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Rosmarinic acid: modes of antimicrobial and antibiofilm activities of common plant polyphenol

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Abstract

Emerging antimicrobial resistance and side effects associated with antibiotics overuse are driving the need for the novel antimicrobial therapeutics with the natural compounds considered as an appealing alternative. Rosmarinic acid is abundantly present in medicinal plants. This study aimed to elucidate its possible role as an inhibitor of planktonic and biofilm microorganism growth, along with its antifungal mechanisms. In this study, rosmarinic acid showed promising anticandidal (MIC 0.1-0.2 mg/mL) and antibacterial (MIC 0.002->0.8 mg/mL) activity. To some extent it was able to prevent cell attachment and eradicate established biofilms. Rosmarinic acid antifungal mechanisms included reduction of mitochondrial activity, alteration in membrane integrity, and slight inhibition of protease production but not binding to ergosterol. Its antibiofilm activity was moderately linked to the reduction in exopolysaccharide production. Considering the wide antimicrobial and antibiofilm of spectrum of rosmarinic acid, it could be further explored as an antimicrobial agent along with range of medicinal plants with rosmarinic acid as the dominant compound. Nevertheless, rosmarinic acid could serve as the basis for the development of antimicrobials employing novel mechanisms of activity.

Keywords: Rosmarinic acid; polyphenolic compound; anticandidal; antibiofilm, mode of action.

1. Introduction

There is the rising incidence of fungal infections worldwide with the significant contribution of *Candida* spp. in their frequency (Osman et al., 2020). Mortality rates as a consequences of invasive candidiasis are up to 55% and are frequent among the intensive care patients (Logan et al., 2020). Oral cavities are an anatomical site suitable for *Candida* spp. infections with oral candidiasis being linked to abundant use of broad-spectrum antibiotics, immunosuppressive agents, catheters, indwelling medical devices and organ transplant patients (Vila et al., 2020) with incidence rates going up to 20-31% in some groups of patients (Patil et al., 2015). Current antifungal drugs are not able to efficiently fight these fast adapting yeasts (Hokken et al., 2019), driving the need for the development of novel therapeutics.

Candida albicans is the dominant cause of invasive candidiasis followed by *C. glabrata, C. parapsilosis* and *C. tropicalis* (Ricotta et al., 2020). Although these yeasts are normally considered as harmless, under certain conditions they can cause life treating diseases – with the cell attachment to biotic and abiotic surfaces as the first stage of infection (Ciurea et al., 2020). *Candida* spp. have developed a range of adaptation traits in order to successfully avoid host immune system reactions and establish infection. These virulence factors include biofilm formation, dimorphism, production of toxin candidalysin and secretion of hydrolytic enzymes such as proteases (Staniszewska, 2019). Biofilm formation starts by cell attachment and continues by colonization and polysaccharide production. When cells are embedded in a matrix

they are protected from environmental factors including antibiotics to which they gain resistance (Chandra and Mukherjee, 2015).

Candida is frequently accompanied with bacteria including *Streptococcus* sp., *Staphylococcus aureus* and *Enterococcus faecalis* (Montelongo-Jauregui and Lopez-Ribot, 2018). Yeast and bacterial communities are responsible for range of conditions such as oropharyngeal diseases (Koo et al., 2018) and urinary tract infections (Shing et al., 2020) emphasizing the demand for novel antimicrobial agents suitable for both bacteria and fungi.

A range of molecules including ones of natural origin have been studied along with synthetic compounds (Ogris et al., 2021; Zoidis et al., 2021), with the search for novel antimicrobials continuing. Rosmarinic acid is a naturally occuring polyphenol abundantly present in herbs, spices and medicinal plants like *Nepeta nuda* (Smiljkovic et al., 2018a), *Melissa officinalis* (Caleja et al., 2018), *Lavandula pedunculata* (Lopes et al., 2018). This molecule is an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid. It has numerous biological activities such as antiviral, antibacterial, antioxidant and anti-inflammatory activities (Luo et al., 2020).

This study aimed to elucidate the antimicrobial spectrum of rosmarinic acid. This encompasses its inhibitory potential towards both fungal and bacterial pathogens, its antibiofilm capacity towards a range of *Candida* spp. including its most common member *Candida albicans* as well as the potential of rosmarinic acid to act as membrane disrupting agent, inhibitor of mitochondrial activity, and inhibitor of protease production in yeast cells.

