



# Antifungal activity of *Myrtus communis* against *Malassezia* sp. isolated from the skin of patients with pityriasis versicolor

Aleksandra Barac<sup>1,2</sup> · Matthew Donadu<sup>3</sup> · Donatella Usai<sup>3</sup> · Vesna Tomic Spiric<sup>1,4</sup> · Vittorio Mazzarello<sup>3</sup> · Stefania Zanetti<sup>3</sup> · Ema Aleksic<sup>5</sup> · Goran Stevanovic<sup>1,2</sup> · Natasa Nikolic<sup>2</sup> · Salvatore Rubino<sup>3</sup>

Received: 8 August 2017 / Accepted: 13 November 2017 / Published online: 20 November 2017  
© Springer-Verlag GmbH Germany, part of Springer Nature 2017, corrected publication December 2017

## Abstract

The increasing incidence of fungal infections and antifungal resistance has prompted the search for novel antifungal drugs and alternative agents. We explored the antifungal activity of *Myrtus communis* essential oil (EO) against *Malassezia* sp. isolated from the skin of patients with pityriasis versicolor. These broad-spectrum antimicrobial activities of *M. communis* EO and its potent inhibiting activity on *Malassezia* growth deserve further research with aim to considerate this EO as candidate for topical use in treatment of skin diseases.

**Keywords** Antifungal activity · *Myrtus communis* · Essential oil · *Malassezia* · Pityriasis versicolor

## Introduction

*Malassezia* species are lipophilic yeasts that comprise part of the normal microflora of the skin of humans and warm-blooded animals [1]. However, this yeast can also cause lesions with distinct absence of inflammation despite the heavy fungal load (pityriasis versicolor/PV), or it can be involved with diseases that lead to characteristic inflammation, or even systemic infections under suitable environmental conditions [1, 2]. Among 14 *Malassezia* species, *Malassezia furfur* is one of the main causative agents of PV [1, 2].

PV or tinea versicolor is the most common disease caused by *Malassezia* yeasts and it is characterized by the development of hypo or hyperpigmented scaly spots, being more frequent in the upper trunk [1, 3]. The clinical aspect of the

lesions and the residual hypochromia or achromia that the disease may cause leads to great social stigma, so the proper topical antifungal treatment is required [2].

The increasing incidence of fungal infections and antifungal resistance has prompted the search for novel and effective antifungal drugs and alternative agents [3, 4]. As one of the most promising groups of natural products, essential oils (EO) are made up of many different volatile compounds and considered as potential targets for the development of safe natural antifungals [4]. In recent years, EO obtained from different plants has been documented to have the ability to effectively inhibit the growth of phytopathogenic fungi in vitro and in vivo [4, 5]. However, there is still need for development of new effective antifungal products, especially for local fungal infections that do not require systemic antifungal therapy, such as skin diseases. In addition, use of natural antifungal products could prevent the development of resistance on antifungal drugs.

It has been documented that *Myrtus communis* EO has fungicidal activity against the *Rhizoctonia solani*, *Fusarium solani*, *Colletotrichum lindemuthianum*, *Aspergillus flavus*, *A. ochraceus*, *Fusarium culmorum*, *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis* [6–8]. Additionally, this EO has broad antimicrobial activity; there are studies in the literature that report the antimicrobial activity of *M. communis* EO against *Escherichia coli* O157:H7, *Bacillus cereus*, *Listeria monocytogenes*, and *Streptococcus iniae*, antiplasmodial and insecticidal activity [5, 7, 8]. The

✉ Vittorio Mazzarello  
abarac@jdc.org

<sup>1</sup> Faculty of Medicine, University of Belgrade, Dr Subotica 1, 11000 Belgrade, Serbia

<sup>2</sup> Clinic for Infectious and Tropical Diseases, Clinical Centre of Serbia, Bulevar Oslobođenja 16, 11000 Belgrade, Serbia

<sup>3</sup> Department of Biomedical Sciences, University of Sassari, V. le San Pietro 43/C, 07100 Sassari, Italy

<sup>4</sup> Clinic for Allergology and Immunology, Clinical Centre of Serbia, Dr Koste Todorovica 2, 11000 Belgrade, Serbia

<sup>5</sup> Faculty of Stomatology, University of Business Academy, Zarka Zrenjanina 179, 26000 Pancevo, Serbia

antifungal effect of *M. communis* EO against *Malassezia* species has not been investigated yet, although it could have great impact for topical treatment of skin diseases caused by *Malassezia* species.

