
Acta Scientiarum. Biological Sciences
ISSN: 1679-9283
eduem@uem.br
Universidade Estadual de Maringá
Brasil

Guerra Lund, Rafael; Serpa, Rosana; da Silva Nascente, Patrícia; Aver Ribeiro, Gladis; Freitag, Rogério Antonio; Burkert Del Pino, Francisco Augusto
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Acta Scientiarum. Biological Sciences, vol. 34, núm. 4, octubre-diciembre, 2012, pp. 437-442
Universidade Estadual de Maringá
.png, Brasil

Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1806-2563
ISSN on-line: 1807-8664
Doi: 10.4025/ actascibiolsci.v34i4.8959

# *In vitro* study on the antimicrobial effect of hydroalcoholic extracts from *Mentha arvensis* L. (Lamiaceae) against oral pathogens

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**ABSTRACT.** *In vitro* tests could be a valuable tool for the evaluation of medicinal plants' antimicrobial activity. *Mentha arvensis* of the Lamiaceae family is one of the most frequently traditional plants used in Brazil. Hydroalcoholic extracts of *M. arvensis* were analyzed for antimicrobial activity on *Streptococcus mutans*, *Streptococcus sobrinus* and *Candida albicans*. Three different assays (agar diffusion, broth macro- and micro-dilution methods) were used to evaluate antimicrobial activity. Although hydroalcoholic extracts of *M. arvensis* did not show any antibacterial effect, its antifungal activity against *C. albicans* was revealed. According to the micro-dilution broth assay, MIC of the hydroalcoholic extract from leaves of *M. arvensis* on *Candida albicans* strains ranged between 625 and 2500 μg $mL^{-1}$. Results suggest that *M. arvensis* hydroalcoholic extract may be considered a potentially antifungal agent against *C. albicans*, and a possible item for human antibiotic therapy. However, further biological tests on the plant's efficacy and side-effects are necessary before its use on humans.

**Keywords:** *Mentha arvensis*, antimicrobial activity, *Streptococcus mutans*, *Candida albicans*.

## Estudo *in vitro* do efeito antimicrobiano dos extratos hidroalcólicos de *Mentha arvensis* L. (Lamiaceae) contra patógenos orais

**RESUMO.** Testes *in vitro* podem ser uma ferramenta valiosa para a avaliação da atividade antimicrobiana de plantas medicinais. *Mentha arvensis* é uma das plantas medicinais brasileiras mais frequentemente utilizadas e pertence à família Lamiaceae. No presente estudo, extratos hidroalcólicos de *M. arvensis* foram analisados quanto à sua atividade antimicrobiana sobre *Streptococcus mutans*, *Streptococcus sobrinus* e *Candida albicans*. Três diferentes ensaios (métodos de difusão em ágar, macro e microdiluição em caldo) foram utilizados para avaliação da atividade antimicrobiana. Embora os extratos hidroalcólicos de *M. arvensis* não demonstraram qualquer efeito antibacteriano, eles apresentaram atividade antifúngica contra *C. albicans*. Baseado no ensaio de microdiluição em caldo, a CIM do extrato hidroalcólico das folhas de *M. arvensis* sobre cepas de *C. albicans* variaram de 625 a 2500 μg $mL^{-1}$. Estes achados sugerem que o extrato hidroalcólico de *M. arvensis* pode ser considerado um agente antifúngico em potencial contra *C. albicans*, e um possível candidato para antibioticoterapia humana. Contudo, mais testes biológicos sobre a eficácia e efeitos adversos desta planta são necessários antes do seu uso em humanos.

**Palavras-chave:** *Mentha arvensis*, atividade antimicrobiana, *Streptococcus mutans*, *Candida albicans*.

## Introduction

Plants in traditional medicine for the treatment of various illnesses are widespread and a number of naturally produced herbal formulations are available for infectious diseases (BALBANI et al., 2009). While many of these herbal medicines may not produce any significant results, and some may even be potentially toxic and dangerous to people, demands and applications of formally accepted and/or informal herbal drugs are increasingly popular (MESQUITA et al., 2009). Therefore scientific tests on traditionally used herbs for the treatment of different infections could be valuable sources for new natural antibiotics.

*Mentha arvensis* L., popularly known as 'Vique', is consumed in Brazil mainly for its antiseptic, insect repellent, carminative, antispasmodic, diaphoretic and anti-inflammatory properties. Traditionally, the infusion of this herb is used for stomachache and vomiting (MATOS, 2000). *M. arvensis* L. is a species of great economic interest among medicinal and aromatic plants due to its essential oils which are a rich source of menthol, with several industrial

applications in oral health care products, flavorings, aromatic food and drinks, perfumeries and pharmaceutical products (IMAI et al., 2001; SRIVASTAVA et al., 2002).

