Original Research Article

Phytochemical analysis and antifungal activity of aqueous leaf extract of *Trema guineensis* (ulmaceae), ~~a plant from the~~ Ivoirian ~~pharmacopoeia~~

ABSTRACT

Trema guineensis is a woody plant distributed in tropical forest which leaf, and back extracts are traditionally used for the treatment of various diseases including fever, bronchitis, and gastrointestinal disorders. Previous studies have highlighted their antibacterial activity. So, present work was designed to examine the phytochemical and antifungal properties of aqueous crude leaves extract of *T. guineensis*. The bioactive components extracted from the leaf was tested against pathogenic fungi using the agar tube dilution method. Antifungal activity of aqueous leaves extracts was carried out against selected pathogenic fungal strains as *Aspergillus fumigatus, Cryptococcus neoformans* and *Candida albicans*. The phytochemical analyses of the aqueous crude extract revealed the presence of secondary metabolites widely reported as antifungal such as flavonoids, saponins, quinones, alkaloids, polyphenols.The aqueous crude leaves extract of *T. guineensis* was effective in inhibiting the fungal growth. ~~Extract from leaves~~ were active against *A. fumigatus, C. neoformans* and *C. albicans* with MIC and MFC ranged from 20 to 200 mg/mL and 100 to 400 mg/mL respectively. The efficient antifungal activity of *T. guineensis* from the present investigation revealed that aqueous leaf crude extract of the selected plant had a moderate potential to inhibit the growth of pathogenic fungal strains. This finding showed that the aqueous extract of *T. guineensis* exerted an antifungal effect on *C. albicans, A. fumigatus* and *C. neoformans* and supports its traditional use in herbal medicine.

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Keywords: Trema guineensis, Antifungal activity, Aqueous extract, minimum inhibitory concentration (MIC).

1. INTRODUCTION

Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. In many developing countries, traditional medicine is one of the primary healthcare systems [1;2]. Herbs are widely exploited in the traditional medicine and their curative potentials are well documented [3]. About 50% to 60% of pharmaceutical products are natural origin or synthesized from natural products [4]. The use of medicinal plants has gained a wide recognition as a result of its safety, low cost and effectiveness [5]. However, scientific studies have been conducted only to a limited extent with few medicinal plants [6].

In Ivory Coast, traditional medicines are increasingly sought from traditional practitioners and herbalists for the treatment of various diseases. Among the plants used, *Trema guineensis* (Ulmaceae) is a woody plant distributed in the west central part of Ivory Coast. The leaves are locally used for the treatment of various diseases including cardiac failure and constipation [7]. The *in vivo* anti-inflammatory activity of this plant has been demonstrated by [8]. ~~Elsewhere in West Africa, South-West Nigerian communities used the leaf and back extracts of *T. guineensis* for the treatment of fever, bronchitis, pneumonia, and gastrointestinal disorders.~~ Its phytochemical analysis indicated the presence of several

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secondary metabolites such as polyphenols, alkaloids, flavonoids, saponosids and tannins [9], ~~which presence~~ could confer to the plant, several pharmacological activities including the analgesic activity. Previous studies conducted by Akinyemi *et al.,* [9, 10] revealed that aqueous of *T. guineensis* had antibacterial activity against strains of three food borne pathogens that resisted conventional orthodox antimicrobials.

In recent times, no scientific report regarding the antifungal activity of *Trema guineensis* extract has been published. After scrutiny of published literature showing its medicinal importance, the present protocol has been outlined regarding the antifungal activity on this plant aqueous extracts. The aims of this study were therefore to evaluate antifungal activity of aqueous extract of *T. guineensis* against some pathogenic fungi (*C. albicans, A. fumigatus* and *C. neoformans*) and to identify which components are deeply involved in this activity.

2. MATERIAL AND METHODS

2.1 Plant collection and authentication

Fresh leaves of *T. guineensis* (*Ulmaceae*) were collected from Korhogo and Abidjan (Ivory Coast) in february 2017. The botanical authentication of this plant was done by the herbarium of National Floristic Center of University FELIX HOUPHOUET BOIGNY (Abidjan, Ivory Coast), where a voucher specimen was conserved with reference number 8536; 10881 and 13968. The collected leaves were hand plucked aseptically and cleaned for debris using tap water and then rinsed ~~in sterile~~ distilled water. The leaves were shade dried at room temperature. The dried leaves were powdered using an electric blender (IKAMAG RCT®) and powdered samples were stored in airtight glass containers protected from sunlight for subsequent extraction and further bioassay.