Given that, the aim of this study was to explore the antifungal activity of *M. communis* EO against *Malassezia* species isolated from the skin of the patients with PV.

## Materials and methods

### Study population

Study included 41 patients with PV that had not received any treatment for PV 2 weeks prior their enrolment. Inclusion criterion was a confirmed presence of PV and exclusion criteria were the presence of other skin or systemic diseases. The clinical diagnosis of PV was based on the clinical picture—the presence of coppery brown, paler than surrounding skin, or pink patches on trunk, neck, and/or arms, and uncommon on other parts of the body.

### Study design

This was a prospective case-series study, which was conducted at the Department of Biomedical Sciences, University of Sassari, Sardinia, and at the Faculty of Medicine, University of Belgrade. The study was approved by the Ethics Committee of the Clinical Centre of Serbia under the number 3177/2016.

### Data collection

All subjects had a complete clinical history, while physical examinations and necessary procedures from the skin were done aiming to confirm the diagnosis. Trained mycologists carried out laboratory analysis and were engaged in data collection and data entry.

### Sampling procedure for isolation of *Malassezia* yeast

For the isolation, identification and determination of density of *Malassezia*, samples were taken from four different locations: the surface layers of the back, thorax, arms and facial skin (area of 4 cm<sup>2</sup>), using adhesive tape (Suprasorb\_F, Lohmann Rauscher, Austria) on the skin for 20 s. Four samples were taken from the skin of thorax glabella or paranasal region.

Isolation of *Malassezia* was made by applying the sticky side of the tape to the surface of the selective culture media containing fatty acids with a higher carbon number. Two different selective media were used mDixon agar and

Leeming–Notman agar (LNA) for the isolation of lipophilic species, and standard mycological medium Sabouraud dextrose agar (SDA) was used for the isolation of *M. pachydermatis*. Media with samples were incubated for 10 days at 32 °C under aerobic conditions and growth on culture was observed daily. A preliminary identification of the lipophilic *Malassezia* species was done by macroscopic detection of colonies on mDixon and LNA, and by the lack of growth of colonies on SDA.

### Identification of *Malassezia* yeast

Identification of isolated strains was performed by examining microscopy cell characteristics and by testing physiological and biochemical characteristics (catalase production and Tween assimilation). *Malassezia* colonies were suspended in 0.9% NaCl and examined under the microscope to detect shape and size of yeast cells and width of budding yeast base. Production of the enzyme catalase was tested by adding a drop of 3% H<sub>2</sub>O<sub>2</sub> to the culture suspension. The suspension was observed microscopically and the production of gas bubbles was considered as a positive catalase reaction. Tween diffusion test was used for further identification of *Malassezia*. This method is based on the ability of different species to exploit a specific pattern of Tween as the only source of lipids in the SDA plate (Tween 20, 40, 60 and 80).

### Chemical composition of EO

The composition of EO *M. communis* was investigated by LC–MS/MS according to the previously reported method. Main compounds of *M. communis* EO are presented in the Table 1. Standards of the compounds were purchased from Sigma-Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland) or from ChromaDex (Santa Ana, CA, USA). The Agilent 1200 series liquid chromatograph coupled with Agilent series 6410B electrospray ionization triple-quadrupole mass spectrometer and controlled by MassHunter Workstation B.03.01 software was used for analysis. Components were separated using a Zorbax Eclipse XDB-C 18 4.6 mm × 50 mm × 1.8 μm (Agilent Technologies, Santa Clara, CA, USA) reversed phase column.

### Retention indexes

A hydrocarbon mixture of *n*-alkanes (C<sub>9</sub>–C<sub>22</sub>) was analyzed separately under the same chromatographic conditions used on the HP-5MS and the VF-Wax capillary columns to calculate the retention indexes with the generalized equation,  $I_x = 100[(tx - tn)/(tn + 1 - tn) + n]$ , where *t* is the retention time, *x* is the analysis, *n* is the number of carbons of alkane

**Table 1** Main compounds of *M. communis* essential oil

Compound	RT	A%	RI
$\alpha$ -Pinene	9.31	0.38	932.06
Limonene	13.19	0.60	1024.68
1,8-Cineole	13.50	10.27	1032.01
Linalool	16.51	3.78	1103.10
Terpinolene	16.69	1.41	1107.48
<i>cis</i> Verbenol	18.01	0.91	1140.34
<i>trans</i> Verbenol	18.21	0.95	1145.24
Camphor	18.31	1.91	1147.62
$\alpha$ -Terpineol	20.23	7.12	1195.22
Nerol	21.71	5.97	1234.07
Geraniol	22.23	0.63	1247.83
Linalyl acetate	22.69	3.66	1260.16
Myrtenyl acetate	25.27	7.00	1330.30
Terpinyl acetate	26.08	1.01	1353.08
Neryl acetate	26.63	3.40	1368.57
Geranyl acetate	27.40	16.36	1390.15

A results are expressed as relative percentage area

RT retention time, RI retention index

that elutes before analysis and  $n + 1$  is the number of carbons of alkane that elutes after analysis.

