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**ORIGINAL PAPER**

# *In vitro* evaluation of the antimicrobial properties of some plant essential oils against clinical isolates of *Prototheca spp.*

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## Abstract

*This paper aimed to evaluate the effectiveness of Satureja hortensis and Abies alba essential oils in inhibiting the growth/survival of two Prototheca species involved in human and animal pathology. Eight P.zopfii isolates from cow mastitic milk samples, two P.zopfii isolates from bovine feces, and one P. wickerhamii referent strain (RE-4608014ATCC16529), from American Type Collection, were submitted to antifungal susceptibility testing by broth microdilution assay following the CLSI guidelines for yeasts. Satureja hortensis (savory) and Abies alba (fir) oils seemed to exert an interesting activity against all Prototheca tested isolates. Both natural products tested proved to be more active against P. wickerhamii. Difficulties in treating protothecosis using classical drugs and the antimicrobial effect of some essential oils extracted from various plants species are stimulating the interest to continue the investigations in using these natural products in therapy.*

**Keywords**: *Prototheca*, savory oil, fir oil, therapy

## Introduction

The genus *Prototheca* includes unicellular achlorophyllous microalgae, spherical, oval or even kidney in shape, with dimension ranging to 3-30 μm in diameter, that reproduce by formation of a variable numbers of sporangiospores within a sporangium [6, 18, 20]. Mobility was not observed in *Prototheca*.

*Prototheca spp.* is ubiquitous in nature and widely distributed all over the world. The organism had first been isolated from the slime flux of trees and over the following decades from a wide variety of sources including sewage, soil, plants and many fresh and salt water sources, even from water-supply systems.

The taxonomic position of *Prototheca* algae has for long been disputed, but currently they display the following integration: *Eucariota* domein, kingdom *Viridiplantae*, phylum *Chlorophyta*, class *Trebouxiophyceae*, order *Chlorellales*, family *Chlorellaceae*, genus *Prototheca* [21]. Of the five known species of the genus – *P. zopfii*, *P.wickerhamii*, *P.stagnora*, *P. ulmea* and *P. blaschkeae* - only *P. wickerhamii* and *P. zopfii* are considered pathogenic, yet their pathogenic potential is low [11, 14]. Until now, they are the only known plant causative agent of human and animal infections [11, 21, 22].

Usualy *P.wickerhamii* causes humans protothecosis manifested as localized skin lesions, while *P.zopfii* produces infections in animals, particularly in cows and dogs. Pharmacological protocol for protothecosis therapy has not been developed yet either in human or in veterinary medicine. Several antifungal agents such as: Amphotericin B, Ketoconasole, Itraconasole, Fluconasole, Nystatin, Polymixin B, Gentamicine and Neomicine

are applied for treatment; however, the observed effects are variable [13, 26]. Effective therapy of protothecal mastitis has not yet been developed and the only measure to control the spread of infection in the herd is exclusion of the infected animals [14].

Resistance to antimicrobial agents has become an increasingly important and pressing global problem [5] and is the main reason for an extended research for new drugs to treat *Prototheca* infections. Plants represent a good source of novel antimicrobial molecules [3, 4, 10, 19]. Therefore the antimycotic activity was investigated in case of essential oils of *Satureja hortensis*, and *Abies alba*, plants of special importance due to there rich content in active compounds.

The aim of this work was to evaluate *in vitro* susceptibility of *Prototheca* isolates to essential oils of *Satureja hortensis* (savory) and *Abies alba* (fir), as an alternative to classical antifungal therapy.

## Materials and Methods

**Microorganisms utilized.** A total of eight *P.zopfii* isolates from mastitic cows milk samples (Fig. 1 and 3), two *P.zopfii* isolates from bovine feces and one *P.wickerhamii* referent strain (RE-4608014ATCC16529), from American Type Collection (Fig. 2 and 4) were used for the present study. All isolates were kept in *Prototheca* Collection of Microbiology Department, Faculty of Veterinary Medicine Cluj-Napoca. The organisms were identified following the usual methods based on their cultural, microscopic and biochemical features (carbohydrates assimilation and growth at 27-37ºC)

**Essential oils.** Plants essential oils tested were found and extracted from the Romanian flora. Essential oils of savory (*Satureja hortensis*) - family *Lamiaceae* and fir (*Abies alba*) - family *Pinaceae* were purchased from commercial sources as standardized products, recommended in various affections of yeast etiology.