*Mutans streptococci* are the main etiologic agents of dental caries in humans (LOESCHE, 1986; NAPIMOGA et al., 2005; TANZER et al., 2001) and *Streptococcus sobrinus* and *S. mutans* are the most frequently isolated bacteria from the human oral cavity (LOESCHE, 1986; NAPIMOGA et al., 2005). Moreover, research indicates that the coexistence of *S. sobrinus* and *S. mutans* is an important factor in the development of dental caries (BERKOWITZ, 2006; TANZER et al., 2001).

Fungal infections, such as candidiasis, also deserve attention since *Candida* sp. is an important emergent nosocomial pathogen causing severe morbidity and mortality in immunocompromised patients (DIEMOND et al., 2008). Modern therapies and management such as bone marrow or solid-organ transplants and new and more aggressive chemotherapy have resulted in a rapidly increasing number of immunosuppressed patients (AGUADO; AYATS, 2008). These patients now survive longer and become highly susceptible to life-threatening fungal infections. Concomitant with the increased incidence of fungal infections, there has been a high increase in the use of antifungals for the treatment of both systemic and localized fungal infections (RITCHIE et al., 2009). Consequently, the extensive use of antifungal agents has accelerated the development of antifungal drug resistance followed by frequent therapeutic failures and increasing mortality rates (FERA et al., 2009; LOEFFLER; STEVENS, 2003).

The antimycotic drugs available for the treatment of systemic fungal infections are limited (11 active compounds) and may be divided into four classes: (i) polyene macrolides; (ii) azole derivatives; (iii) DNA and RNA inhibitors; and (iv) 1,3-β-glucan synthase inhibitors (echinocandins) (FERA et al., 2009; LOEFFLER; STEVENS, 2003; RITCHIE et al., 2009). Hence, there is a great demand for new agents with a wide spectrum of activity and reduced toxicity.

The relevance of this study is based on the increasing trend for the use of hydroalcoholic extracts in phytotherapy and by the fact that *Mentha* species are found and used worldwide for medicinal and industrial purposes.

Few investigations on the antimicrobial activity of the different parts of this *Mentha* species have been carried out and even fewer about oral microorganisms. Current study evaluates the antimicrobial activity of hydroalcoholic extracts from the stems and leaves of *M. arvensis* L. ('Vique') against *Streptococcus mutans*, *S. sobrinus* and *Candida albicans*.

## Material and methods

### Medicinal plant

Fresh aerial parts of *M. arvensis* (stems and leaves) were collected in July 2006 in Pelotas, State of Rio Grande do Sul, in southern Brazil. A voucher specimen was deposited at the Herbarium of the Federal University of Pelotas (Pelotas, State of Rio Grande do Sul, Brazil) under code number PEL24603, and identified by Dr. Maria Antonieta Décio da Costa (a botanist from the Catholic University of Pelotas). Plants were collected in their entire, rolled up in paper and packed in cardboard pouches. The plants were then cleaned, their respective vegetable parts separated, dried in a stove with air circulation at 40ºC for three days.

The stems and leaves were harvested during the flowering phase, washed and dried at 40ºC for 72h, and ground into powder. The dried stems and leaves were ground by tissue grinder and Soxhlet-extracted sequentially with 217.5 mL and 480 mL of 70% ethanol, respectively.

### Preparation of the plant's hydroalcoholic extracts

Further, 14.3 g of dried stem powder were macerated with 3 x 72.5 mL ethanol 70%, v $v^{-1}$, at room temperature (~25ºC) for 9h. At every 3h, the extract was filtered and 72.5 mL of the corresponding hydroalcoholic solution were added to the residue. The supernatant formed the crude hydroalcoholic extract obtained from the stems of *M. arvensis* (SMA). The hydroalcoholic extract obtained from leaves was produced from the maceration of 25.5 g of dried leaves powder with 3 x 160 mL of ethanol 70%, v $v^{-1}$, at room temperature (~25ºC) for 9h. The supernatant formed the crude hydroalcoholic extract obtained from leaves of *M. arvensis* (LMA). The supernatant was then concentrated under reduced pressure in rotavapor and lyophilized.

The lyophilized extracts were re-suspended in their corresponding hydroalcoholic solution (70%, v $v^{-1}$) at concentrations 5.87% (w $v^{-1}$) for SMA and 10% (w $v^{-1}$) for LMA, prior to the performance of the assays.