2.2. Aqueous extract preparation

The powder of *T. guineensis* was used to prepare aqueous extract. Briefly, 100 g of the powder was soaked in 1 L of distilled water for 24 hours at room temperature, under shaking using a mixer. The extracts obtained were filtered successively twice with absorbent cotton then once on Whatman filter paper number 1. After that, the resulting filtrate was concentrated under vacuum by using a Büchi rotary evaporator at 50°C [14]. The dark obtained powder constituted the aqueous extract (AqEx) used for the phytochemical screening and *in vitro* antifungal tests.

2.3. Fungi

Fungi strains, *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans* were obtained from the Laboratory of Mycology / Pasteur Institute, Côte d'Ivoire. These strains have been isolated from patients with deep mycoses. The fungi cultures were cultivated in Sabouraud agar (Bio-RAD/ref: 64494, lot: 7C2219) and incubated for 48 h at 30°C.

2.4 Phytochemical screening

The extracts were subjected to preliminary phytochemical testing to detect for the presence of different chemical groups of compounds. Air-dried and powdered plant materials were screened for the presence of saponins, tannins, alkaloids, flavonoids, terpenoids, steroids, quinones by chemical test.

2.4.1 Test for Alkaloids

0.5 g of extract was diluted to 10 mL with acid alcohol, boiled and filtered. To 5 mL of the filtrate was added 2 mL of dilute ammonia. 5 mL of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 mL of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and

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Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids [11].

2.4.2 Tests for Flavonoids

Few fragments of magnesium metal ribbon (3-4 pieces) was added to 1 mL of the extracts, followed by drop wise addition of concentrated hydrochloric acid. Formation of pink or red color indicated the presence of flavonoids [12].

2.4.3 Test for quinones

A quantity (0.5 g) of the extract was boiled with 10 mL of sulphuric acid (H_2SO_4) and filtered while still hot. The filtrate was shaken with 5 mL of chloroform. The chloroform layer was pipette into another test tube and 1 mL of dilute ammonia was then added. The resulting solution was observed for color changes [11].

2.4.4 Test for Saponin

The 2 mL of distilled water was added to extracts suspended in ethanol and was shaken vigorously. The formation of profuse foam layer indicated the presence of saponins [12].

2.4.5 Test for Tannins

Extracts were treated with 1 mL of 5% ferric chloride. The presence of tannin was indicated by the formation of bluish black or greenish black precipitate [13].

2.4.6 Test for Terpenoids

5 mL of each extract was added to 2 mL of chloroform and 3 mL of concentrated H_2SO_4 to form a monolayer of reddish brown coloration of the interface was showed to form positive result for the terpenoids [11].

2.4.7 Test for polyphenols

Two milliliters of extract were added a drop of alcoholic solution of ferric chloride at 2%. The appearance of a dark green or lighter or darker blue color indicated the presence of polyphenolic derivatives.

2.5 Assay for antifungal activity

The inhibitory effect of *T. guineensis* leaf aqueous extract against fungi's strains was measured by agar tube dilution method and minimum inhibitory concentration (MIC) [15]. Sabouraud agar (10 mL) was dispensed in screw capped tubes or cotton plugged test tubes. Then, Sabouraud agar was loaded with plant extracts using the method of the double dilution agar slopes. Tests tubes consisted of eight tubes containing plant extract and two controls tubes without plant extracts (one serving as control for the growth of germs; the other without germs serving as a witness sterility controls of the culture medium). For the eight tests tubes, concentrations of plant extract ranged from 400 to 3.125 mg/mL binding by a geometrical reason of ½. After incorporation of extract in agar, all tubes were autoclaved at 121°C for 21 min. The tubes containing the media were then allowed to solidify in slanting position at room temperature. Standard drug Ketoconazole USP (20 mg) was used as a positive control. The tubes containing solidified media and plant extract were inoculated with a previously prepared inoculum (10^5 cells/mL), obtained from culture cells of fungus on day 2. The tests tubes were then incubated at 30 °C for 48 hours. After this period, the colonies were counted and growth in experimental tubes was determined. Growth in the eight tubes of each experimental series was assessed as a percentage of survival compared to 100% survival in the control tube growth control [16]. The formula of this calculation has been mentioned below. Control experiments were carried out under similar condition by using Ketoconazole USP (20 mg) for antifungal activity as standard drugs. Treatment of