2. Material and methods

2.1. Microorganisms and culture conditions

Candida species used were clinical isolates *C. albicans* 475/15, *C. albicans* 13/15, *C. albicans* 17/15, *C. albicans* 527/14, *C. albicans* 10/15, *C. albicans* 532/15, *C. krusei* H1/16, *C. glabrata* 4/6/15 and ATCC strains *C. albicans* ATCC 10231, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019. Clinical *Candida* strains were isolated from oral cavities of patients at the Clinic of Otorhinolaryngology, Clinical Hospital Centre Zvezdara, Belgrade, Serbia after obtaining informed written consent. Strains were determined on CHROMagar plates (Biomerieux, France) and grown on Sabouraud Dextrose Agar/Broth (Merck, Germany).

The following Gram-positive and Gram-negative bacteria were used: *Micrococcus luteus* (dT_9/2), *Rothia mucilaginosa* (oT_22/2), *Streptococcus agalactiae* (oT_20/1), *Streptococcus anginosus* (oT_26), *Streptococcus dysgalactiae* (oT_21/2), *Streptococcus oralis* (oT_5), *Streptococcus parasanguinis* (oT_3), *Streptococcus pyogenes* (dT_14), *Streptococcus salivarius* (dT_12), *Staphylococcus aureus* (oT _ 4), *Staphylococcus hominis* (oT_14/2), *Enterobacter cloacae* (oT_18), and *Stenotrophomonas maltophilia* (A_12). These bacteria were maintained on Blood Agar (Torlak, Belgrade, Serbia). They were obtained from the tonsillar tissue of patients after obtaining informed written consent, at Otorhinolaryngology clinic at Clinical Hospital Center Zvezdara, Belgrade, Serbia. All tested isolates were identified by matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry (VITEK MS bioMerieux, Marcy l'Etiole, France).

Tested microorganisms were deposited at the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" - National Institute of Republic of Serbia, University of Belgrade.

2.2. Anticandidal activity

Minimal inhibitory and minimal fungicidal concentration (MIC/MFC) were determined for rosmarinic acid (0.0031 – 0.8 mg/mL) and ketoconazole (0.00039 – 0.1 mg/mL) in Sabouraud Dextrose Broth growth medium (Torlak, Serbia) as previously described (Ivanov et al., 2020). Briefly, fresh overnight yeast cultures were adjusted to a concentration 1.0×10^5 CFU/well with the use of sterile saline. The microplates were incubated at 37 °C for 24 h, after which the MIC and MFC were determined. The MIC values were considered as the lowest concentrations without microscopically observed growth compared to the control (untreated yeast cells). For microscopic determination of growth, we used inverted microscope Nikon Eclipse TS2 (Amsterdam, Netherland) and examined the fungal growth in the wells of 96-well microtiter plates. Following the serial subcultivations of 10 µL into microtiter plates containing 100 µL broth per well, as well as subsequent incubation at 37 °C for 24 h, the lowest concentrations with no visible growth were defined as the MFC values. Ketoconazole was used as a positive control. Both rosmarinic acid and ketoconazole were obtained from Sigma–Aldrich, Germany.

2.3. Antibacterial activity

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined by a serial microdilution of rosmarinic acid (0.0031 – 0.8 mg/mL) in 96 well microtiter plates following the protocol previously described (Kostić et al., 2017).

Briefly, the rosmarinic acid was serially diluted in Tryptic Soya Broth medium (Torlak, Serbia) and inoculated with bacteria (1×10^5 CFU/mL). The results were observed after incubation at 37°C for 24 h. The color p-iodonitrotetrazolium chloride (0.2 mg/mL, SigmaAldrich, Germany) was added to the wells of the microtiter plates and incubation continued for further 30 min. Minimal inhibitory concentration was indicated by a decrease in the color reaction. The minimum bactericidal concentration (MBC) was determined by serial sub-cultivation of a 2 µL sample into microtiter plates containing 100 µL of broth per well and further incubation for 24 h at 37 °C. The lowest concentration with no visible growth was defined as the MBC.

Amoxicillin with clavulanic acid (Hemofarm, Vršac, Serbia) was used as positive control (0.0002 – 0.056 mg/mL).

2.4. Inhibition of cell attachment

Strains used for inhibition of cell attachment assay and biofilm eradication assay were: *C. albicans* 475/15, *C. albicans* ATCC 10231, *C. krusei* H1/16, *C. glabrata* 4/6/15, *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019.