### Preparation of *Malassezia* cell suspension

*Malassezia* cell suspension was prepared direct from mDixon, LNA or SDA agar using a sterile cotton swab from the surface of the colonies and re-suspended in 0.9% NaCl with 0.1% Tween 20 by mixing thoroughly using vortex mixer. After settling of the larger particles, suspensions were adjusted to 106 colony forming units per 1 ml (CFU/ml), and then diluted in RPMI 1640 (2% glucose) in a ratio of 1:10 (105/ml).

### Antifungal effect assessment of EO using microdilution method

Antifungal effect of EO on *Malassezia* strains was tested using EUCAST broth microdilution method [9]. The final volume of the microtiter plate wells was 200  $\mu$ l (100  $\mu$ l/well of EO solution and 100  $\mu$ l/well spore suspension). Final concentrations of EO were 0.12, 0.5 and 1%, and final inoculum size 104/well of *Malassezia* cells. Appropriate control including no addition of EO was included: 100  $\mu$ l/well RPMI 1640 and 100  $\mu$ l/well *Malassezia* strains were added to each well where EO was not added. Microtiter plates were incubated at 35 °C for 5 days under aerobic conditions. Cell growth was investigated by measuring absorbance at 620 nm after 5 days incubation at 35 °C. Minimum inhibitory concentration (MIC) was considered as the lowest concentration

that inhibits visible growth of fungal spores in the well. Contents of all wells with no visible growth after 5 days were subcultured on mDixon, LNA and SDA plates in aim to determine minimum fungicidal concentrations (MFC). MFC was considered as the lowest concentration that kills all the fungal spores (at which no growth occurred on the SDA plates).

### Data analysis

Descriptive and inferential statistical analysis were used for the evaluation of the data using the Statistical Package for Social Science (SPSS) (SPSS version 17.0; SPSS, Chicago, IL, USA). Data were expressed as mean  $\pm$  SD and counts or percentages (%), where appropriate. The Mann–Whitney test was used for non-parametric data, and the Chi-square test was used for categorical variables. A *p* value of < 0.05 was assumed to be significant.

## Results

### Patient's demographic data and isolated strains

In the PV population ( $n = 41$ ), there were 22 men (~ 54%) and 19 women (~ 46%) aged 20–80 years (mean  $43 \pm 16$ ). A total of 86 yeast colonies were isolated from 41 patients with PV. Seven different *Malassezia* species were identified as follows: *Malassezia furfur* (42.5%, 37/86), *M. sympodialis* (23.5%, 20/86), *M. slooffiae* (13.9%, 12/86), *M. globosa* (7.5%, 7/86), *M. obtusa* (6%, 5/86), *M. japonica* (4%, 3/86) and *M. restricta* (2.5%, 2/86). The most and least infected sites were back (74.5, 31/41) and forehead (11.8%, 5/41), respectively.

### Inhibition of fungal growth with EO

Inhibition of fungal growth with *M. communis* EO was effective in: 96% (36/37) of isolated *M. furfur*, 83% (16/20) of *M. sympodialis*, 78% (9/12) of *M. slooffiae*, 78% (5/7) of *M. globosa*, 75% (4/5) *M. obtusa*, 73% (2/3) of *M. japonica* and 62% (1/2) of *M. restricta*.

In other cases, *Malassezia* had shown growth despite EO presence.

### MIC and MFC

MIC<sub>90</sub> values range from 15.625 to 600  $\mu$ l/ml. The less MIC and MFC of *M. communis* EO were 31.25 and 62.5  $\mu$ l/ml, respectively. Results of inhibitory effect of *Myrtus communis* against different *Malassezia* species are presented in Table 2.

