

Comparison of the Antifungal Efficacy of Four Concentrations of *Minthostachys mollis* (Muña) Essential Oil against *Candida albicans*: An *In Vitro* Study

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ABSTRACT

Aim and objective: This paper aims to compare the antifungal efficacy of four concentrations of *Minthostachys mollis* essential oil (MEO) against *Candida albicans* ATCC 10231.

Materials and methods: This study was conducted *in vitro*. Ten kilograms of *M. mollis* (Muña) were collected in the city of Tarma, Peru. The plant was then dried in the shade at room temperature (21°C), and the essential oil was obtained through distillation. *C. albicans* ATCC 10231 was cultured at a MacFarland scale of 0.5, which corresponds to a concentration of 3×10^8 CFU/mL. Each plate was filled with one of the four MEO concentrations (25, 50, 75, or 100%), dimethyl sulfoxide (negative control), or fluconazole (positive control), a known antifungal agent. After incubation, each plate was examined using the Kirby-Bauer method.

Results: Compared to MEO 25%, MEO 50%, and MEO 75%, MEO 100% had the highest antifungal efficacy at 24, 48, and 72 hours of evaluation, with an average of 18.9 ± 0.7 , 18.2 ± 0.7 , and 17.0 ± 0.4 mm, respectively. However, fluconazole had higher efficacy (27.9 ± 0.5 , 27.5 ± 0.5 , and 23.7 ± 0.7 mm, respectively). *Post hoc* analysis showed that there were significant differences between all concentrations of the MEO groups and their respective positive and negative control groups ($p < 0.001$).

Conclusion: Among the MEO groups, the pure concentration (MEO 100%) had the highest antifungal efficacy. However, fluconazole presented greater efficacy, and the differences were statistically significant.

Clinical significance: This research allowed to know the efficacy of this natural resource against one of the most prevalent fungi in the oral cavity. Therefore, a line of research could be opened to deepen its potential benefits for oral health.

Keywords: Antifungal efficacy, *Candida albicans*, *Minthostachys mollis*.

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INTRODUCTION

In recent years, fungal infections have increased in frequency and importance; they are often associated with high mortality and are generally caused by bloodstream infections due to *Candida* species.¹⁻⁴ Worldwide, oral candidiasis prevalence rates of 32.5, 83, and 88% have been reported in patients with brain damage, denture wearers, and children with human immunodeficiency virus (HIV), respectively. Oral candidiasis is characterized by white lesions located in any region of the oral mucosa such as the tongue, floor of the mouth, tonsils, etc. These whitish lesions usually present an elevation with erythematous areas, causing pain and discomfort to speak and eat.^{2,3}

In addition, the few drugs currently available have significant toxicity that can worsen the systemic response. In addition, in recent years, there have been reports of antifungal resistance, so it is important to identify therapeutic alternatives such as phytotherapy. Patients often gravitate toward phytotherapeutics, as they are free of synthetic and chemical additives. However, these agents lack rigorous scientific backing; therefore, it is necessary to demonstrate the therapeutic effects of phytotherapeutics considering the well-being and safety of the patient.^{4,5}

Muña, scientifically known as *Minthostachys mollis*, is an aromatic shrub native to South America and of great pharmacological interest because of its essential oils.⁶⁻⁸ In the Andes, it is used not only as a condiment or tea but also as popular medicinal plant; it has received increasing attention in pharmacology and modern medicine. Muña is a folkloric plant that is often used to treat decompensation due to changes in altitude, flatulence. Its main components include

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phosphorus, calcium, menthone, etc. This prodigious plant stands out for its multiple benefits for the human being; although scientific evidence is still scarce, it is attributed with certain anti-inflammatory and antimicrobial properties.⁶⁻¹⁰

However, there is little scientific evidence regarding this natural resource. Therefore, the objective of the present study was to compare the antifungal activity of different concentrations of *M. mollis* essential oil (MEO) against *Candida albicans* ATCC 10231.

