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Research Article

# Potentiating Antifungal Activity of Fluconazole or Nystatin with Methanol Bark Extract of *Harungana madagascariensis* Stem Bark

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**ABSTRACT**

The therapeutic failures and the increasingly high costs of treating resistant bacterial infections calls for alternative means of care. The present study was initiated to evaluate the antifungal properties of the *Harungana madagascariensis* methanol barks extract and potent interactions with some usual drugs. The extract was prepared by maceration of the dry stem bark powder in methanol. Phytochemical analysis was carried out by colorimetric assays. Antifungal activity as well as interactions between extract, Nystatin or Fluconazole was evaluated by broth microdilution method. The therapeutic efficacies of *H. madagascariensis* extract and Fluconazole – extract mixture as compared to Fluconazole extract was studied in experimental models of oral and vulvovaginal candidiasis in rats by oral route. Results showed that methanol extract of *H. madagascariensis* stem barks had antifungal activity ranging from 128 to 1024 µg/ml. This extract also had a fungicidal activity on all of the tested yeasts. Moreover, the extract presented *in vitro* synergetic and additive interactions with Nystatin and Fluconazole. In both oral and vaginal infection model, all the treatment significantly reduced ($P < 0.05$) the number of colony formant unit (UFC) of *C. albicans* compared with untreated control. Moreover, significant decrease in the percentage of animals showing positive cultures was observed in rats treated with Fluconazole-extract mixture. In both therapeutic efficacy studies, the histological findings confirmed the microbiological results. The results of this study constitute a base for the usage of *H. madagascariensis* stem barks in association with Fluconazole to overcome yeast infection.

**Keywords**: *Harungana madagascariensis*, antifungal activity, synergy, oral candidiasis, vulvovaginal candidiasis.

## INTRODUCTION

Over the past two decades, the prevalence of fungal infections has increased significantly due to the growing number of populations at high risk. Candida species are the most commonly isolated fungal pathogens causing morbidity and mortality in patients with impaired immunity, accounting 80% of fungal infections[1]. These infections ranged from mucosal candidiasis, including oropharyngeal and esophageal candidiasis, which is frequently observed in immunocompromised patients, to vulvovaginal candidiasis, which affects a large number of women[2]. Oropharyngeal candidiasis is the most common opportunistic infection strongly correlated with impairment of the immune system[3]. Vaginal candidiasis is widespread because it has been estimated that 75% of all women will experience an episode of *Candida* vaginitis once in their lifetime, with up to 5% showing recurrence[4]. In recent years, azole agents have become the drugs of choice for treating oropharyngeal and vulvovaginal candidiasis. Recent studies indicated the possibility of treatment failures associated with some *C. albicans* and related species by expression of efflux pumps that reduce drug accumulation, alteration of the structure or concentration of antifungal target proteins, and alteration of membrane sterol composition[5]. For a long time, polyene drugs including Amphotericin and Nystatin were the only therapeutic options for invasive fungal infections. Nowadays, Nystatin remains the more available antifungal drugs in more developing countries. The still unacceptably high morbidity rate associated with some resistant mycosis indicates that alternatives to existing therapeutic options are needed. Plants are a major source of biomolecules with pharmacological properties recognized worldwide including antimicrobial activity. They could exhibit beneficial interactions with conventional drugs in the treatment of microbial infections.

*H. madagascariensis* is a plant of Hypericaceae family. It is used in traditional medicine for the treatment of microbial and non-microbial diseases. Previous studies confirm the use of aqueous root extract in the treatment of drug-induced hepatotoxicity[6]. They also highlighted the antibacterial activity[7-9]. The present findings were conducted to explore the antifungal activity of *H.*

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*madagascariensis* stem bark and potent potentiating activity with Fluconazole and Nystatin.

## MATERIALS AND METHODS

*Plant material*

Fresh *H. madagascariensis* stem barks were collected at Mount Khalla, *Yaoundé* (Cameroon) in April 2014. The identification of the plant was ascertained morphologically at the Cameroon National Herbarium where voucher sample were deposited under the registration number N° 4224 HNC.

*Microorganisms*

Six yeast strains including *Candida albicans* ATCC 1663, *Candida albicans* 9003, *C. krusei*, ATCC 6258, *Candida glabrata* CIP35, *Candida parapsilosis* ATCC 22019, *Cryptococcus neoformans* IP 96026 and one clinical isolate of *Candida albicans* and *Cryptococcus neoformans* were considered in the study. The isolates were obtained from the Pasteur Center, Cameroon.