### Test of microorganisms

The *in vitro* antimicrobial activity of *M. arvensis* hydroalcoholic extracts was tested against the oral streptococci *Streptococcus mutans* UA159 and *S. sobrinus* 6715 and *Candida albicans* strains. Gram-positive bacteria were obtained from the

Department of Pharmacology, Anesthesiology and Therapeutics, Faculty of Dentistry, University of Campinas (UNICAMP) (Piracicaba, State of São Paulo, Brazil). Two of the four yeasts *C. albicans* tested were clinical isolates from the oral cavity of children who were attended at health clinics of Pelotas, State of Rio Grande do Sul, Brazil, and two were pure collection strains: ATCC 18804 and ATCC44858. This study was approved by the Ethics Committee of the Pelotas Dental School (Document no. 036/2006).

### Antimicrobial activity assays

#### Agar diffusion assay

The disk diffusion method evaluated the antibacterial activity of *M. arvensis* hydroalcoholic extracts (KOO et al., 2000; LUND et al., 2009). A suspension of the tested bacteria (0.1 mL of $10^8$ cells $mL^{-1}$) was spread on solid media plates. The microorganisms were seeded on pour plate in BHI agar and incubated for 18-24h. The oral streptococci grown on brain-heart infusion agar were suspended in sterile brain-heart infusion broth. The suspension was adjusted spectrophotometrically (OD 660 nm) to match the turbidity of a McFarland 0.5 scale (1.5 x $10^8$ CFU $mL^{-1}$). A 400 $\mu$L portion of each tested suspension was mixed with 40 mL brain-heart infusion agar at 45ºC, and poured on a previously set layer of Mueller Hinton agar. The nutritive media were prepared according to the manufacturer's instructions. All agar plates were prepared on 90 mm petri dishes with 22 mL of agar and a final depth of 4 mm. The inoculum procedure provided a semi-confluent growth of the microorganisms tested. Four sterilized stainless-steel cylinders of 8.0 x 10.0 mm (internal diam. 6 mm) were placed on each inoculated agar plate. Either the tested extracts or the controls (40 $\mu$L) were placed inside the cylinders. The plates were kept for 2h at room temperature to allow the diffusion of the agents through the agar. Afterwards, the plates were incubated at 37ºC under microaerophilic conditions (5-10% $CO_2$) for 24-48h. Cylinders with 40 $\mu$L of 70% ethanol (EtOH) and 0.12% chlorhexidine digluconate were used respectively as negative and positive controls.

Inhibition zones of microbial growth around the cylinder containing the extracts were measured, after incubation time, with a ruler, and the results expressed in millimeters. The plates used for each treatment were chosen randomly and each extract was processed in triplicate. Three replicates were made for each of the tested bacteria. The experiment was repeated twice.

#### Broth dilution assay (MIC)

The antimicrobial activity of *M. arvensis* hydroalcoholic extracts was determined by the minimum inhibitory concentration (MIC), according to Koo et al. (2000) and Duarte et al. (2005). For MIC determination, the starting inoculum was 5 x $10^5$ CFU $mL^{-1}$. Two-fold dilution series of extracts (concentrations ranging between 8.15 and 521.78 µg $mL^{-1}$ for SMA and between 13.89 and 888.89 µg $mL^{-1}$ for LMA) were tested. The control vehicles were 70% ethanol, v $v^{-1}$ (positive control; final ethanol concentrations in the culture medium of 0.650, v $v^{-1}$) and 0.12% chlorhexidine digluconate (positive control). MIC was defined as the extract's lowest concentration that had restricted the growth to a level lower than optical density (OD) of 0.05 at 660 nm (no visible growth). Three replicates were made for each concentration of the tested extracts for MIC assay, in each experiment. The experiment was repeated three times.

#### Antifungal activity assay (MIC)

The isolates were obtained from the oral cavity of children and adults from a hospital clinic in Pelotas, State of Rio Grande do Sul, Brazil. The samples were cultivated in Sabouraud agar-dextrose with chloramphenicol. Antifungal susceptibility of *C. albicans* against the crude hydroalcoholic extract from leaves of *M. arvensis* was determined by CLSI broth microdilution method (CLSI, 2005).