MATERIALS AND METHODS

Study Design

The study was of an *in vitro* experimental type. Physicochemical and microbiological analyses were performed at the Analytical Control Center of the Faculty of Pharmacy and Biochemistry of the Universidad Nacional Mayor de San Marcos, Lima-Peru. The sample size was calculated from a mean comparison formula, where a confidence level of 95% and power of 80% were considered. The mean and standard deviation parameters were obtained from a previous pilot study. A total of 84 specimens were determined, divided among the seven groups as follows: Group I, MEO 25%; Group II, MEO 50%; Group III, MEO 75%; Group IV, MEO 100%; Group V, Fluconazole; Group VI, Dimethyl sulfoxide; and Group VII, distilled water.

Specimen Preparation

Ten kilograms of *M. mollis* (Muña) plant were collected in the city of Tarma (location 11°2444" S 75°4104" W) in the Department of Junín, Peru (Fig. 1A). The study of the plant was carried out by the Faculty of Pharmacy and Biochemistry (CENPROFAMRA) of the Universidad Nacional Mayor de San Marcos, with the following classification:

- Division: Magnoliophyta
- Class: Magnoliopsida
- Subclass: Asteridae
- Order: Verbenales
- Family: Lamiaceae
- Genus: *Minthostachys* (Benth.) Spach
- Species: *clinopodium*

The plant was then dried under shade at room temperature for 7 days, until the dry sample was obtained. The essential oil was obtained by the steam entrainment method. For which, the solvent diethyl ether ($C_2H_5)_2O$ was used to obtain the separation using water vapor volatilizing the components of the vegetable matter. The samples were then preserved in hermetically sealed bags for transport to the laboratory. The dried leaves were selected and subjected to steam distillation in a distillation system consisting of an 8-L boiler, hopper, refrigerant and connection hoses; water and oil were obtained after 3 hours of distillation. Considering its immiscibility properties and the difference in density between water and the essential oil, the distillate was separated using a

glass decanting funnel. Finally, 10 mL of *M. mollis* essential oil was obtained and stored in an amber-colored glass bottle and refrigerated at 4°C (Fig. 1B).

Culture Medium

Ten milliliters of Sabouraud broth (30 g for 1 L of distilled water) were prepared in a test tube and autoclaved. Fifty milliliters of Sabouraud agar was prepared for the activation phase (52 g for 1 L of distilled water). The sterilized agar was then cooled in a water bath at 45–50°C and poured into sterile Petri dishes. Then, 900 mL of Mueller Hinton agar was prepared for the analytical phase. Once tempered, the preparation was poured into sterile glass Petri dishes to give a uniform bottom of approximately 4 mm, corresponding to 25–30 mL for 90 mm diameter plates. The plated agar was allowed to solidify at room temperature.

Strain Reactivation

The strain was refrigerated at 4–8°C. A colony was taken with the seeding loop into a tube with sterile Sabouraud broth and placed in an incubator at 35°C for 24 hours. Turbidity showed growth in the strains. Sabouraud broth was seeded onto plates with Sabouraud agar. The mixture was incubated at 35°C for 24 hours. Pure colonies of *C. albicans* ATCC 10231 were diluted in a test tube containing 10 mL of sterile saline (0.9% sodium chloride), so that the resulting solution had a turbidity corresponding to the McFarland scale of 0.5, which represents a concentration of 3×10^8 CFU/mL.

Inoculation and Seeding

One hundred microliters of the prepared inoculum (1×10^8 CFU/mL) were added to the plates with Mueller Hinton agar. Using a Drigalski spatula, the inoculum was spread throughout the plates in such a way as to obtain homogeneous growth; the loop was slid on the plate in a parallel and compact manner, covering the entire surface of the plate. The procedure was repeated by rotating the plate 60° two more times. The plates were carefully seeded from edge to edge to prevent difficulty when taking readings. The plates were allowed to dry for 3–5 minutes before the wells were made. The punch was sterilized and flamed in a burner to make the wells. The wells should be more than 15 mm from the edge of the plate and should be distributed in such a way that there is no overlap of the inhibition halos. Each group was loaded with approximately 40 µL of each well. Dimethyl sulfoxide was used as a negative control, and fluconazole (2 mg/mL) was used as a positive control.