*Experimental animals*

*In vivo* experiments were performed using *Wistar* albino adult rats of both sexes, 10 to 12 weeks old (200 ± 30 g). They were fed with a standard diet. Food and water were given *ad libitum* throughout the experimental period. Animals were maintained at room temperature (22 ± 2°C) and were handled according to standard protocols for the use of laboratory animals as recommended by the Guide for the Care and Use of Laboratory Animals, Washington.

*Extract preparation*

*H. madagascarienis* stem bark were air-dried for three weeks and powdered to coarse particles. Two hundred and fifty grams of powder were macerated during 48 hours, using 2.5 l methanol. Upon filtration, the extract was concentrated under reduced pressure at 45°C using Rota vapor Buchi R205 to yield a paste of 14.50%. The paste was further incubated 48 hours at 45°C.

*Preliminary phytochemical screening*

The secondary metabolite classes such as alkaloids, anthocyanins, quinones, flavonoids, phenols, saponins, tannins, sterols and triterpenes were screened according to phytochemical methods previously described by Harbone[10].

*In-vitro antifungal susceptibility testing*

Antifungal susceptibility tests were performed by broth microdilution method in 96 microtitres plates[11]. In short, stock solution of the plan extract was prepared in 5% Dimethylsufoxide (DMSO). Serial two-fold dilutions of the extract were performed to obtain a final concentration of 1024µg/ml in the first well. Fungal suspension (100 µl) in Sabouraud broth culture medium was added in each well and the plates were incubated 48 h at 35°C. 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 during MIC assay and incubated further for 48 hours at 35°C. The lowest concentration from which negative growth was recorded was considered as MFC. Fluconazole and Nystatin were used as reference antifungal drugs.

The extract was further tested in association with Nystatin and Fluconazole at MIC/2 and MIC/4, MIC/8, MIC/16 and MIC/32 using checkerboard method[12,13]. All assays were performed in triplicate and repeated thrice. Fractional Inhibitory Concentration (FIC) was calculated as the ratio of $MIC_{Extract}$ in combination with reference drug/$MIC_{Extract}$ alone and the interpretation made as follows: synergistic (<0.5), indifferent (0.5 to 4), or antagonistic (>4).

*In-vivo antifungal susceptibility testing*

*Oral candidiasis*

Oral infection in male rats was induced basically as reported by Martinez et al.[14] with slight modifications. Rats were immunosuppressed one week before the first inoculation and continued throughout the experiment by administering dexamethasone (Sigma-Aldrich, German) in drinking water at 0.5 mg per liter. Also, a 0.1% aqueous solution of tetracycline hydrochloride (Sigma-Aldrich, German) was given to animals 7 days before inoculation. Experimental infection was established by inoculation of the oral cavities of the rats three times in 48 hours interval with 0.1 ml of yeast suspension containing 5 $x10^8$ cells of *C. albicans* ATCC 1663. Oral inoculation was performed by means of a cotton swab rolled twice over all parts of the mouth.

*Vulvovaginal candidiasis* (VVC)

The animal model of VVC was established based on previously described models[15] with slight modifications. Briefly, estrus was induced with intraperitoneal administration of estradiol (Sigma-Aldrich, German) at 4 mg/kg daily for 9 days before inoculation. Furthermore, animals were immunosuppressed with dexamethasone as described earlier. Aqueous solution of tetracycline hydrochloride 0.1% was given to experimental animals throughout the experimental period. Intravaginal infection was established with $10^7$ yeast cells (*C. albicans* ATCC 1663) per 0.1 ml of sterile physiological saline water after three time inoculation at 48 hours intervals.

*Treatment*

In both cases, infected animals were randomly distributed into five groups of 5 animals each two days after the last inoculation. For oral candidiasis model, rats were treated by topical application in the oral cavity twice a day for eight consecutive days with 0.5 ml plant extract (2 mg/ml) (Group I), mixture of plant extract and Fluconale (1 mg/ml; ½, ½) (Group II) and Fluconazole (1 mg/ml) (Group III) in viscous 0.8% agar solution as excipient.