Fir essential oil (*Albies alba*) is recommended in therapy due to its content in poliphenol such as: pinen, free therpenic acids, dipentene, phelandren, silvestren, cadiden, and acetates 5-7% bornyl and citronenil acetates.

Savory (*Satureja hortensis*) is a well-known medicinal and aromatic plant, cultivated worldwide, based on antispasmodic, antidiarrhea, antioxidant and antimicrobial activities. The main compounds of the essential oil are carvacrol, thymol, p-cymene, β-pinen and γ – terpinene [8, 9].

**Antimicrobial assay.** The antimicrobial activity of both plant essential oils were determined by broth microdilution assay following the CLSI (formerly NCCLS - The National Committee for Clinical Laboratory Standards) guidelines based on documents M27-A2 for yeasts [17].

Previous to testing *Prototheca* isolates were subcultured in glucose agar medium during 48 hours, in aerobiotic conditions, at 37ºC. After subculture, the alga inoculum was prepared in saline sterile solution, so that the turbidity of the suspension was similar to 0.5 Mc Farland standard ($1x10^8$ cfu/mL). Tests were performed in sterile U-bottom 96-well plates. Ten different dilutions of each essential oil (32, 16, 8, 4, 2, 1, 0,5, 0,25, 0,125, 0,0625 µL/mL) in RPMI 1640 medium without sodium bicarbonate (Sigma), supplemented with 2% glucose were prepared. Tween 80 (final concentration 0.2% v/v) was also included to facilitate oil solubility. 100µL of each dilution were mixed with an equal volume of algae suspension. The positive growth control wells without essential oil (antimicrobial agent) and the negative control wells (without *Prototheca* suspension) were also prepared. The plates were incubated for 48 h, at 37ºC, under aerobiotic conditions. Tests were performed in duplicate. Microplates were read visually.

**Interpretation of results.** The minimal inhibitory concentration (MIC) was defined as the lowest concentration of essential oils that completely inhibited the visible growth of microorganism (no turbidity) when compared to the control. After that 5µl of each well were transferred in glucose agar plates and than incubated for 48 h, at 37ºC. The lowest concentration which did not show any visible growth on the agar plates was considered minimal algicidal concentration (MAC).

As yet unknown "break points" useful in interpreting anti-*Prototheca* effect of essential oils of savory (*Satureja hortensis*) and fir (*Abies alba*) the only considerations are those relating to the values and frequency of MICs. Excel program (*Microsoft Office XP 2007*) was used for calculating these indicators.

## Results and Discussions

The current necessity of discovering new antimicrobial compounds in all fields of microbial control has stimulated researches regarding the antimicrobial properties of plant essential oils. In the present work *P.zopfii* isolates and *P. wickerhamii* referent strain were used in order to evaluate antimicrobial effect of two essential oils: *Satureja hortensis* and *Abies alba*. The tested *Prototheca* isolates, their sources of isolation and also the MIC and MAC values for both essential oils, determined using a broth microdilution method, are summarized as values in table 1.

Aligianis *et al*., (2001) [1] who tested the inhibitory effect of *Origanum vulgare* essential oil on some yeast strains, proposed a classification for the antimicrobial activity of plant products, based on the MIC results as follows: strong inhibitors – MIC up to 0.5 µL/mL; moderate inhibitors MIC between 0.6 and 1.5 µL/mL; weak inhibitors - MIC above 1.6 µL/mL.

Thus, based on this classification, the results showed that the essential oil of *Satureja hortensis* (savory) had a substantial inhibitory effect against most of *P. zopfii* isolates collected from mastitic cow milk samples, noted by the values of MIC that are between 0.25 – 0.5 µL/mL. Moreover as we can observe in table 1, three *P. zopfii* mastitic cow milk isolates as well as those from feces were moderate susceptible to this product, MICs being 1 µL/mL.