Ten dilutions of the crude hydroalcoholic extract from leaves of *M. arvensis* were prepared with concentrations between 9.75 and 5,000 µg $mL^{-1}$. The solution containing each isolate was transferred in 100 $\mu$l aliquots into each well of the sterile plates, already with 100 $\mu$l of the solution containing the dilution of the extract tested. Wells 11 and 12 contained the positive control (100 $\mu$L of Sabouraud dextrose agar and 100 $\mu$L of the half-inoculum solution) and the negative control (200 $\mu$L of the same culture medium). The plates were incubated at 37ºC for 96 hours. The readings were made by visually comparing the growth of the yeast on wells 1 to 10 with wells with positive control (well 11). The lowest concentration that produced a relative significant inhibition of yeast growth for positive control was identified as the MIC of the drug.

## Results and discussion

Results for antibacterial activity revealed that the hydroalcoholic extracts from stems and leaves of . *arvensis* did not show halo inhibition (Table 1), MIC and minimal bactericidal concentration (MBC).

**Table 1.** Means of inhibition zones of bacterial growth (mm) against *S. mutans* and *S. sobrinus*.

| Treatments | *S. mutans* UA159 | *S. sobrinus* 6715 |
|---|---|---|
| *M. arvensis* (leaf) | 0 | 0 |
| *M. arvensis* (stem) | 0 | 0 |
| Ethanol 70% (Negative control) | 0 | 0 |
| 0.12% Chlorhexidine (Positive control) | 16mm | 18mm |

In the macro-dilution broth assay, there was no effect of *M. arvensis* extracts against oral streptococci in the concentrations tested (between 8.15 and 521.78 μg $mL^{-1}$ from the stem's extract and between 13.89 and 888.89 μg $mL^{-1}$ for the leaves' extract).

The crude hydroalcoholic extracts from dried stems and leaves of *M. arvensis* were also assayed for antifungal properties with macro- and micro-dilution assays against clinical isolates of opportunistic pathogenic yeast from the oral cavity (*C. albicans*). Antifungal activity with MIC ranging from 156.3 to 2,500 $\mu$g $mL^{-1}$ was reported (Table 2).

**Table 2.** Minimum Inhibitory Concentration (MIC) of the hydroalcoholic extract from the leaves of *M. arvensis* against strains of *C. albicans* (microdilution broth technique).

| *Candida albicans* | *Mentha arvensis* L. |
|---|---|
| | MIC ($\mu$g $mL^{-1}$) |
| Clinical isolate 1 | 625 |
| Clinical Isolate 2 | 1,250 |
| ATCC 18804 | 156.3 |
| ATCC 44858 | 2,500 |

The development of susceptibility tests for antimicrobials is comprised within the history of advancements achieved in antibacterial therapy. In fact, *in vitro* susceptibility tests, including the main known methodologies such as agar diffusion and broth dilution, were first used by Flemming in 1939 during the investigation on penicillin's potential therapeutics. The introduction of new types of chemotherapies and antibiotics and the recognition of penicillin-resistant bacteria made it mandatory for microbiology laboratories to perform susceptibility tests (SIDRIN; ROCHA, 2004).

The tests of *in vitro* evaluation of antifungal activity use the same methods to evaluate antibacterial activity. In general, the techniques performed in microbiological laboratories are known as broth dilutions, agar dilutions and agar diffusion. The methods' principle is the exposure of defined inoculates of microorganisms to known concentrations of the drug to be tested under excellent conditions for microbial development and the verification whether the bacteria or fungus growing is observed. The final interpretation of the dilution tests in liquid and/or solid medium identifies the lower concentration of the drug that inhibited the growth of the tested microorganism.

The diffusion method is the most employed method in this kind of research despite some limitations. It is a model with low credibility for samples that are difficult to diffuse in the media because there is no relationship between their solubility in water, diffusion power, and antimicrobial study. In some cases, diffusion techniques may be used for antimicrobial screening, but they may not be used as a definitive method due to lack of relationship between MIC rates and inhibition diameters (RIOS et al., 1988). The agar diffusion assay is a qualitative non-standardized method useful only to detect but not to compare antimicrobial properties of different samples. Comparison of inhibition halos sizes of different extracts may not be used to determine relative antimicrobial potencies, since a more diffusible but less active extract could give a larger diameter than a non-diffusible but more active extract (LUND et al., 2009).

The Clinical and Laboratory Standards Institute (CLSI) defined two standardized methods of broth microdilution as antifungigram, the M27A2 and the M38P protocols, for some yeasts and filamentous fungi respectively. The CLSI (CLSI, 2005) recommends the use of RPMI 1640 culture medium in the performance of antifungigrams with yeasts (*Candida* spp. and *Cryptococcus* spp.) (REX et al., 2001).

