T_{2-2} extract, it presents the same value but comparison made based on the value of IC_{50} reveal that the T_{4-2} extract is the most active (IC_{50}= 5.87µg/mL against 10µg/mL for T_{2-2}). Furthermore the comparison based on the values of MFC of T_{4-2} extract to those of the crude extracts, it emerges that this extract is still 8 times more active than T_{Aq} and 4 times more active than its basic extract (T_0). Therefore from the analysis of the whole of these results, it has been revealed that this extracts T_{4-2} has the best antifungal properties.

In addition, comparing the results of the most active extract (T_{4-2}) to those on the *in vitro* growth of *Cryptococcus neoformans* with the hexane and acetate extracts of *Mitracarpus frigidus*, reveal that the extract T_{4-2} is still 13 times and 3 times more active than these hexane extracts (MFC = 313µg/mL) and acetate extract (MFC = 78µg/mL) respectively [8]. Moreover, statistical analysis performed using data from the extracts tested is characterized by an analysis of variance to a factor. It's about the effect of the concentration of the extract tested on the survival of the organism tested. These statistics data with the completely randomized is accurate α = 5% level of confidence ($p < 0.05$).For each sample tested, calculated F (Fcal) is always strictly less than the theoretical F (Ftab): (Fcal <Ftab). We accept the null hypothesis Ho at 95% confidence level. There are not significant differences between the different tests for each extract.

Moreover, a previous study has revealed that the most active extract T_{4-2} is very rich in Polyphenols and in quinones. These compounds would be at the origin of the antifungal activity of this extract and even the plant [25]. The antifungal activity of phenols and the quinones were proved by other researcher [7,8,13].

5. CONCLUSION

This study confirms the use of this specie of Combretaceae in the traditional medicine. It reveals its antifungal potential and was highlighted:

- The tested isolate *Cryptococcus neoformans* is sensitive to the drugs according to dose-response relationship.
- The crude extract hydro-alcoholic is the most active (2 times more active than the aqueous extract).
- Among partition extracts, the most active is the extract T_{4-2}. It is still 4 times more active than its basic extract T_0.

- The obtaining method of T_{4-2} constitutes the best way of the principle active selection and concentration.
- The aqueous phases of various partitions hold the best performance.
- The most active extract T_{4-2} is rich in polyphenols and in quinones. They would be at the origin of the antifungal activity.

It would be thus desirable to pursue this works by committing a series of chromatography which could certainly allow to optimize the antifungal activity and to determine the chemical compounds of this activity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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