In the VVC model, treatment was administered by gavage, twice daily. The first group received no treatment. The second group was treated with *H. madagascarienis* bark extract at 200 mg/kg, the third group with 50 mg/kg *H. madagascarienis* bark extract and 50 mg/kg Fluconazole, and the last group was treated with Fluconazole at 200 mg/kg. In both cases, control group consisted of infected and untreated animals.

*Evaluation of therapeutic efficacy*

The evaluation of the therapeutic efficacy of the treatment was assessed by microbiological and histopathological evaluation. Oral and vaginal samples were collected each

Table 1: Minimal Inhibitory Concentrations (MIC), Minimal Fungicidal Concentrations (MFC) and MFC/MIC ratios of *H. madagascariensis methanol bark extract*

| Extract and antibiotics | Extract | | | Fluconazole | | | Nystatin | | |
|---|---|---|---|---|---|---|---|---|---|
| | Antimicrobial parameters (µg/ml) | | | | | | | | |
| yeasts | CMI | CMF | CMF/CMI | CMI | CMF | CMF/CMI | CMI | CMF | CMF/CMI |
| *C. albicans* ATCC 1663 | 256 | 256 | 1 | 1 | 4 | 4 | 1 | 1 | 1 |
| *C. albicans* | 1024 | 1024 | 1 | 4 | 16 | 4 | 4 | 16 | 4 |
| *C. albicans* ATCC 9003 | 512 | / | / | / | / | | 8 | 16 | 2 |
| *C. neoformans* | 1024 | 1024 | 1 | 4 | 8 | 2 | 4 | 2 | 2 |
| *C. neoformans* IP 96026 | 256 | 256 | 1 | 1 | 4 | 4 | 4 | 16 | 4 |
| *C. glabrata* CIP 35 | 256 | 256 | 1 | 1 | 16 | 16 | 1 | 1 | 1 |
| *C. parapsilosis* ATCC 22019 | 256 | 512 | 2 | 2 | 4 | 2 | 2 | 16 | 8 |
| *Candida krusei* ATCC 6258 | 128 | 512 | 4 | / | / | / | 4 | 16 | 4 |
| *C. albicans* ATCC 9003 | 512 | / | / | / | / | / | 1 | 1 | 1 |

*/= undetermined*

Table 2: Fractional Inhibitory Concentrations (FIC) of *H. madagascariensis* bark methanol extract associated with Fluconazole or Nystatin.

| | Plant extract + Fluconazole | | | | | Plant extract + Nystatin | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Yeasts | FIC2 | FIC4 | FIC8 | FIC 16 | FIC 32 | FIC2 | FIC4 | FIC8 | FIC 16 | FIC 32 |
| *C. albicans* ATCC 1663 | 0.5 | 0.5 | _ | _ | _ | 0.5 | 4 | _ | 4 | _ |
| *C. albicans* | 0.125 | 0.125 | _ | _ | _ | 0.0625 | 0.125 | 1 | _ | _ |
| *C. neoformans* | 0.125 | 0.125 | _ | 1 | 1 | 0.125 | 0.125 | 1 | 1 | _ |
| *C. neoformans* IP 96026 | 0.5 | 0.5 | 4 | 4 | 4 | 0.25 | 0.5 | 4 | 4 | 4 |
| *C. glabrata* CPI 35 | 0.5 | 0.5 | 1 | 1 | 1 | 0.5 | 1 | 4 | 4 | 4 |
| *C. parapsilosis* ATCC 22019 | 0.25 | 0.25 | 4 | 4 | 2 | 0.25 | 0.25 | 4 | 4 | 4 |

*CIF 2, 4, 8, 16, 32= Fractional inhibitory concentrations of H. madagascariensis bark methanol extract in addition with Fluconazole or Nystatin at MIC/2, MIC/4, MIC/8, MIC/16, MIC/32 -= could not be found. Synergy activity (FIC≤0.5), additive activity (FIC 0.5 FIC< 1), indifferent (1<FIC < 4) and antagonism (FIC>4).*

two days by rolling a sterile cotton swab over the oral cavity or within the vagina. Samples were then suspended in 4 ml of sterile physiological saline water and cultured in triplicate on Sabouraud dextrose agar supplemented with 0.05 mg/ml actidione and gentamycin (Sigma-Aldrich, German). Plates were incubated at 37°C for 48 h. The yeast count was expressed as log10 CFU per milliliter. Twenty-four hours after the last treatment, experimental rats were anesthetized with diazepam and sacrificed. Tongues or vaginas were aseptically removed. These organs were fixed by immersion in neutral buffered 10% formalin solution. Serial cross sections were further made in formalin for 12 h and small pieces were subjected to haematoxylin-eosin staining[16]. Pathological observations were performed on gross and microscopic basis. Histological plates were encrypted for analysis by a histopathologist.

