The antifungal activity of methanol and ether extracts of the leaves of *Leonotis nepetaefolia*

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Abstract

A survey done on Rwandese traditional healers revealed that *Leonotis nepetaefolia* has got many therapeutic uses in Rwanda. This research was carried out on the leaves of this plant to verify its efficacy in the treatment of antifungal diseases. Preliminary phytochemical analysis of leaves of the plant indicated the presence of tannins, flavones, quinones and saponins. The antifungal test of the crude methanol and the crude ether extracts was realized and revealed that crude methanol extract was more active than crude ether extract on *Candida albicans* and *Malassezia fulfur* growth. The minimum inhibitor concentration (MIC) of the crude methanol extract were 4.12 mg/ml and 2.38 mg/ml respectively for the growth of *C. albicans* and *M. fulfur* while those obtained from crude ether extract were 4.95 mg/ml and 3.7 mg /ml respectively for *C. albicans* and *M. fulfur*. Phytochemical screening, physical and biological methods were used to partially characterize the antifungal products contained in the crude methanol extract. Open column chromatography was used to fractionate these compounds and led to seven separated fractions and to the test of each fraction for antifungal activity. Fractions F_3 and F_7 inhibited completely the growth of *C. albicans* and *M. furfur*. The MIC of F_3 were found at 1.45 mg/ml and 2.38 mg/ml respectively for *C. albicans* and *M. fulfur* while those of F_7 were 1.92 mg/ml and 1.45 mg/ml respectively for *C. albicans* and *M. fulfur*. Phytochemical screening revealed that tannins and quinones compounds are responsible of this activity.

Key words*: Leonotis nepetaefolia,* leaves, natural products, phytochemical screening, chromatography, *Candida albicans*, *Malassezia fulfur*, antifungal activity.

Résumé

Un aperçu fait sur les guérisseurs traditionnels rwandais a indiqué que le nepetaefolia de *Leonotis* a beaucoup d'utilisations thérapeutiques au Rwanda. Cette recherche a été effectuée sur les feuilles de cette usine pour vérifier son efficacité dans le traitement des maladies antifongiques. L'analyse phytochimique préliminaire des feuilles de l'usine a indiqué la présence des tannins, des flavones, des quinones et des saponines. L'essai antifongique du méthanol brut et des extraits d'éther bruts a été réalisé et a indiqué que l'extrait brut de méthanol était plus en activité que l'extrait d'éther brut sur la croissance d'albicans de candida et de fulfur de Malassezia. La concentration minimum en inhibiteur (MIC) de l'extrait brut de méthanol étaient de 4.12 mg/ml et de 2.38 mg/ml respectivement pour la croissance des albicans de C. et du fulfur de M. tandis que tels obtenus à partir de l'extrait d'éther brut étaient de 4.95 mg/ml et de 3.7 mg/ml respectivement pour les albicans de C. et le fulfur de M. Des méthodes de criblage, physiques et biologiques phytochimiques ont été employées pour caractériser partiellement les produits antifongiques contenus dans l'extrait brut de méthanol. La chromatographie sur colonne ouverte a été employée pour fractionner ces composés et mené à sept a séparé des fractions et à l'essai de chaque fraction pour l'activité antifongique. Le F-3 et le F7 de fractions ont empêché complètement la croissance des albicans de C. et du furfur de M. La MIC du F-3 ont été trouvées à 1.45 mg/ml et à 2.38 mg/ml respectivement pour les albicans de C. et le fulfur de M. tandis que tels de F7 étaient de 1.92 mg/ml et de 1.45 mg/ml respectivement pour les albicans de C. et le fulfur de M. Le criblage phytochimique a indiqué que les composés de tannins et de quinones sont responsables de cette activité.

Mots clés *:* Nepetaefolia de Leonotis, feuilles, produits naturels, criblage phytochimique, chromatographie, albicans de candida, fulfur de Malassezia, activité antifongique.

INTRODUCTION

Leonotis nepetifolia belongs in Angiospermae phylum to Labiatae (Lamiaceae) family. It is known as Lion's Ear in English and as Igicumucumu in Kinyarwanda. Entheogenicly it is reported that *Leonotis nepetifolia* is stronger in active constituent than cousin *Leonotis leonorus* especially when smoked. In Namibia the leaves of this plant are used by women for menstrual problems as they are antispasmodic and stop bleeding. They are also purgative, and have been used to induce abortion. Decoctions of *L. nepetifolia* have also been used to promote menstruation in amenorrhea **[1]**. In Madagascar, the plant is used to treat rheumatism and dermatophyte diseases **[2]**. In the Guiana, people use this plant to deal with cramps and diarrhoea, and as diuretic, for skin stones, skin diseases, swelling, thrush, uterine contractions, wounds, and yaws. According to Rwangabo **[3]** and Ntezurubanza **[2]**, respectively the ash and aqueous extract of the leaves of this plant are used to treat different diseases including dermatophyte and mixed skin infections. A survey done on Rwandese traditional healers showed that the leaves of the plant taken alone or in association of other indigenous plants are used to treat many fungal diseases. However, few data on scientific proof of its efficacy are available.