experimental data was used to determine the antifungal parameters (MIC, MFC, IC_{50}). Values of IC_{50} (concentration producing 50% inhibition) were determined on the survival curves of microorganism's strains established with Graph Pad software, U.S.A. The MIC values were taken as the lowest concentration that inhibited the visual growth of the tested organisms. Minimum fungicidal concentration (MFC) was determined by culturing 20 µL of the mixed broth culture from the tubes with no visible turbidity on Sabouraud at 30ºC for 48 h on the MIC assay. The MFC was defined as the lowest concentration completely inhibiting the fungi's growth. [17, 18]. The mechanism of antibiosis (fungicidal or fungistatic) of the extracts was calculated using the ratio of MFC/MIC [19] to elucidate whether the observed antifungal effect was fungicidal or fungistatic. When the ratio of MFC/MIC is ≤ 2.0, the extract is considered fungicidal or otherwise fungistatic. If the ratio is ≥ 16.0, the extract is considered ineffective. All assays were carried out in three replicates to ensure accuracy.
Formula to calculate the survival percentage:

$$S = \frac{n}{N} \times 100$$

S: The survival (%)
N: Number of colonies in one experimental tube and
n: Number of colonies in the witness tube of growth control

2.6 Statistical analysis

All experiments were repeated at least in triplicate and the results were presented as the average value with standard deviation.

3. RESULTS

The results of the preliminary phytochemical screening of aqueous plant leaf extracts of *T. guineensis* revealed the presence of various bioactive agents. The leaves aqueous extracts were found to contain compounds such as flavonoids, polyphenols, quinones, saponins and alkaloids ~~but leaves extracts were negative for tannins~~ and terpenoids (Table 1).

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Table 1 : Phytochemical analysis of *T. guineensis* leaves extracts.

Sample	Tannin	Alkaloid	Polyphenol	Flavonoid	Terpenoid	Saponin	Quinone
***Trema guineensis* leaf**	-	+	+	+	-	+	+

+: Present, **-**: Absent

The antifungal activity of the aqueous extracts of *Trema guineensis* were studied in different concentrations (3.125; 6.25; 12.5; 25; 50; 100; 200 and 400 mg/mL) against three fungal strains (*Aspergillus fumigatus*, *Cryptococcus neoformans*, *Candida albicans*). Antifungal effect of *T. guineensis* extract of was quantitatively assessed by measuring minimum inhibitory concentration (MIC, mg/mL), minimum fungicidal concentration (MFC, mg/mL), IC_{50} (mg/mL), and the efficacy ratio (MFC/MIC). The results of the antifungal activities are presented in Table 2. The assays showed that the fungi exhibited varied susceptibilities to

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the extract at different concentrations used. The fungi were sensitive to the aqueous extract tested in a dose-response relationship. The survival percentage of fungal germs decreased with increasing concentration of aqueous extracts (figure 1). The three fungal strains were sensitive to the aqueous extract tested. Thus, the sensitivity of aqueous extract decreases in the following order: *C. albicans* > *C. neoformans* > *A. fumigatus. A. fumigatus* is the most sensitive seed of the aqueous extract (MIC = 50± 0.00 mg/mL). *C. neoformans* has moderate sensitivity (MIC = 100 ± 0.00 mg/mL), whereas *C. albicans* has low sensitivity (MIC = 200 ± 0.00 mg/mL) (Table 2).

Table 2 : Antifungal parameters crude aqueous extract of *T. guineensis* against clinical fungi isolates (*C. albicans, C. neoformans and A. fumigatus*).

