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Activity of Methanol/Methylene Extract Mixtures from *Monodora myristica* (Gaertn), *Xylopia aethiopica* and *Eremomastax speciosa* (Hochst.) Against *Candida albicans*

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ABSTRACT

Background: Ethnopharmacological studies have been framework of well established biological properties of medicinal plants. Most of these scientific studies are performed on individual plants but natural medicine treatments most often use mixtures of plants. The present study was undertaken to investigate the activity of the methanol/methylene chloride extract mixture from *M. myristica*, *X. aethiopica* and *E. speciosa* against *Candida albicans* isolates. **Materials and Methods:** The plant extract was prepared by maceration in methanol/methylene chloride. phytochemical analysis was performed by chemical reaction method. The broth microdilution method was used to evaluate the *in vitro* activity against ten isolates of *C. albicans*. The *in vivo* antifungal activity of *X. aethiopica*: *M. myristica* mixture (1:1) was evaluated using a *Candida albicans* induced gastrointestinal infection in a rat model. **Results:** The results of the phytochemical tests indicate that alkaloids and triterpenes were present in all extracts, other classes of chemicals being selectively present. Extracts and their mixtures displayed various degrees of antifungal activities. *Xylopia aethiopica*: *M. myristica* mixture (1:1) with MIC values ranging from 32-256 μg mL^{-1} was found to have the best antifungal activity. This mixture at 200 and 400 mg kg^{-1} b.wt. were able to progressively and significantly reduce the fungal load in the faeces of the infected rats within the treatment period. **Conclusion:** The results of this study provide an important basis for the use of methanol/methylene chloride extract mixture from *M. myristica* and *X. aethiopica* to control infectious diseases caused by *C. albicans.* Further studies need to be carried out as to establish the role of *E. speciosa* in the mixture.

Key words: Antifungal activity, *Candida albicans*, dietary plants, medicinal plant, plant mixtures

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INTRODUCTION

Candida infections are a problem of growing clinical importance. The incidence of these infections has increased dramatically over the past two to three decades[1,2]. *Candida albicans* is the most common human pathogenic species implicated in a broad spectrum of diseases such as skin, mucosal and systemic infections (candidiasis)[3]. This microorganism is implicated in life threatening infections in immunocompromised individuals or when natural barriers are damaged. The patterns of antifungal susceptibilities vary almost as greatly as the organisms themselves. In addition, the widespread use of antifungal agents may have contributed to a shift in species distributions via the emergence of inherently resistance.

The use of medicinal plants in traditional medicine has generated a lot of interest and concern about their efficiency and safety margin. Medicinal plants have become the frame work of many scientific studies[4]. They have led to the discovery of new molecule or extract with well established activities against human diseases. Nevertheless, these useful results hide the

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ethnopharmacological relevance. Although, several useful results of individual plant extract and their compound have been reported, many plants with well known ethnopharmacologiacl activities as a mixture have been failed to be active as individual or revealed weak in biological activities. Therefore, there is a real need to emphasize on the evaluation of the whole formulation of traditional medicine instead of single components as several different types of positive interactions between different components of medicinal plants have been reported[5,6].

Monodora myristica fruits, *Xylopia aethiopica* fruits and *Eremomastax speciosa* leaves are a well known mixture used in the West region of Cameroon to overcome nappy rash usually associated with yeasts.

Monodora myristica is a widespread and attractive small tree with very decorative flowers, widely distributed from Africa to Asia. It belongs to the custard apple family of flowering plants called Annoneceae[7]. Its seeds are aromatic and are employed after grinding into a powder as condiments in food providing a flavor resembling that of nutmeg. Previous studies on this plant have revealed its antioxidant properties[8] and a short term safety of the seed oils[9].