This paper describes our investigation on the effect of crude methanol and crude ether extracts as well as fractions obtained from methanol extract on two fungi such as *M. furfur* and *C. albicans*. These two fungi are widely known in Rwandese fungal diseases. Research on fungal diseases has been neglected because people consider these as benign. However, some researches done in this domain showed that when not treated, they could conduct to complication and in some cases to the death **[4]**. Many antifungal drugs are available. Unfortunately those are not accessible by all patients because they are expensive and associated with secondary effect **[4, 5]**. The data from traditional medicine, when well exploited, could give answer to these problems. In this study *L. nepetaefolia* indicated that it could be used in the treatment of mycoses.

2. MATERIALS AND METHODS

Plant material

The leaves of *L. nepetaefolia* (Figure 1) were collected at Mamba (Butare town in Rwanda) during sun season (July, 2004). They were well identified by Dr Ntaganda Charles, botanist and lecturer of Sciences Faculty, National University of Rwanda and a voucher specimen number 20349 was deposited in the Rwandese National Herbarium in the Centre de Recherche en Phytomédicaments et Sciences de la vie (IRST, Butare). Leaves are then washed with distilled water and left to dry at room

temperature. Then, they were milled into powder using electric blinder, Janke-Kunker.

Figure 1: *Leonotis nepeatafolia*

Phytochemical screening

Standard methods for photochemical screening **[3, 6, 7-11]** were used to identify the main groups of compounds found in the leaves of *L. nepetaefolia* and to partially characterize the active fractions. Mayer, Dragendorff and Wagner reagents were used to test the presence of alkaloids while Salkowski and Liebermann Buchard reagents are used for the presence of terpenoids sterols. The presence of Quinones and flavonoids were evaluated respectively with Borntrager and Willstater reactions and Saponosides were identified using foam test. The presence of anthocyane was evaluated by both, acid and basic reagents while tannins were evaluated by heavy metals reagents (CuSO4 and $FeCl_3$).

Preparation of extracts
250 g of powder were macerated in 650 ml of sprit ether during three days under stirring. The filtrate was recuperated and the solvent was evaporated under diminished pressure at 30° C to obtain fatty yellow extract (crude ether extract). This process was repeated three times on the same plant material. The residue obtained after filtration was left to dry before to be submitted to another extraction with methanol as solvent. The evaporation of methanol under diminished pressure at 40° C left the methanol extract as the green residue.

Fractionation of methanol extract
Methanol extract was fractionated using open column chromatography, CC. The stationary phase and mobile phase were respectively silica gel and sprite ether/ethyl acetate (70:30). This CC was monitored by thin layer chromatography, TLC (silica gel 60 F254, Merck). Seven fractions were obtained from this column chromatography and tested for antifungal activity.

Preparation of drugs
250 mg of crude methanol extract or each fraction were resolved in 1 ml of methanol and completed to 5 ml with physiological saline. In the case of ether extract, the same mass was resolved in 1 ml of dimethyl sulfoxide (DMSO) and completed as previously done. Different volumes of drugs ranging from 0.4 ml to 1.1 ml were used to test the antifungal efficacy of each extract.

Microorganisms
Two pathogenic fungi, *C. albicans* and *M. fulfur*, used during this study, were isolated by Microbiology department of the University clinical laboratory, National University of Rwanda. Strains suspended in sterile saline solutions (0.9 %) were obtained from the laboratory and directly used without doing any other manipulation.

Medium culture and Antifungal essay
To test for antifungal activity, the agar dilution method with superficial inoculation was used. 45.5 g of Sabouraud medium culture were dissolved in 1 l of distilled water. The solution obtained was sterilized at 118° C for 15 min. 10 ml of the sterilised solution containing different concentration of inhibitors was poured in Petri dish and left open to solidify in a laminar flow hood. Triplicate Petri dishes were used for each test experiment. Inoculate of *C. albicans* or *M. fulfur* were applied to the surfaces of the plates with steel replicator. Control Petri dishes without any inhibitors as well as those containing 1ml of methanol or DMSO were also inoculated in the same time of the test experiment. All Petri dishes were then incubated at 37° C for 5 days. The growth inhibition of fungi was evaluated over the control experiments.