Currently several developed countries have their own standard committees, such as the very dynamic CLSI in the USA. This technique has been adapted by several authors and any method that promotes similar results to the above protocol must conform itself to the technique so that its use may be accepted. The standardization of antifungal susceptibility tests started early in the 1990s, with the initiative of the NCCLS, when the macro- and micro-dilution broth methods were proposed for yeasts *C. albicans* and *Cryptococcus neoformans*, which amplified the clinical use of these tests, with special reference to the microdilution broth, and facilitated their use in epidemiological surveillance programs in human medicine (ESPINEL-INGROFF et al., 2005; PFALLER, 2005; TORTORANO et al., 1998).

The prevalence of dental caries and the occurrence of oral candidiasis and its clinical importance in immune-compromised patients justified this study with *Streptococcus mutans* and *S. sobrinus*, and four different strains of the yeast *C. albicans*, respectively. Current investigation comprised the antibacterial activity of the crude hydroalcoholic extracts from the aerial parts (stems and leaves) of *Mentha arvensis* evaluated on oral bacteria and yeasts.

Controls used to evaluate the efficacy of plant compounds are usually standard antibiotics as indicated for each microorganism. However, there is no agreement on the acceptance level for plants when compared with standards. In fact, some authors even provide higher rates (DUARTE et al., 2005).

Aligiannis et al. (2001) proposed a classification for plant materials based on MIC results: strong inhibitors – MIC up to 0.5 mg $mL^{-1}$; moderate inhibitors – MIC between 0.6 and 1.5 mg $mL^{-1}$; and weak inhibitors – CIM above 1.6 mg $mL^{-1}$. Duarte et al. (2005) have established the concentration of 2 mg $mL^{-1}$ as the highest concentration acceptable so that a vegetable extract may potentially have antimicrobial activity. In current study, based on the MIC values hereby and on the investigations by Aligiannis et al. (2001) and Duarte et al. (2005), it has been observed that the hydroalcoholic extract from the leaves of *M. arvensis* was potentially fungistatic, although it has an inhibitor behavior ranging from moderate to weak against the *C. albicans* yeasts tested. Another study on the antifungal properties of 70% hydroalcoholic extract from leaves of *M. arvensis* demonstrated that this extract was inactive against *C. albicans* strains. However, its essential oil presented moderate activity against *C. albicans*.

Phytochemical analysis of several species of the genus *Mentha* showed that their essential oils are a rich source of menthol, with several industrial applications, such as in oral health care products, flavorings, aromatic foods and drinks, perfumeries and pharmaceutical products (MATOS, 2000; MESQUITA et al., 2009).

Despite progress in antimicrobial therapies, many problems remain to be solved for most antifungal and antibacterial drugs available. For example, several chemical agents have been tested to restrict the harmful effects of the dental biofilm to the host, such as fluoride, antibiotics and antiseptics, particularly chlorhexidine, which is very efficacious and has different types of use as adjunctive or temporary replacement in the biofilm's mechanical control. However, the chronic ingestion of fluoride by children causes dental fluorosis, the indiscriminate use of antibiotics may cause antimicrobial resistance to this drug, and chlorhexidine causes dental discromies, unpleasant taste, palate alterations and mucous membrane erosions which stimulate the development of new antimicrobial agents (SARI; BIRINCI, 2007; ZANATTA et al., 2007).

Furthermore, when antifungal drugs available are taken into consideration, azole drugs, especially fluconazole, are widely used against *C. albicans* infection. Not surprisingly, repeated fluconazole therapy for antifungal infections in patients could be associated to an increase in azole resistance. It is therefore very important to find antifungal drugs with new chemical structures and pharmacological mechanisms of action (FERA et al., 2009; PEREA et al., 2001).

Current research revealed that the hydroalcoholic extracts from dried stems and leaves of *M. arvensis* didn't show any antibacterial effects against *Mutans streptococci*, whereas the hydroalcoholic extract from dried leaves of *M. arvensis* presented antifungal properties and thus an *in vitro* fungistatic effect. However, this antifungal activity was detected with MIC between 156.3 and 2,500 $\mu$g $mL^{-1}$ (Table 2) which classified the extract from moderate to weak inhibitor against the *C. albicans* yeasts tested.

## Conclusion

Current assay suggests that *Mentha arvensis* hydroalcoholic extract is a potentially antifungal agent on the *Candida* species and a possible candidate for human antibiotic therapy. However, further biological tests on the efficacy and side-effects of the plant are necessary prior to its use on humans.

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*Received on December 3, 2009.*
*Accepted on March 20, 2011.*