Xylopia aethiopica (Annonaceae) commonly known as "African guinea pepper" is widely spread in tropical Africa, Zambia, Mozambique, Angola and Cameroon[10]. It is used as a spice and possesses great nutritional and medicinal values in traditional medicine[11]. The aromatic roots of the plant are used to arrest bleeding in addition to antiseptic properties. The plant is also used in decoction to treat dysentery, bronchitis, ulceration, skin infection and female sterility. From several studies, *X. aethiopica* has shown a wide range of biological activities, such as antibacterial[12,13], antifungal[14,15], antiplasmodial[16], anti-inflammatory, antitumoural and insecticidal effect among others[12].

Eremomastax speciosa (Acathaceae) is an erect multi-branched tropical herb that grows in the forest[17]. Due to its multifunctional use, it is now grown in farmlands around living houses. It is commonly referred to in Cameroon as "blood plant" due to its reputed use in the treatment of cases of anaemia. It is also used for the treatment of various stomach complaints and information obtained from traditional practitioners suggested that it possesses antiulcer effects. Previous works on this plant have highlighted the positive effects on ulcer formation, gastric secretion and haematoprotective[18]. Moreover, the anti-diarrhea properties[19] and the antimicrobial activity are well documented[20].

Although, ethnopharmacological data are in accordance with the antifungal activity of the mixture of *M. myristica*, *X. aethiopica* and *E. speciosa* extracts, the exact proportion of each extract in the mixture for best activity is unknown. Therefore, the present study was undertaken to evaluate the antifungal activity of extract mixtures from these plants.

MATERIALS AND METHODS

Plant material, collection and extraction: The herbal sample consisted of two different Cameroonian dietary plants namely *Monodora myristica* and *Xylopia aethiopica* and one Cameroonian medicinal plant, *Eremomastax speciosa*. The dietary plants were purchased in Dschang market, West region of Cameroon while *E. speciosa* was harvested in Dschang. They were further identified at the National Herbarium (Yaoundé, Cameroon) by referring to the voucher specimen 16419/SRF-Cam (*X. aethiopica*, 36228/HNC (*E. speciosa*), 2949/SFR-Cam (*M. myristica*).

Each plant was dried at room temperature and the powdered material was then weighed (200 g), it was later soaked in 1 L of methanol-methylene chloride (1/1; v/v) for 48 h and filtered using Whattman No. 1 filter paper. The filtrate obtained was concentrated under reduced pressure at 50°C in a rotary evaporator to obtain the crude extract. The crude extracts were further kept at 4°C until further uses.

Preliminary phytochemical screening: The plant materials were screened for the presence of different classes of secondary metabolites by chemical reaction method using standard procedure that was based on those described by Sofowora[21]. These included alkaloids, flavonoids, phenols, saponins, tannins, anthocyanins, anthraquinones, sterols and triterpenes.

Yeast isolates and *in vitro* susceptibility testing: Ten yeast isolates of *Candida albicans* were used for this experiment. These microorganisms were isolated from patient's stools with stomach disorder at the Dschang District Hospital and maintained on Sabouraud Agar (SDA) slant at the Laboratory of Microbiology and Antimicrobial substances, University of Dschang, Cameroon.

Yeast suspensions of about 1.5×10^8 CFU mL^{-1} (McFarland turbidity standard No. 0.5) were prepared from 48 h culture in normal saline and diluted in Sabouraud broth culture medium (SDA, Conda, Madrid, Spanish) to obtain a 2.5×10^5 UFC mL^{-1} inoculum[22]. The *in vitro* antifungal activity of the plant extracts and their mixture was performed by determining the Minimum Inhibitory Concentrations (MIC) using broth microdilution method[23]. The stock solutions of each plant extract were prepared in 5% tween 80. The mixtures were performed by combining of two plant extract solutions at different ratios 1:1; V:V, 2/3:1/3; v:v, 1/3:2/3; v:v) or three (1/3:1/3:1/3; v:v:v; 2/4:1/4:1/4; v:v:v, 1/4:2/4:1/4; v:v:v, 1/4:2/4:2/4; v:v:v). The antifungal susceptibility tests of each extract solution and the extract mixtures solutions were performed in 96 well microtitre plates. Serial two-fold dilutions of the extract were performed to obtain a final concentration ranging from 1024-8 µg mL^{-1} in a total volume of 100 µL/well. Fungal suspension (100 µL) in Sabouraud broth culture medium was seeded into all the wells to a final volume of 200 µL/well. The plates were incubated at 35°C for 48 h. Minimum Inhibitory Concentrations (MIC) were defined as the lowest concentrations of extract required to prevent the visual growth of the fungi at the end of the incubation time.