**Table 2** Inhibition effect of *Myrtus communis* against different *Malassezia* species

Fungi (n)	EO			
	<i>M. communis</i>			
	Range	Median MIC ( $\mu\text{l/ml}$ )	Range	Median MFC ( $\mu\text{l/ml}$ )
<i>M. furfur</i> (37)	15.625–31.25	31.25	31.25–62.5	62.5
<i>M. sympodialis</i> (20)	31.25–62.5	62.5	62.5–125	125
<i>M. slooffiae</i> (12)	15.625–31.25	31.25	31.25–62.5	62.5
<i>M. globose</i> (7)	15.625–31.25	31.25	62.5–350	350
<i>M. obtuse</i> (5)	15.625–62.5	62.5	62.5–125	125
<i>M. japonica</i> (3)	15.625–31.25	31.25	31.25–62.5	62.5
<i>M. restricta</i> (2)	31.25–125	125	125–600	600

EO essential oils, MIC minimum inhibitory concentration, MFC minimum fungicidal concentrations

## Discussion

EOs are widely used in folk medicine and cosmetic industry, but only in recent years they have been recognized as a potential antimicrobial agent that could be used in the treatment of infectious diseases [1, 2]. Up to our knowledge, this is the first study aimed to analyze inhibitory effect of *M. communis* EO against *Malassezia* sp. Our data showed that median MIC and MFC of *M. communis* tested against ten isolates were 31.25 and 62.5  $\mu\text{l/ml}$ , respectively, while the highest inhibiting activity was shown on growth of *M. furfur* (96%) and *M. sympodialis* (83%). It can be concluded that the broad-spectrum antimicrobial activity of *M. communis* EO includes also potent inhibition on *Malassezia* growth placing this EO as a candidate for topical use in the treatment of skin diseases.

*Myrtus communis*, belonging to the *Myrtaceae* family, is a shrub that is growing in different parts of the world, including Sardinia [5, 6]. People traditionally are using the leaves and fruits of myrtle as stomachic, hypoglycaemic, anti-cough, antimicrobial, and anti-haemorrhage remedy [6–8, 10]. The EO obtained from myrtle leaves were found to be efficient in lung disorders and wound healing, and they showed antimicrobial, antioxidant, and anti-mutagenic properties [6–8]. The main constituents of Italian myrtle oil were found to be 1,8-cineole and  $\alpha$ -pinene [10].

Ghasemi Pirbalouti et al. evaluated the antimicrobial activity of *M. communis* extracts and EOs against *Escherichia coli* O157:H7, *Bacillus cereus*, *Listeria monocytogenes*, and *C. albicans*, and both the extracts and EOs showed to be effective growth inhibitors of *C. albicans* (83.67 and 91.16%, respectively) and bacterial pathogens [5]. Moreover, these authors revealed the positive

antibacterial activity of *M. communis* against *Streptococcus iniae*, as well as its antiplasmodial and insecticidal activities [5]. The growth inhibition of clinical isolates of *Candida* by *M. communis* EO was also studied [7], while there is no study on growth inhibition of clinical isolates of other fungi, as *Malassezia*. Our study revealed potent therapeutical activity of *M. communis* on different types of skin diseases caused by *Malassezia* sp. The highest inhibiting activity was shown on growth of *M. furfur* (96%) and *M. sympodialis* (83%). Our results suggest that the *M. communis* EO may replace antifungal drugs in the treatment of fungal infections of the skin, mucous membranes and fight against dandruff.

Antimicrobial activity of plant extracts is considered significant if MIC values are below 100  $\mu\text{l/ml}$  for crude extract and moderate when MICs vary from 100 to 625  $\mu\text{l/ml}$  [7, 8]. In this study, *M. communis* significantly inhibited the growth of all the tested *Malassezia* sp. with median MIC and MFC 31.25 and 62.5  $\mu\text{l/ml}$ , respectively. This study indicates that the crude extract of *M. communis* had an in vitro antifungal activity against pathogenic fungal strains isolated from the skin. These results should be confirmed by in vivo research of efficacy of this EO on the skin redness, lesion severity, and *Malassezia* occurrence on the skin. There are only a very few references in the literature that refer to the efficacy of *M. communis* on the human skin diseases, but no one refers to PV and *Malassezia* sp. These studies showed that the statistically significant recurrence of hair growth in the dermatophytes infected areas in treatment models compared with control group. While clotrimazole cured *T. mentagrophytes* and *M. canis* infection on days 21 and 17, respectively; *M. communis* extract cured *T. mentagrophytes* and *M. canis* infection on days 9 and 13, and days 9 and 11, respectively [8–10].