Different effectiveness of savory essential oil among *P. zopfii* isolates could be explained by the behavior of each strain that probably present virulence factors, determining resistance to this natural unconventional product. Essential oil of *Abies alba* appeared to be mainly ineffective *in vitro* on all *Prototheca zopfii* tested isolates, values of MICs being between 1 - 4 µL/mL.

Both essential oils proved to induce a strong inhibitor effect upon *P. wickerhamii* referent strain tested (MICs 0.25 µL/mL and 0.125 µL/mL), the highest efficacy being registered for *Abies alba* essential oil.

Some researches regarding the antimicrobial properties of essential oils have found different results of MIC when using different methods for its determination, so that the microplate bioassay has always shown the lowest MIC values [7, 24]. Different results could be related mainly to the variation of well size, medium composition as well as the volatility of the essential oil [27].

**Table 1.** Minimal inhibitory concentration (MIC) and minimal algicidal concentration MAC (% v/v) for the tested essential oils established by broth microdilution method

| *Prototheca* tested isolates | Source of isolation | Plants tested | | | |
|---|---|---|---|---|---|
| | | *Satureja hortensis* | | *Abies alba* | |
| | | MIC (µL/mL) | MAC (µL/mL) | MIC (µL/mL) | MAC (µL/mL) |

| | | | | | |
|---|---|---|---|---|---|
| *P.zopfii* 1 | mastitic cow milk | 0.25 | 0.25 | 1 | 1 |
| *P.zopfii* 2 | mastitic cow milk | 1 | 1 | 2 | 2 |
| *P.zopfii* 3 | mastiticow c milk | 0.25 | 0.25 | 2 | 2 |
| *P.zopfii* 4 | mastitic cow milk | 0.5 | 0.5 | 4 | 4 |
| *P.zopfii* 5 | mastitic cow milk | 1 | 1 | 1 | 1 |
| *P.zopfii* 6 | mastiticow c milk | 0.5 | 0.5 | 4 | 4 |
| *P.zopfii* 7 | mastitic cow milk | 0.25 | 0.25 | 2 | 2 |
| *P.zopfii* 8 | mastitic cow milk | 1 | 1 | 1 | 1 |
| *P.zopfii* 9 | bovine feces | 1 | 1 | 2 | 2 |
| *P.zopfii* 10 | bovine feces | 1 | 1 | 2 | 2 |
| *P. wickerhamii* | human skin | 0.25 | 0.25 | 0.125 | 0.125 |