Minimum Fungicidal Concentrations (MFC) were determined by sub-culturing 10 µL aliquots of the medium drawn from wells which did not show any growth after incubation during MIC assay and incubated for 48 h at 35°C. The lowest concentration of the antifungal agent from which negative growth was recorded was considered as MFC.

***In vivo* antifungal susceptibility testing:**

Experimental animals: Experiments were performed using male Wistar albino adult rats between 10-12 weeks old that tested negative for the *Candida* genus in their stools. They weighed 130-200 kg. These animals were bred in the animal house of the Department of Biochemistry, University of Dschang, Cameroon. The animals were fed with a standard rat diet. Food and water were given *ad libitum* to all animals throughout the experiment. Animals were maintained at room temperature (22±2°C) and were handled according to standard protocols for the use of laboratory animals. The studies were in accordance to the ethical guidelines of Committee for Control and Supervision of Experiments on Animals (Registration No. 173/CPCSEA, dated 28 January, 2000), Government of India, on the use of animals for scientific research.

Experiment design: The antifungal activity was evaluated as previously described by Takakura[24] with slight modifications. Twenty five rats were divided into five groups of 5 each comprising two control group and three treatment groups. The first control group received 5% tween 80 and the second control group was treated with fluconazole (200 mg kg^{-1} b.wt.). The treatment groups received each of the following doses of *M. miristica* and *X. aethiopica* mixture (1:1; v: v) at 100, 200 and 400 mg kg^{-1} b.wt.

Prior to rat inoculation, they were initially treated with tetracycline (Terramicina, Laboratórios Pfizer Ltda., Guarulhos, SP, Brazil) (1 g L^{-1}) through their drinking water for three consecutive days, for any bacterial infection. For inoculation purpose, rats were fasted for 12 h. Inoculate consisted of 1 mL of 48 h old culture of *Candida albicans* (LEV 85), standardized at Mc Farland turbidity n° 9, corresponding to 25×10^8 UFC mL^{-1}. Six hours later, food and water were provided *at libitum*. Forty eight hours after inoculation, yeast load were investigated in each animal faeces. For this purpose, 0.7 g of rat faeces was dissolved in 25 mL of NaCl 0.9% and serial two fold dilution were made. 25 µL of each dilution were then spread in duplicate on corn meal agar. *Candida* colonies were counted on plates exhibiting 30-300 colonies to determine Colony-Forming Units (CFU) mL^{-1} and yeast load was evaluated and expressed as CFU g^{-1} of faeces following 48 h incubation time at 35°C. The treatment trough oral route started after the establishment of infection and proceeded once every three days during 18 consecutive days.

Statistical analysis: Data of the CFU of *C. albicans* isolated in the faeces of rats was subjected to the one way analysis of variance (ANOVA) and recorded as Mean±SD and where differences existed, means were separated using Waller Duncan test at $p<0.05$ significant level.

RESULTS

Phytochemical screening: Freshly prepared extracts were subjected to a preliminary phytochemical screening for various constituents. Alkaloids and triterpenes were present in all extracts, other classes of chemicals being selectively present (Table 1).