Fungi strains	Extract	*Antifungal parameters* (mg/mL)			MFC/MIC	Antifungal effect
		MIC	MFC	IC_{50}		
Aspergillus fumigatus	AqEx	50 ± 0.00	100 ± 0.00	2.94 ± 0.003	2	Fungistatic
	Ket	0.156 ± 0.00	0.313 ± 0.00	0.078 ± 0.0001	2	Fungistatic
Cryptococcus neoformans	AqEx	100 ± 0.00	200 ± 0.00	8.82 ± 0.003	2	Fungistatic
	Ket	0.039 ± 0.00	0,039 ± 0.00	0.012 ± 0.00	1	Fongicidal
Candida albicans	AqEx	200 ± 0.00	400 ± 0.00	14.70 ± 0.006	2	Fungistatic
	Ket	0.039 ± 0.00	0.039 ± 0.00	0.012 ± 0.00	1	Fongicidal

AqEx : Aqueous extract ; Ket : ketoconazole

In addition, taking into account both MFC and IC_{50}, the aqueous extract presented the highest sensitivity with *A. fumigatus* (MFC = 100 ± 0.00 mg / mL , IC_{50}= 2.94±0.00 25 mg/mL) followed respectively by *C. neoformans* (MFC = 50 mg/mL, IC_{50} = 8.82±0.003 mg/mL) and *C. albicans* (MFC = 400 mg/mL, IC_{50} = 14.33±0.006 mg/mL).

Regarding the antifungal effect of *T. guineensis* aqueous extract and ketoconazole, the results recorded indicate that the MFC/MIC activity varies between 1 and 2. Ketoconazole showed fungicidal action on the strains tested excepted on *A. fumigatus,* while aqueous extract of *T. guineensis* showed fungistatic action against the three strains studied (*A. fumigatus, C. neoformans* and *C. albicans*)**.** This study also showed that, ketoconazole standard antifungal molecules was more active than water crude extracts of *T. guineensis* against the three fungi strains as revealed by the MICs and MFCs values calculated (Table 2).

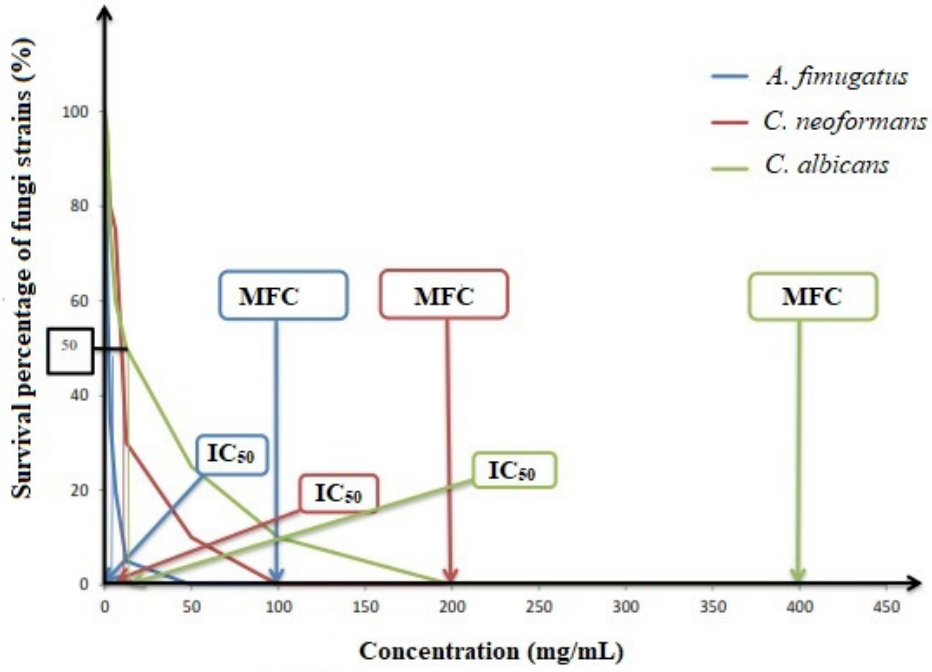

Figure 1 : : Effect of aqueous extract on *in vitro* growth of *A. fumigatus, C. neoformans and C. albicans.*