RESULTS

Phytochemical screening

Phytochemical identification realized on the powder as well as on the crude extracts from the leaves of *Leonotis nepetafolia* showed that they contain quinones, saponosides, flavonoids and tannins. The high amount of quinones was remarked in both methanol and ether extracts while saponins were more well extracted by methanol compared to ether. Tannins and flavonoids found in methanol extract were completely absent in ether extract (Table 1).

Table 1: Phytochemical screening of crude powder, methanol and ether extracts of the leaves of *Leonotis nepetaefolia*

group tested / ***Sample***	Alkaloids	Terpénes-.Stérols	Quinones	Saponosides	Flavonoïdes	Anthocyanidins	Tannins
Crude powder	-	-	+ +	+ +	+ +	-	+ +
EE	*	*	+ +	+	-	*	-
ME	*	*	+ +	+ +	+ +	*	+ +

EE_L: Ether extract
EM_L: Methanol extract
+: few amount of compounds detected
+ +: high amount of compounds detected
-: None detected
*****: test not realized

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Antifungal activity of crude methanol (ME) and crude ether (EE) extracts

Crude methanol and crude ether extracts of the leaves of *Leonotis nepetafolia* showed antifungal activity against *C. albicans*. Methanol extract inhibited the growth of *C. albicans* at the concentration of 4.12 mg /ml while ether extract showed the same activity at the concentration of 4.95 mg/ml. Dose of 2.38 mg /ml of ME inhibited the growth of *M. fulfur* while the same activity was found from ether extract (EE) at the dose of 3.7 mg / ml. Control experiments including 1 ml of solvents, methanol or DMSO, did not show any activity against the growth of all those fungi. All these results were observed in comparison with control experiment (Table 2). The determination of minimal inhibition concentration (MIC) was done using the following mathematical formula :

$$\frac{Q \times 250}{5(10+Q)}$$

This in accord that we dissolved 250 mg of crude extract in 5 ml of solution and from this, Q ml was diluted in 10 ml of culture medium. From this mathematical formula we found that the MIC of ME were 4.12 mg/ml and 2.38 mg/ml for *C. albicans* and *M. fulfur* respectively, while MIC of EE

were 4.95 mg/ml and 3.70 mg/ml respectively for *C. albicans* et *M. fulfur* (Table 2).

Table 2: Effect of crude methanol and ether extracts at different doses on the growth of *C. albicans and M. fulfur*

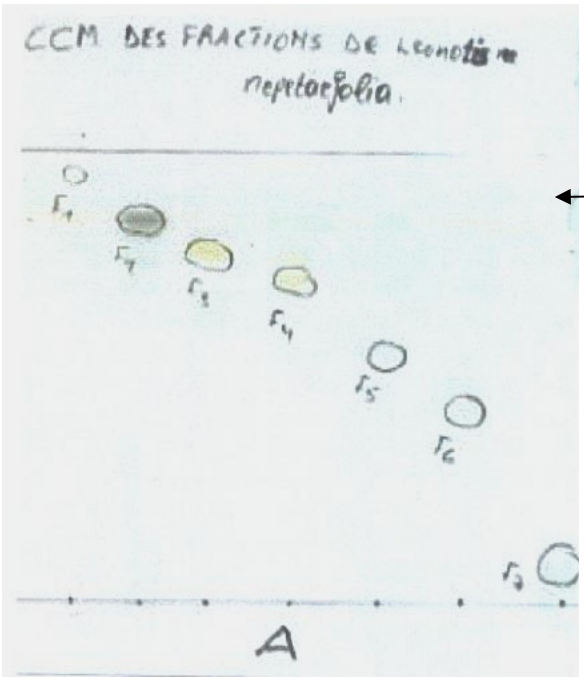
		Microorganisms	
Compounds	**Doses (mg /ml)**	***Candida albicans***	***Mallassezia fulfur***
ME	1.92	ND	+
	2.38	ND	-
	2.83	ND	-
	3.27	+ +	-
	3.7	+	-
	4.12	-	-
	4.55	-	-
	4.95	-	-
EE	3.27	+ +	+
	3.7	+	-
	4.12	+	-
	4.55	+	-
	4.95	-	-
MET	1 ml	+ + +	+ + +
DMSO	1ml	+ + +	+ + +
CTL	-	+ + +	+ + +

ME: Methanol extract
EE: Ether extract
MET: methanol
DMSO: Dimethyl sulfoxide
CTL: Control

-: No growth observed
+: small growth was observed
+ +: fungi were grown in high quantity
+ + +: maximum growth
ND: experiment not done

Isolation and partial identification of active agents

Methanol extract was fractionated by column chromatography to give seven fractions characterized by their retardation factors on TLC which are 0.93, 0.87, 0.80, 0.74, 0.63, 0.42 and 0.10 respectively for F_1, F_2, F_3, F_4, F_5, F_6 and F_7 (Figure 2) and their colours in visible light.