Table 1: Phytochemical composition of methanol-methyl chloride extract of *Xylopia aethiopica*, *Eremomastax speciosa* and *Monodora myristica*

Parameters	Alkaloids	Anthocyanins	Anthraquinones	Flavonoids	Phenols	Saponins	Sterols	Tannins	Triterpenoids
Xylopia aethiopica	+	-	-	-	+	+	-	+	+
Eremomastax speciosa	+	-	-	+	-	-	+	-	+
Monodora myristica	+	-	+	+	+	-	-	-	+

+: Present, -: Absent

Table 2: Minimal inhibitory concentrations, minimal fungicidal and MFC/MIC ratios of the plant extracts, and mixtures on *Candida albicans* isolates

Parameters	*Xylopia aethiopica*	*Monodora myristica*	*Eremomastax speciosa*	a	b	c	d	e	Nystatin
LEV85									
MIC	1024	512	1024	128	>1024	>1024	>1024	>1024	2
MFC	>1024	>1024	1024	1256	>1024	>1024	>1024	>1024	
MFC/MIC			1	2					
LEV 77									
MIC	512	512	1024	128	>1024	256	>1024	>1024	16
MFC	1024	>1024	1024	256	>1024	>256	>1024	>1024	
MFC/MIC	2		1	2					
LEV130									
MIC	512	512	1024	128	>1024	256	>1024	>1024	8
MFC	1024	1024	1024	256	>1024	>1024	>1024	>1024	
MFC/MIC	2	2	1	2					
LEV132									
MIC	128	128	1024	32	256	256	512	512	8
MFC	512	512	1024	128	512	512	512	512	
MFC/MIC	4	4	1	4	2	2	1		
LEV66									
MIC	512	512	512	128	256	256	256	256	2
MFC	>1024	>1024	1024	512	>1024	512	>1024	>1024	
MFC/MIC			2	4		2			
LEV65									
MIC	512	512	1024	128	512	256	512	512	8
MFC	>1024	>1024	1024	512	512	>1024	512	512	
MFC/MIC			1	4	1		1	1	
LEV5									
MIC	512	1024	1024	128	>1024	256	>1024	>1024	8
MFC	1024	1024	1024	512	>1024	>1024	>1024	>1024	
MFC/MIC	2	1	1	4					
LEV84									
MIC	512	128	512	32	>1024	256	>1024	>1024	2
MFC	1024	1024	>1024	128	>1024	>1024	>1024	>1024	
MFC/MIC	2	8		4					
LEVE5									
MIC	512	1024	1024	256	>1024	256	>1024	>1024	4
MFC	1024	1024	1024	512	>1024	>1024	>1024	>1024	
MFC/MIC		1	1	2					
LEV2"									
MIC	512	128	512	64	>1024	256	>1024	>1024	8
MFC	>1024	1024	>1024	128	>1024	>1024	>1024	>1024	
MFC/MIC		8		2					

a: *X. aethiopica*: *M. myristica* (1:1; v:v), b: *X. aethiopica*, *M. myristica* (2/3:1/3; v:v), c: *X. aethiopica*, *M. myristica* (1/3:2/3; v:v), d: *X. aethiopica*: *E. speciosa* (1:1; v:/v), e: *X. aethiopica*: *E. speciosa* (2/3: 1/3; v/v), *X. aethiopica*: *E. speciosa* (1/3: 2/3; v/v), *M. myristica*: *E. speciosa* (1:1 ; v:/v), *M. myristica*: *E. speciosa* (1/3:2/3; v/v), *M. myristica*: *E. speciosa* (2/3: 1/3; v/v), *X. aethiopica*, *M. myristica*, *E. speciosa* (1/3:1/3:1/3; v:v:v), *X. aethiopica*: *M. myristica*: *E. speciosa* (2/4:1/4:1/4; v/v/v), *X. aethiopica*: *M. myristica*: *E. speciosa* (1/4:2/4:1/4; v/v/v) and *X. aethiopica*: *M. myristica*: *E. speciosa* (1/4:2/4:2/; v/v/v4) was found to be almost inactive, MIC: Minimum inhibitory concentration, MFC: Mininum fungicidal concentration