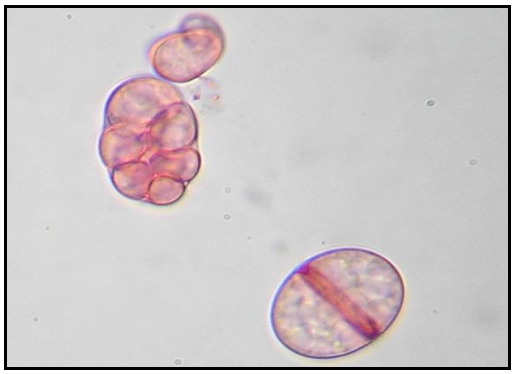

**Figure 1.** *P. zopfii* cells (Fuchsin stain, x1000)

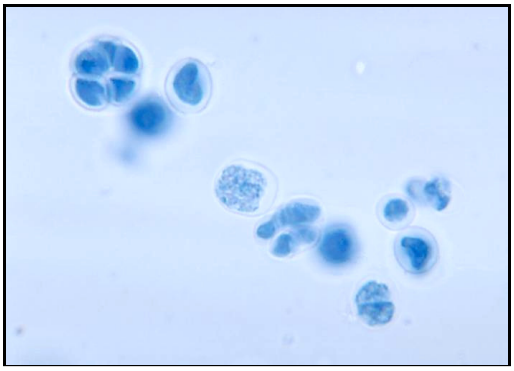

**Figure 2.** *P. wickerhamii* cells (Methylene blue stain, x1000)

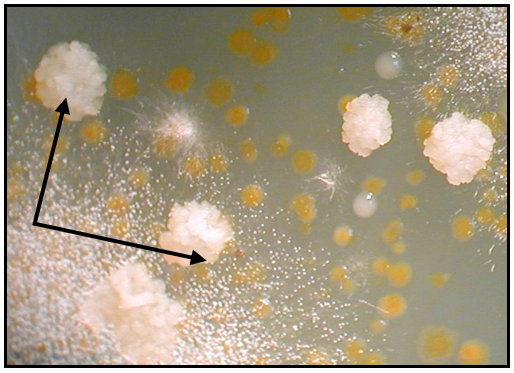

**Figure 3.** *P. zopfii* (black arrows) - 2 days colonies isolated from milk on Smith Baskerville medium, stereo glass x 1,6

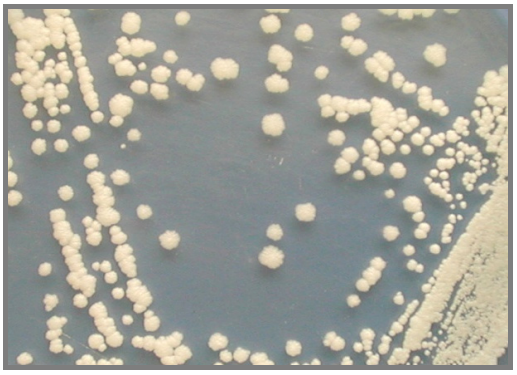

**Figure 4.** 3 days *Prototheca wickerhamii* colonies developed on glucose agar medium, stereo glass x 1,6

The frequency of MICs of *Satureja hortensis* and *Abies alba* essential oils in case of both *Prototheca* species are presented in table 2 and 3.

**Table 2.** The frequency of MICs of essential oil of *Satureja hortensis* for two *Prototheca* species tested

| Concentration (µL/mL) | 0.0625 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 |
|---|---|---|---|---|---|---|---|---|---|---|
| Frequency (%) of *P.zopfii* isolates | 0 | 0 | 30 | 20 | 50 | 0 | 0 | 0 | 0 | 0 |
| Frequency (%) of *P.wickerhamii* strain | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 3.** The frequency of MICs of essential oil of *Abies alba* for two *Prototheca* species tested

| Concentration (µL/mL) | 0.0625 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 |
|---|---|---|---|---|---|---|---|---|---|---|
| Frequency (%) of *P.zopfii* isolates | 0 | 0 | 0 | 0 | 30 | 50 | 20 | 0 | 0 | 0 |
| Frequency (%) of *P.wickerhamii* strain | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Variability of the percentage of inactivated strains depends on plant species from which the product was obtained, on their chemical composition and also on the concentration tested, as observed in fig. 5 and 6. The main characteristics of MICs are their low values, which can be an advantage for the use in therapy of the products based on active natural compounds.

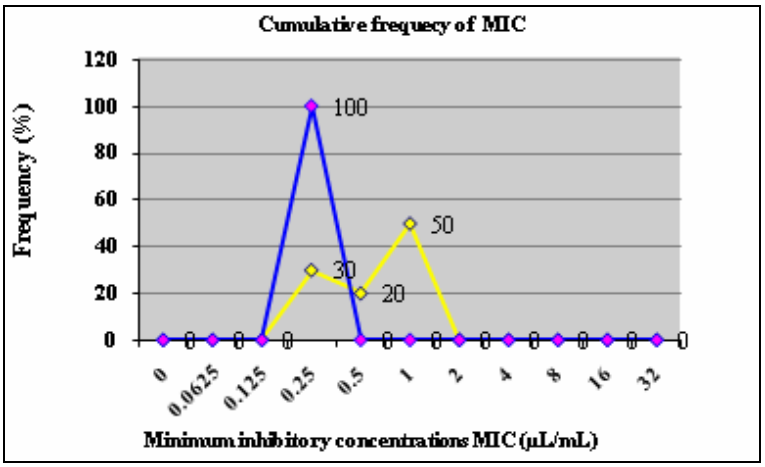


**Figure 5.** Comparative graphical representation of cumulative frequency of MICs for essential oil of *Satureja hortensis* on two *Prototheca* species. *P. zopfii* – yellow line, *P. wickerhamii* – bue line

As shown in Fig. 5 for *Saturenja hortensis* essential oils at MIC of 0.25%, *P. wickerhamii* tested strain was completely inactivated. In case of *P. zopfii*, majority of the tested strains (50%) were inactivated at concentration of 1%, 30% at MIC of 0.25% and only 20% at a MIC of 0.5%.