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4. DISCUSSION

~~The search for new antifungal drugs is very necessary in the face of current resistance of pathogens to the present chemotherapy [20].~~ Antifungal activity of aqueous crude leaf extract of *T. guineensis* have been evaluated in the present research work. The *in vitro* antifungal activity of plant extract is a first step toward the development of new potential drugs. The results obtained from this investigation showed that the highest antifungal activity was exhibited by the standard drugs Ketoconazole USP and the lowest by the aqueous extracts. Thus, the aqueous crude extract of *T. guineensis* revealed antifungal activity against the three fungal strains (*A. fumigatus, C. neoformans* and *C. albicans*) in different degree. *A. fumigatus* was more sensitive to aqueous extract (MIC = 50± 0.00 mg/mL) than *C. neoformans* (MIC = 100 ± 0.00 mg/mL and *C. albicans* (MIC = 200 ± 0.00 mg/mL). The basis of varying degree of sensitivity of test organisms of fungi may be due to the intrinsic tolerance of microorganisms and the nature and combinations of phytocompounds presents in the crude extracts. This result also supported

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the reports of other researchers that plants extracts were very effective in the treatment of candidiasis [21, 22]. However, this activity, compared to other should be considered moderate. In fact, the analysis revealed that water extract of *T. guineensis* (MFC = 400 mg/mL) is less active on *C. albicans* than aqueous extract of *Terminalia catappa* (MFC = 0.78 mg/mL) [23]. Similarly, previous studies of Ouattara *et al.* [24], have shown a highly effective inhibition by *Terminalia ivorensis* (MFC= 0.195 mg/mL) on pathogenic fungi *A. fumigatus*. The moderate's values recorded for the plant extracts may be attributed to the fact that the extracts being in crude form, contain very small amounts of bioactive compounds. Probably, a more refined preparation would have antifungal activity at a lower concentration. It is known that the potency of the plant extracts depends on the solvent used, and this may be due to the degree of solubility of the bioactive constituents. Thus, it has been documented that different solvents have diverse solubility capacities for different phytochemicals [25]. The above results also showed that aqueous crude extracts of *T. guineensis* had fungistatic action against all fungi's strains. It is worthy of note that MFC values obtained for the extracts against fungal strains are higher than MIC, indicating that the extract is fungistatic. The phytochemical screening revealed the presence of various bioactive agents such as flavonoids, polyphenols, saponins, alkaloids and quinones. In a previous finding, Akinyemi *et al.* [9], reported the presence of additional phytoconstituents, such as tannins in leaf extract of *T. guineensis*. There is therefore a difference between our results and those of these authors at the level of the major phytochemical components of *T. guineensis*. Such difference may be explained by several factors. Indeed, according to Sofowora [26], the composition of plant in secondary metabolites varies according to the geographical location, the organ taken, the period of sampling, the time of sampling, the storage conditions, and the solvent for extraction. The degree of the antifungal activity of the extract may be accounted for by the presence of flavonoids as indicated in the phytochemical screening of *T. guineensis* [9]. The detected compounds in this extract may be responsible for antimicrobial observed activity of the plant and thus justifying it traditional use as medicinal plants for many diseases. This agrees with previous reports that associated the antifungal activity of the plant extracts to the presence of flavanoids, steroids, alkaloids and triterpenoids and other natural polyphenolic compounds or free hydroxyl groups [27, 28].

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4. CONCLUSION

The present study focused on the effect of *T. guineensis* leaf ~~water~~ crude extract on *C. albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. ~~and showed its effective~~ antifungal activity. This study justified the claimed uses of leaves in the traditional system of medicine to treat various infectious disease caused by the microbes. This study will add value to the medicinal properties of this plant and urge this plant to be taken forward as

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novel agents against drug resistant microbes. However, further studies are needed to better evaluate the potential effectiveness of the crude extracts as the antifungal agents. More research is required to toxicity testing, isolate active compounds, elucidate the structures of this plant to develop new antifungal and antimicrobials therapeutic principles.

Comment [aa16]: This study showed that this plant could be used as new antimicrobial agents instead of drugs.

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