***In vitro* antifungal activity:** The antifungal activity of *M. myristica*, *X. aethiopica* and *E. speciosa* extract and their mixtures against *C. albicans* are presented in Table 2. *Monodora myristica* extract with MIC ranging from 128-1024 μg mL^{-1} was found to be more active on the tested yeasts compared to *X. aethiopica* extract. Whereas, *E. speciosa* extract was found to be less active. *Xylopia aethiopica*: *M. myristica* mixture (1:1) with MIC ranging

Table 3: Fungal burden of feces in a rat model of gastro-intestinal candidiasis at various time intervals upon treatment with *Xylopia aethiopica* and *Monodora myristica* extracts mixture

		Xylopia aethiopica and *Monodora myristica* extracts mixture (mg kg^{-1})			Nystatin (mg kg^{-1})
Days	Negative control	100	200	400	200
3	855.00±16.91^{a}	837.16±3.76^{b}	865.00±5.37^{a}	863.33±8.42^{a}	852.33±7.23^{a}
6	890.16±21.64^{a}	872.33±4.55^{b}	836.66±3.44^{c}	824.66±4.46^{d}	668.16±8.91^{e}
9	961.16±14.33^{a}	920.44±6.97^{b}	803.83±4.62^{c}	744.66±13.29^{d}	465.83±9.87^{e}
12	961.05±7.29^{a}	880.33±11.46^{b}	757.00±8.83^{c}	641.00±17.01^{d}	273.66±15.47^{e}
15	861.66±7.34^{a}	869.50±8.73^{a}	653.5±14.90^{b}	525.33±21.57^{c}	151.5±11.00^{d}
18	864.84±6.24^{a}	843.66±7.26^{b}	572.33±5.01^{c}	425.00±9.93^{d}	97.00±5.18^{e}

Data is expressed as Mean±SEM, n = 5, values for a given group in a line followed by same letter as superscript are not significantly different according to waller Duncan multiple comparison procedure ($p<0.05$)

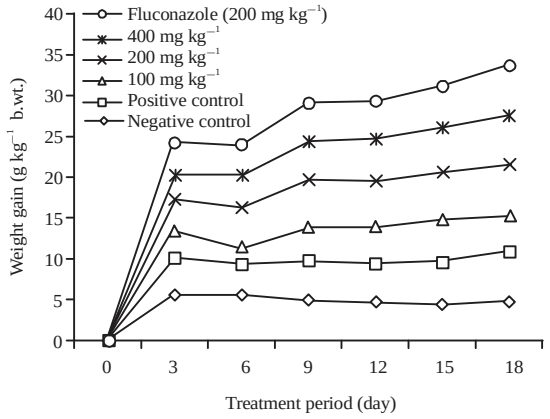

Fig. 1: Effect of the doses of *Xylopia aethiopica* and *Monodora myristica* extracts mixture on weight gain of rat model of gastro-intestinal candidiasis as a function of treatment period

from 32-256 μg mL^{-1} was found to have antifungal activity on all the tested fungi. This activity was highest compared to the individual extract of all the three plants and all the other mixture proportions. The MICs values of this mixture were four fold less than the MBC values, indicating that the fungicidal effect could be expected.

***In vivo* antifungal activity:** Animals inoculated with *C. albicans* isolate showed weight lost upon infection (data not shwon). Administration of *X. aethiopica* and *M. miristica* mixture was able to gradually increase the body weight, suggesting probable progressive recovery of the infected animal (Fig. 1).

Xylopia aethiopica and *M. miristica* mixture (1:1) antifungal activity on gastro-intestinal rat model of yeast infection are presented in Table 3. This results revealed that the extract mixture at 200 and 400 mg kg^{-1} b.wt. were able to progressively and significantly reduced the fungal load in the feces of the infected rats within the treatment period. The best activity was achieved at 400 mg kg^{-1} b.wt. after 18 days treatment, suggesting that total recovering could be expected from this treatment.