## RESULTS

*Phytochemical analysis*

Freshly prepared *H. madagascariensis* bark extract were subjected to a preliminary phytochemical screening. The results revealed the presence of phenols, alkaloids, flavonoids, tannins, sterols, saponins, anthocyanins and quinones. However, triterpenes could not be detected.

*In vitro* antifungal activity

All tested yeasts were sensitive to the methanol extract of *H. madagascariensis* bark with MIC values ranging from 128 mg/ml to 1024 mg/ml (Table 1). *C. krusei* was found to be more sensitive (128 µg/ml). *C. albicans* ATCC 1663, *C. neoformans* IP 96026 and CIP 35, *C. glabrata* and *C. parapsilosis* ATCC 22019 have comparable sensitivity with value equal to 256µg/ml.

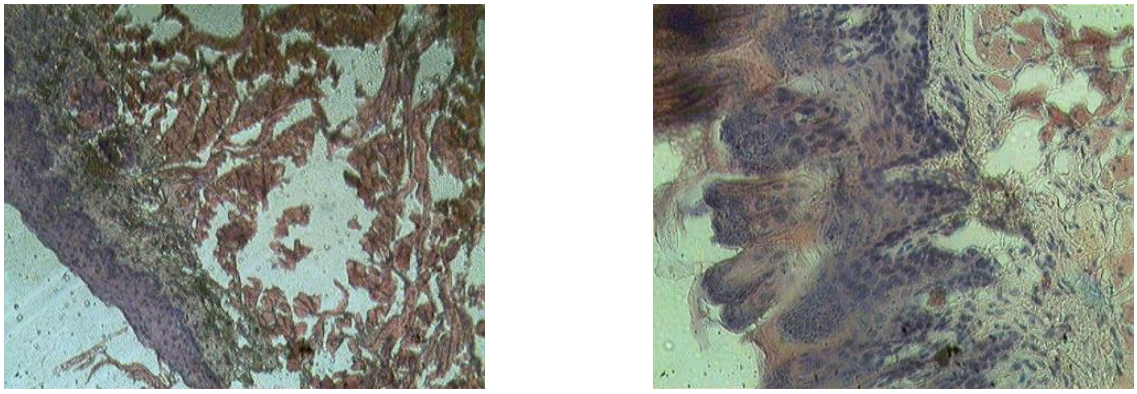
The methanol extract of *H. madagascariensis* bark in combination with Fluconazole and Nystatin revealed on all tested yeasts, additive or synergistic interaction with dilutions of these antifungal corresponding to MIC/2 and MIC/4. Beyond these dilutions, the interaction was either additive or indifferent on all the microorganisms (Table 2).

*In vivo antifungal activity*

*Therapeutic efficacies of H. madagascariensis extract in association with Fluconazole against experimental oral C. albicans infections*

The therapeutic efficacies of the *H. madagascariensis* extract in association with Fluconazole against experimental oral *C. albicans* infections were investigated by microbiological and pathological studies. Infected and untreated animals showed extensive colonization of the epithelium of the dorsal surface of the tongue by numerous hyphae (Figure 1). Treated animals showed multiple regenerative areas of the covering epithelium, and no histological evidence of *C. albicans* within the epithelium of the tongue was seen in animals treated with Fluconazole or in association with *H. madagascariensis* extract.