Fig 1A to C: (A) Selection of Muña leaves; (B) Obtaining essential oil of Muña; (C) Inhibition halos of Muña essential oil 25, 50, and 75%

Incubation and Reading

The sample plates and controls were placed in an incubator at 35°C for 24, 48, and 72 hours. After incubation, each plate was examined using the Kirby-Bauer method (Fig. 1C). The resulting inhibition zones were uniformly circular in a homogeneous layer of microbial growth. The diameters of the complete inhibition zone were measured in millimeters and passed through the center of each well. Measurements were performed in triplicate for each well using a digital vernier (Mitutoyo, Japan). The values of the measurements were averaged and rounded to the nearest natural number.

Statistical Analysis

A descriptive analysis of the data was performed, presenting summary measures (mean and standard deviation) for each group. The normality of the data for each group was evaluated using the Shapiro-Wilk test. Comparative analysis between groups was performed using ANOVA. A multivariate linear regression analysis was then performed. All analyses were interpreted by establishing a $p < 0.05$, using the Stata® 15 statistical software.

RESULTS

Compared to other concentrations, MEO 100% had the highest antifungal efficacy at 24, 48, and 72 hours of evaluation, with an average of 18.9 ± 0.7 , 18.2 ± 0.7 , and 17.0 ± 0.4 mm, respectively. However, fluconazole had higher efficacy (27.9 ± 0.5 , 27.5 ± 0.5 , and

23.7 ± 0.7 mm, respectively). All groups had a normal distribution ($p > 0.05$). The comparative analysis showed that there were significant differences between all the antifungal efficacies of all concentrations of MEO and fluconazole ($p < 0.001$) (Table 1).

The *post hoc* analysis showed that there were significant differences between all concentrations of MEO groups and their respective positive and negative control groups ($p < 0.001$) (Table 2).

The mean of antifungal efficacy was 3.3 mm more (CI: 2.9–3.7) for MEO 50% compared to MEO 25%. The average antifungal efficacy was 5.7 mm more (CI: 5.3–6.1) in MEO 75% compared to MEO 25%. The average antifungal efficacy was 9.8 mm more (CI: 9.4–10.2) in MEO 100% compared to MEO 25%. Finally, the mean antifungal efficacy was 18.8 mm more (CI: 18.4–19.2) in fluconazole compared to MEO 25%. All groups were statistically significant ($p < 0.001$) (Table 3).

DISCUSSION

Oral candidiasis is a common fungal disease usually caused by an overgrowth of *C. albicans* in the oral cavity. Recommended treatments include proper oral hygiene, use of topical agents, and systemic medications.^{11,12} The widespread use of antifungals both prophylactically and therapeutically has led to the emergence of secondary resistance and the displacement of sensitive strains by more resistant ones.^{13–15}

Table 1: Comparison of the antifungal effect of MEO in different concentrations

	MEO 25%		MEO 50%		MEO 75%		MEO 100%		Fluconazole		Dimethylsulfoxide		Distilled water		p*	p**
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
24 hours	9.1	0.5	12.4	0.7	14.8	0.5	18.9	0.7	27.9	0.5	6	0	6	0	0.05	0.01
48 hours	8.3	0.5	12.1	0.5	14.4	0.5	18.2	0.7	27.5	0.5	6	0	6	0	0.05	0.01
72 hours	7.3	0.5	9.0	2.7	12.4	0.7	17.0	0.4	23.7	0.7	6	0	6	0	0.05	0.01