DISCUSSION

Each of the extracts tested in the present study displayed antifungal activity on the ten yeast isolates tested. This suggests that they possess broad spectrum activities on *Candida albicans* species. However, differences were observed between antifungal activities of the extracts. These differences could be due to the differences in the chemical composition of extracts since secondary metabolites of plants have many effects including antifungal and antibacterial[4,14]. The phytochemical analysis of *E. speciosa* and *X. aethiopica* extract was in accordance with previous results[25,26]. The phytochemical composition of *M. myristica* was in accordance with previous works[8,27], but slight differences were observed with results reported by Ekeanyanwu[28]. Indeed, saponins and tannins could not be found in the extract. The variability of preliminary phytochemical results could be attributed mainly to the chemical reaction method commonly used to identify the phytochemical groups of constituents. In fact, plant extracts are usually colored and this may mask specific color of some particular phytochemical group. The origin of the plant material and the nature of the solvent for extraction are other factors that may affect the composition. Moreover, the distribution of these phytochemical groups varied from one organ to another. Since, the secondary metabolites are mainly responsible for the biological activity, the present results constitute a scientific base for the promotion of standardization of phytomedicine for reproducibility with time and space.

Xylopia aethiopica was found to have moderate activity on *C. albicans* isolates. These results correlate

with the finding of previous workers[12,14]. The antifungal activity of *M. myristica* and *E. speciosa* is reported here for the first time.

Researches on combination of plant extracts are very limited and few studies have been reported. The secondary metabolites from plants are good sources for combination therapy. *Xylopia aethiopica*: *M. myristica* mixture (1:1) with MIC values ranging from 32-256 μg mL^{-1} was found to have the best antifungal activity on the tested isolates. This result could express either an additive or synergetic effect of both extract. Similar results were reported on honey and some plant extracts[29,30]. Antimicrobial activity of plant extracts are routinely classified on the basis of susceptibility tests that produce MIC in the range of 100-1000 μg mL^{-1} [31]. The activity is considered to be significant if MIC values are below 100 μg mL^{-1} for crude extract and moderate when the MICs vary from 100-625 μg mL^{-1}. Based on this scale, the mixture was found to express significant activity on almost 33.33% of the tested isolates.

The *in vivo* antifungal activity support the results achieved *in vitro*. The mixture at 200 and 400 mg kg^{-1} b.wt. were able to progressively and significantly reduce the fungal load in the feces of the infected rats within the treatment period, suggesting a progressive recovery of animals from infection. The mixture is therefore a good candidate that may be used to overcome fungal diseases associated with *Candida albicans* species. The results are relevant since, *C. albicans* is the leading primary agent causing superficial and often fatal disseminated infections in immunocompromised patients[32]. This valorizes ethnopharmacological data as a baseline for the establishment of phytomedicine and therefore highlights the importance of plant mixture in phytomedicine[6]. Most remedies used in traditional medicine are made with combination of plants. Some plant constituents are added mainly to attenuate the side-effects of others, for example ginger to prevent nausea[33]. Others possess synergetic effect[28]. Some plant extracts may have an immunomodulatory effect as well as a direct effect on the target cell[5]. Several different types of positive interactions between different components of medicinal plants have been demonstrated. Pharmacokinetic interactions occur, for example between constituents of *Artemisia annua* tea so that its artemisinin is more rapidly absorbed than the pure drug[33]. Therefore, further studies need to be carried out so as to establish the role of *E. speciosa* in the mixture.

Differences in sensibility among isolate were observed. This could be due to their genetic content and it is an evidence for the necessity of antibiogram prior to antimicrobial prescription. It's particularly important because inappropriate antimicrobial drugs enhance microbial resistance[34].

CONCLUSION

The results of the present study support the traditional use of the studied plants in the treatment of yeast infections. They also provide an important basis for the use of methanol/methylene chloride extract mixture to control infectious diseases caused by *Candida albicans.* Further studies need to be carrying out as to establish the role of *E. speciosa* in the mixture.

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