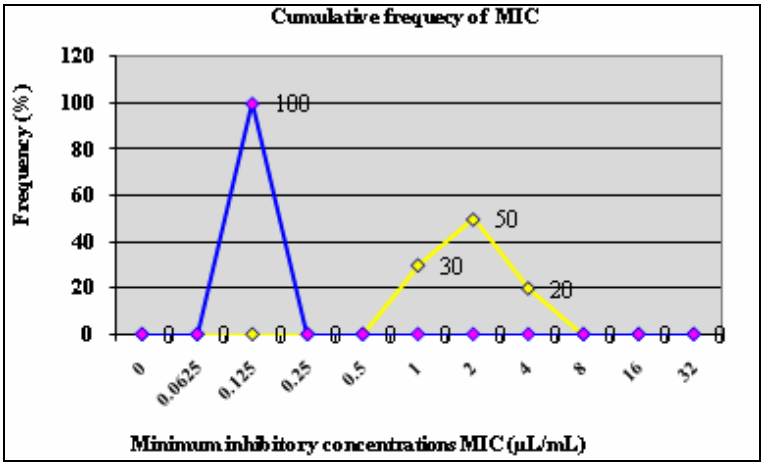


**Figure 6.** Comparative graphical representation of cumulative frequency of MICs for essential oil of *Abies alba* on two *Prototheca* species. *P. zopfii* – yellow line, *P. wickerhamii* – bue line

In Fig. no. 6, it can be observed that *Abies alba* essential oils inactivated *P. wickerhamii* strain at a MIC of 0.125%. Half of *P. zopfii* tested strains were inactivated at a MIC of 2%, 30% at a MIC of 1% and a percentage of 20% at MIC of 4%. So we can say that *Abies alba* essential oil is less effective for *P. zopfii spp*, comparative with *P. wickerhamii*.

The tested essential oils presented antialgicid properties on most of the *Prototheca* clinical isolates. Essential oil concentration varied depending on the climate, soil, collection period, extraction method, etc. [12], explaining high efficiency due to multiple substance compounds acting synergic and inducing a strong antialgicid activity. It is supposed that the active principles (carvacrol, poliphenols) are capable of dissolving algae wall, thus penetrating inside the cell, where they interact with cellular metabolic mechanisms [2]. Cytoplasm membrane disturbance, rupture of proton motive force and cytoplasm content coagulation are some mechanisms involved in the antimicrobial properties of essential oils [25].

In terms of evaluating anti-*Prototheca* effect of these natural products, the performed studies have global precedence, as the literature data are not mentioning the antimicrobial activities of these essential oils.

Yet recently several reports have documented the antimicrobial effects of essential oils extracted from various plant species, such as *Maleleuca alternifolia* (tea three) and *Citrus bergamota* (bergamot) [15, 23].

Our results are encouraging and require further study both in terms of quantifications of the different behavior of *Prototheca* isolates for natural products and also in order to obtain new pharmaceutical forms with adequate bioavailability of the active substance used in human and animal protothecosis therapy. So it is reasonable to say that the antialgae activity of these tested unconventional products could be successfully used in protothecosis therapy. However it is important to note that *in vitro* susceptibility values must be interpreted with caution to conclude *in vivo* clinical efficiency and should be confirmed by experimental studies *in vivo*.

## Conclusions

This study may conclude that the best algicidal effect in case of *P. zopfii* clinical isolates is obtained using products with major compounds like carvacrol and thymol (savory), and besides these poliphenols (fir) in *P.wickerhamii* infections.

In conclusion, difficulties in treating protothecosis with conventional antifungal drugs and the potent *in vitro* activity of essential oils as demonstrated by our studies justifies the increased interest for further investigations on the therapeutic use of these non-conventional natural products.

## Acknowledgements

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