MEO, *minthostachys mollis* essential oil; *Shapiro Wilk test; **ANOVA test

Table 2: Post hoc analysis of antifungal effect of MEO

Groups	MEO 25%	MEO 50%	MEO 75%	MEO 100%	Fluconazole	Dimethylsulfoxide
MEO 50%	0.001	—	—	—	—	—
MEO 75%	0.001	0.001	—	—	—	—
MEO 100%	0.001	0.001	0.001	—	—	—
Fluconazole	0.001	0.001	0.001	0.001	—	—
Dimethylsulfoxide	0.001	0.001	0.001	0.001	0.001	—
Distilled water	0.001	0.001	0.001	0.001	0.001	0.001

MEO, *minthostachys mollis* essential oil

Table 3: Linear regression of the antifungal effect of MEO

Antifungal efficacy	Coef.	Std. Err.	p	[95% confidence interval]	
MEO 25%	Ref.				
MEO 50%	3.3	0.2	0.001	2.9	3.7
MEO 75%	5.7	0.2	0.001	5.3	6.1
MEO 100%	9.8	0.2	0.001	9.4	10.2
Fluconazole	18.8	0.2	0.001	18.4	19.2
Dimethylsulfoxide	-3.1	0.2	0.001	-3.5	-2.7
Distilled water	-3.1	0.2	0.001	-3.5	-2.7
_cons	9.1	0.1	0.001	8.8	9.4

MEO, *minthostachys mollis* essential oil

Peru has an abundant plant diversity, including medicinal plants with established antibacterial and antifungal properties, such as *M. mollis* or "Muña."^{9,10} This study aimed to evaluate the efficacy of *M. mollis* essential oil as an alternative to fluconazole.

According to a study by Cano et al.,¹⁶ monoterpenes and other compounds in Muña essential oil inhibited *M. canis* and *Trichophyton tonsurans* and showed an inhibition diameter of up to 30 mm for the pure (100%) concentration. These results coincide with our findings, which showed an antifungal efficacy around 18.9 mm. Moreover, according to a study by Peña et al.,¹⁷ *M. mollis* essential oil from Huancavelica, Peru, not only has antifungal efficacy but also antibacterial efficacy against *S. aureus* and *Klebsiella pneumoniae*. These findings also coincide with a study by Torrenegra-Alarcon et al.,¹⁸ which identified bacteria sensitive to *M. mollis* essential oil due to the high monoterpene content. Therefore, this plant species is promising for the control of bacterial pathogens.

Another study conducted by Alcalá et al.¹⁹ reported that pure *M. mollis* essential oil (100%) was more effective against *C. albicans* strains than fluconazole and that the antifungal effect of fluconazole was greater than that of *M. mollis* 25%, which was the same as that of *M. mollis* 50%. However, our results showed some variance; all concentrations (25, 50, 75 and 100%) had a lower mean compared to fluconazole.

Another important study was conducted by Alcalá et al.¹⁹ who mentioned that the essential oil of *M. mollis* (100%) was more effective against *C. albicans* strains than fluconazole, in addition, the antifungal effect of fluconazole was greater than that of *M. mollis* 25% and was the same as that of *M. mollis* 50%. However, our results were different since all our concentrations (25, 50, 75, and 100%) had lower mean compared to Fluconazole.

The main limitation of this study was the scarce scientific evidence on the antifungal efficacy of this natural resource in the field of oral health. So, it is difficult to discuss the similarities and differences between studies.²⁰ This is probably since this natural resource is a plant native to the high Andean zones of South America, which makes it difficult to carry out studies in other continents. Another limitation was that only the antifungal efficacy against one strain (*C. albicans*) was evaluated; it would be useful to evaluate other fungi. In this way, the spectrum of action of Muña could be broadened.

The future direction of this study is its contribution to scientific knowledge. By demonstrating the antifungal efficacy of *M. mollis*, our findings allow us to consider this plant a natural source with antifungal potential and thus apply it in the future as an accessible therapeutic alternative to treat *C. albicans* infections.

CONCLUSION

All concentrations of MEO were effective against *C. albicans*; but the pure concentration (MEO 100%) showed the highest antifungal efficacy among the experimental groups. However, fluconazole presented greater efficacy, and these differences were statistically significant.

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