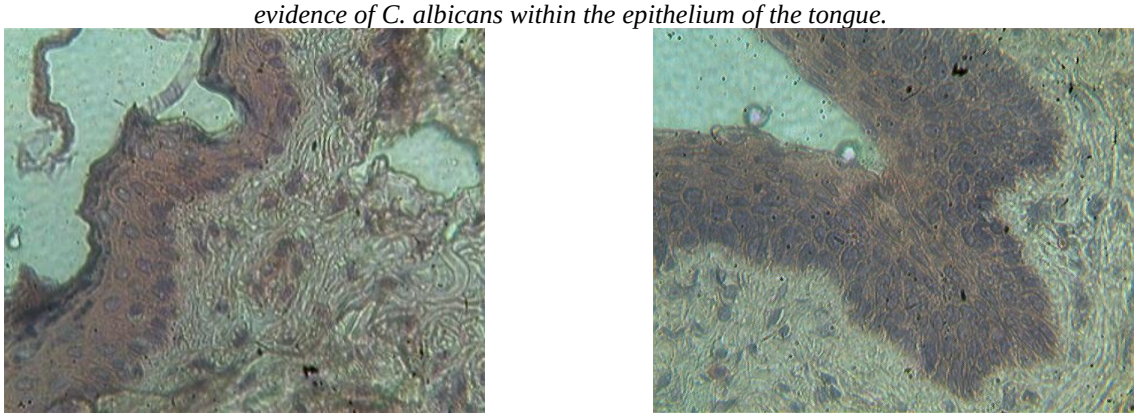
The results of oral wabs culture as a function of treatment and duration is presented in Table 3. The results from



a b

Figure 1: Histopathological analysis in rat's oral candidiasis following treatment with *H. madagascariensis* bark methanol extract.

***a)*** *Control animals showing extensive colonization of the epithelium of the dorsal surface of the tongue by numerous hyphae. b) Treated animals showing multiple regenerative areas of the covering epithelium, and no histological evidence of C. albicans within the epithelium of the tongue.*



a b

Figure 2: Histopathological analysis in rat's vulvovaginal candidiasis following treatment with *H. madagascariensis* bark methanol extract.

*a) Control showing abundant growth of Candida organisms, both as budding yeasts and in the pseudohyphal form B) Treated animal showing restoration of degrade structures and a lack of yeast*.

infected rats indicate that all animals developed oral infection, with a log CFU per sample (mean ±standard deviation) of 4.68±1.04. This infection remained in the untreated rats, although with a certain decrease in the number of organisms when evaluated 24 h after the end of the treatment (1.87±0.70 log CFU/sample). In the treated groups, a significant and progressive decrease in yeast load was observed in the saliva. The decrease was more pronounced with rats treated with the extract associated with Fluconazole. In the 8$^{th}$ day of treatment, all the rats treated with the *H. magascariens* extract had positive yeast culture. Three out of five the rats treated with Fluconazole had positive culture while one out of five of those treated with the *H. madagascariensis* extract associated with Fluconazole had positive culture meaning that this association is more efficient compared to Fluconazole alone.

*Therapeutic efficacies of H. madagascariensis extract in association with Fluconazole against experimental vaginal C. albicans infections*

The vaginal histopathological analysis revealed two major findings (Figure 2). Untreated rats showed abundant growth of *Candida* organisms, both as budding yeasts and in the pseudohyphal form, involving the stratum corneum and the luminal keratin debris accompanied by a thin epithelium, degraded and disorganized cells. On the other hand, treated animals regardless of treatment showed restoration of degraded structures and sometimes a lack of yeasts. The therapeutic effect of *H. madagascariensis* extract in association with Fluconazole, as compared to extract or Fluconazole on the *C. albicans* vaginal burden in infected rats is summarized in Table 4. A significant decrease in the percentage of animals showing positive vaginal cultures was observed in rats treated with both Fluconazole and Fluconazole -extract mixture. All the five animals treated with *H. madagascariensis* extract at 200 mg/kg body weight revealed positive cultures. Two-fifth of animals treated with Fluconazole at 200 mg/kg showed negative culture whereas three-fifth of animals treated with Fluconazole and extract mixture at 100 mg/kg showed positive *Candida* cultures. Animals still positive for vaginal *Candida* presence showed a statistically significant ($P < 0.05$) reduction in fungal burden compared to control as a function of treatment duration. This was more pronounced with Fluconazole and *H.*

*madagascariensis* extract mixture making it the most active.

## DISCUSSION

The methanol extract of the bark of *H. madagascariensis* possess inhibitory properties on tested yeasts, and this could be justified by the presence of several classes of chemical compounds which was identified, since antimicrobial activity of compound from these groups have previously been mentioned[17].