In general, longer treatment periods (up to 4 weeks) and higher concentrations of topical regimens or doses of systemic agents result in higher cure rates, without, however, avoiding the increased relapse rate [3]. Optimal preventive regimens employing other oral antifungals or topical formulations have not been adequately evaluated to date. Our study suggests application of *M. communis* EO in treatment and prophylaxes of PV as safe, less expensive and non-hepatotoxic and non-nephrotoxic treatment. Various pharmacological activities such as antioxidant, anti-inflammatory, anticancer, and antimicrobial effects have been related to *M. communis* [5, 10]. In addition, phytochemical screening of the crude extract of *M. communis* has revealed the presence of tannins, alkaloids, flavonoids, and phenols in these plants. Individual activities of these compounds have also been proven [5, 6, 10]. Various studies have reported the potent antifungal effects of these compounds and their derivatives such as thymol and carvacrol against some pathogenic fungal strains [10].

The lack of this study is use of morphological and biochemical analysis for identification at species level, although, in some countries, new methods are available. Matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) has been reported to be a rapid and reliable diagnostic tool to identify clinically important yeasts, but so far no data have been reported on identification of *Malassezia* isolates with this technique. Implementation of the MALDI-TOF MS system as a routine identification tool will contribute to correct identification of *Malassezia* yeasts with minimal effort and in a short turnaround time, which is especially important for the rapid identification of *Malassezia* in skin diseases and nosocomial outbreaks [10].

## Conclusion

These results suggest that the *M. communis* EO may replace antifungal drugs in the treatment of fungal infections of the skin, mucous membranes and fight against dandruff. The broad-spectrum antimicrobial activity of *M. communis* EO should be further researched by in vivo studies with aim to considerate this EO as candidate for topical use in treatment of skin diseases.

**Acknowledgements** The paper has been published with the support of the Projects of Ministry of Education, Science and Technology of the Republic of Serbia (Nos. III45005 and OI 175025).

## Compliance with ethical standards

**Conflict of interest** There is no financial or any other conflict of interest.

## References

- Gaitanis G, Velegraki A, Mayser P, Bassukas ID. Skin diseases associated with *Malassezia* yeasts: facts and controversies. *Clin Dermatol*. 2013;31:455–63.
- Velegraki A, Cafarchia C, Gaitanis G, Iatta R, Boekhout T. *Malassezia* infections in humans and animals: pathophysiology, detection, and treatment. *PLoS Pathog*. 2015;11:e1004523.
- Sharma A, Rabha D, Ahmed G. In vitro antifungal susceptibility of *Malassezia* isolates from pityriasis versicolor lesions. *Indian J Dermatol Venereol Leprol*. 2017;83:249–51.
- Pekmezovic M, Aleksic I, Barac A, Arsic-Arsenijevic V, Vasiljevic B, Nikodinovic-Runic J, Senerovic L. Prevention of polymicrobial biofilms composed of *Pseudomonas aeruginosa* and pathogenic fungi by essential oils from selected Citrus species. *Pathog Dis*. 2016;. <https://doi.org/10.1093/femspd/ftw102>.
- Ghasemi Pirbalouti A, Izadi A, Malek Poor F, Hamed B. Chemical composition, antioxidant and antibacterial activities of essential oils from *Ferulago angulata*. *Pharm Biol*. 2016;54:2515–20.
- Kordali S, Usanmaz A, Cakir A, Komaki A, Ercisli S. Antifungal and herbicidal effects of fruit essential oils of four *Myrtus communis* genotypes. *Chem Biodivers*. 2016;13:77–84.
- Cannas S, Molicotti P, Ruggeri M, Cubeddu M, Sanguinetti M, Marongiu B, Zanetti S. Antimycotic activity of *Myrtus communis* L. towards *Candida* spp. from isolates. *J Infect Dev Ctries*. 2013;7:295–8.
- Deriu A, Branca G, Molicotti P, Pintore G, Chessa M, Tirillini B, Paglietti B, Mura A, Sechi LA, Fadda G, Zanetti S. In vitro activity of essential oil of *Myrtus communis* L. against *Helicobacter pylori*. *Int J Antimicrob Agents*. 2007;30:562–3.
- Rodriguez-Tudela IL, Donnelly JP, Arendrup MC, Arikan S, Barchiesi F, Bille J, et al. EUCAST technical note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia-forming moulds. *Clin Microbiol Infect*. 2008;14:982–4.
- Aleksic V, Knezevic P. Antimicrobial and antioxidative activity of extracts and essential oils of *Myrtus communis* L. *Microbiol Res*. 2014;169:240–54.