Solvant front

Colour of fractions :
F_1, F_3, F_4 and F_6: yellow
F_2 and F_7: green
F_5: dark greenish

Figure 2: TLC chromatogram of fractions from r

All these fractions were tested for antifungal a[illegible] *albicans* and *M. fulfur*. Fractions F_3 and F_7 inhibited completely the growth of *C. albicans* and *M. furfur*. MIC of F_3 were found at 1.46 mg/ml and 2.38 mg/ml respectively for *C. albicans* and *M. fulfur* while those of F_7 were the 1.92 mg/ml and 1.46 mg/ml respectively for *C. albicans* and *M. fulfur 5* (Table 3).

Table 3: Effect of different fractions from methanol extract on the growth of *C. albicans* and *M. fulfur*

Microorganisms	Doses (mg / ml)												Control
	F_1	F_2	F_3				F_4	F_5	F_6	F_7			
	4.54	4.54	0.98	1.46	1.92	2.38	4.54	4.54	4.54	0.98	1.46	1.92	
Candida albicans	+	+	+	-	-	-	+	+	+	+	+	-	+
Mallassezia fulfur	+	+	+	+	+	-	+	+	+	+	-	-	+

-: *No growth observed*
+: *Maximum growth*

The active fractions were identified using standard methods for quinones, saponins, flavonoids and tannins, which group of compounds, were detected from crude methanol extract (Table 1). Fraction F_3 was found to contain tannin while fraction F_7 was identified as quinonoid compounds (table 4).

Table 4: Partial identification of active compounds

Group of compounds tested	Fractions	
	F3	F7
Quinones	-	+
Saponins	-	-
Flavonoids	-	-
Tannis	+	-

+: *Presence of compound tested*
-: *Absence of compound tested*

Discussions and conclusions

During this study two fungi has been used, *C. albicans* and *M. fulfur*. Those fungi are widely found in human infections and in some cases they can cause serious problems to patients.

C. albicans, an ascomycota subphylum of saccharomycotina is a causal agent of opportunistic oral and vaginal infections in humans. Under normal circumstances, *C. albicans* lives in 80% of the human population with no harmful effects. However under overgrowth it causes a wide variety of infections ranging from mucosal infection in generally healthy persons to life threatening systemic infection in individuals with impaired immunity. Oral and oesophageal Candida infections are frequently seen in Aids patients. Few classes of drugs are effective against these fungal infections and all of them have limitations with regard to efficacy and side effects **[12- 14]**.

M. fulfur is a lipophilic yeasts and causative agent of *Pityriasis versicolor or Pityriasis foliculitis*. Recently it has been implicated as a causative agent of seborrhoea dermatitis, folliculitis, atopic dermatitis and dandruff. Lesions of these fungi are chronic and usually present on the back and chest and, occasionally, on the neck, shoulders, upper arms and face **[15- 17]**.

During this study *L. nepetafolia* showed an antifungal efficacy for the inhibition of fungi growth. Crude methanol extract has been found to be more active than crude ether extract. The difference remarked could be explained, on one hand, by the fact that methanol extracted all groups of compounds found in the crude powder. Quinones, flavonoids, saponins and tannins are soluble in methanol; this is in conformity of the literature **[7, 8, 11]**. The second raison of the difference in activity between these crude extracts could be the presence of tannins in methanol extract, which group, not detected from ether extract. The antifungal assessment realized on the fractions of methanol extract showed that two fractions inhibited the growth of strains. These fractions exhibited their activity more strongly than crude methanol extract. This increasing of activity remarked could be raised from the presence of some products with negative synergistic effect,

in the crude extract. These could be separated during the column chromatography and left free active compounds.

The active fractions were identified as quinones and tannins compounds. The antifungal activity of tannins and quinones has been reported **[18-21]**. *Senna alata* was found to be active against the growth of *Candida albicans*. Quinonoid compounds like antraquinone aglycone and antraquinone glycosides were identified as responsible of the activity. Further more, the antifungal activity of naphtoquinone, another quinonoid compound, from *Tebebuia serratifolia* was also reported. In addition, the efficacy of tannins and quinones in the treatment of *Candida albicans* and *Mallassezia fulfur* diseases was found **[8, 11]**. All these research reports are in conformity of our founding which confirm the therapeutic use of *L. nepetafolia* in Rwandese traditional medicine. In conclusion, our study indicated that *L. nepetafolia* could be used in traditional medicine to treat diseases from *Candida albicans* and *Mallassezia fulfur*. However the toxicity profile of the plant must be done carefully before to confirm this conclusion.

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