During the last twenty years, the frequency of invasive fungal infections and the risk of opportunistic fungal infections had increased especially in immunocompromised patients[18]. Among the opportunistic fungal pathogens, *C. neoformans* and *Candida* germs are commonly associated with fungal infections, causing most common infections. These microorganisms were susceptible to *H. madagascariensis* stem bark methanol extract, showing that the extract is a potential source of antifungal molecules capable of fighting against diseases caused by these microorganisms. Indeed, Iwalewa et al.[19] reported antifungal activity of six extracts (hexane, dichloromethane, ethyl acetate, chloroform, acetone and methanol) bark of *H. madagascariensis* on various microorganisms including *C. neoformans* and *C. albicans*. Five of the eight tested yeasts exhibited MIC values below 500 µg/ml, and three of them out of six showed MICs values between 500 and 1500 µg/ml translating respectively strong and moderate activities of the methanol extract of *H. madagascariensis*. MFC/MIC ratios were in all cases <4 expressing the fungicidal activity of *H. madagascariensis*[20].

The continuing increase of resistance to antifungal drugs on one hand and the development of traditional medicine on other hand raise questions about the possible association for faster and effective struggling against infections. In combination with Fluconazole or Nystatin, the methanol extract of the bark of *H. madagascariensis* presented synergistic and additive effects on all the yeasts tested at MIC/2 and MIC/4. This suggests that these two antifungal reference drugs offer opportunity for association with the methanol extract of *H. madagascariensis* for potentiation of the antifungal activity. The presence of synergy between the methanol extract of *H. madagascariensis* bark and antifungal assumed that the latter would have molecules with complementary mechanisms of action or molecules with similar modes of action with these antifungal drugs. Indeed, Nystatin belongs to the polyene group. It exerts a physical-chemical interaction with membrane sterols, leading to the formation of aqueous pores in the fungal membrane. On the contrary, Fluconazole belongs to the group of azoles which exert their antifungal activity by inhibiting one of the steps in the synthesis of ergosterol, a major component of the fungal membrane[21].

The therapeutic efficacies of *H. madagascariensis* extract and Fluconazole mixture (100 mg/kg body weight) as compared to Fluconazole (200 mg/kg) and *H. madagascariensis* extract (200 mg/kg) was studied in experimental models of oral and vulvovaginal candidiasis in rats. Oral and vaginal candidiasis models have been shown to afford a simple, reliable, and highly reproducible method for studying the efficacies of antifungal agents[22].

Although oral candidiasis is not a dangerous disease in itself, it is the origin of morbidity among human. Its prevalence increases in immunocompromised patients whose prevalence is constantly growing. On the other hand, VVC also affects a large number of otherwise healthy women. It has been estimated that 75% of all women of childbearing age will experience an episode of VVC in their lifetime[23]. Results point out that orally administered, the methanol extract of *H. madagascariensis* and Fluconazole as mixture have led to both candidiasis models studied, a significant reduction in fungal burden during treatment. This adequately showed that the extract of this plant had *in vivo* antifungal properties elucidated *in vitro*.

The extract of *H. madagascariensis* in combination with Fluconazole resulted after eight days treatment on both candidiasis models studied four healings against five compared to three against five with Fluconazole, used as a reference antifungal against candidiasis. This result shows that a complete cure of all animals could be acquired, the healing could be done much more quickly with this mixture compared to Fluconazole eventhough assessed at a reduced dose. The use of reduced doses for maximal activity seems useful because it reduces the risk of side effects following the administration of drug.

Administered at 100 mg/kg orally, the methanol extract of *H. madagascariensis* in combination with Fluconazole had a better result compared to Fluconazole given at 200 mg/kg. This result confirms the potentiator effect resulting in an additive or synergistic effect as observed *in vitro*. This result seems promising because beyond the antifungal activity highlighted, it provides the beneficial effects of the association between herbal and conventional medicines. This is particularly important since the resurgence of microorganisms to conventional antibiotics is growing and calls new challenges.

Animals given *H. madagascariensis* and Fluconazole mixture showed histopathological findings in line with normal mucosal recovery from the induced infectious process thereby, confirming the therapeutic efficiency of the treatment.

The results achieved with the *in vitro* synergetic and additive antifungal activity together with oral and vaginal candidiasis models strongly suggest that Fluconazole and *H. madagascariensis* extract mixture could be promising antifungal agents for the treatment of human oral and VVC Candida infections.

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