Impact of rosmarinic acid on *Candida* attachment as the first stage of biofilm formation was determined as previously described (Smiljkovic et al., 2018b). Rosmarinic acid was applied in concentration range 0.025 mg/mL – 0.1 mg/mL for *C. albicans* 475/15, *C. glabrata* 4/6/15, and *C. parapsilosis* ATCC 22019, while 0.05 – 0.2 mg/mL of rosmarinic acid was tested towards *C. albicans* ATCC 10231, *C. krusei* H1/16, and *C. tropicalis* biofilm formations according to their previously determined range 0.25 MIC - MIC.

Candida spp. cells were incubated for 24 h in 96-well microtiter plates with an adhesive bottom (Sarstedt, Germany) at 37 °C with MIC and subMIC concentrations of the compound. After 24 h, each well was washed twice with sterile PBS (phosphate-buffered saline, pH 7.4). Fixation of adhered cells was done with methanol, after which the plate was air dried and stained with 0.1% crystal violet (BioMerieux, France) for 30 min. Wells were washed with water, air dried, and then 100 µL 96% ethanol (Zorka, Serbia) was added into wells to suspend all the bound stain. Absorbance was read at 620 nm with a Multiskan FC Microplate Photometer (Thermo Scientific). Percentage of inhibition of biofilm formation was calculated by using equation:

$[(A_{620}\text{control} - A_{620}\text{sample}) / A_{620}\text{control}] \times 100.$

Where A_{620}control is the absorbance of the untreated *Candida* biofilm, and A_{620}sample absorbance of the treated sample.\

2.5. Minimal biofilm eradication concentration

Candida spp. cells (1.0×10^5 CFU/well) were incubated in 96-well microtiter plates with an adhesive bottom (Sarstedt, Germany) in YEPD broth (Himedia, India) at 37 °C for 24 h. Preformed 24 h *Candida* spp. biofilms were treated with rosmarinic acid (0.025 – 1.6 mg/mL) at 37 ºC for another 24 h. Biofilms were washed and treated as described above. After crystal violet staining minimal biofilm eradication concentration was determined as a concentration without observed biofilm biomass in the treated wells, in comparison with the biofilm formed by untreated yeast cells.

2.6. Congo red binding assay

The impact of tested compound on exopolysaccharide (EPS) production by *C. albicans* 475/15 biofilm was estimated with some modifications according to the method previously published (Dey et al., 2020). Pre-formed 24 h biofilms in microtiter plates were treated with rosmarinic acid at their MIC, 0.5 MIC and 0.25 MIC concentrations at 37 °C for 24 h. Planktonic cells were then discarded and the adhered cells were washed with PBS. Congo red (1 %, w/v) was added to wells and was kept in dark for 30 min. Excess dye was removed and the bound dye was solubilized with 200 µL DMSO. The absorbance was measured at 490 nm in a microtiter plate reader. Percentage of EPS inhibition was calculated according to the equation:

$$\%inhibition=(OD_{490}(control)- OD_{490}(sample))/ OD_{490}(control)*100$$

where OD_{490}(control) is the absorbance of the untreated biofilm, and OD_{490}(sample) absorbance of the treated sample.

2.7. Protease inhibition

The impact of tested compound on proteolytic activity of *C. albicans* was measured using azocasein as substrate according to the modified method previously described (Abirami et al., 2020). Briefly, *C. albicans* 475/15 was grown in YEPD broth in the presence and absence of rosmarinic acid in MIC concentrations at 37°C for 16 h. After incubation, samples were centrifuged at 9660 g, room temperature (RT), for 10 min (Heraeus biofuge stratos centrifuge,

Thermo electron corporation, MA, USA). Supernatant was mixed with 2% azocasein and incubated at 37°C for 45 min. The reaction was stopped using 10% trichloroacetic acid and centrifuged at 9660 g , RT, for 10 min. After centrifugation, the absorbance of the supernatants was measured at OD_{440} nm. Percentage of inhibition of protease activity was determined according to equation:

$$\%inhibition=(OD_{440}(control)- OD_{440}(sample))/ OD_{440}(control)*100$$

where OD_{440}(control) is the absorbance of the untreated control, and OD_{440}(sample) absorbance of the treated sample.

2.8. Ergosterol binding as potential mode of action

Microdilutions of rosmarinic acid and amphotericin B (positive control) were prepared in the same manner as used for determination of antimicrobial activity, except that ergosterol (400 µg/mL) was added to the rows of the plate (Leite et al., 2015). MIC values were observed after incubation at 37ºC for 24 h, and were compared with the MIC value of samples without ergosterol addition.

2.9. Crystal violet uptake assay

Alteration of membrane permeability was detected by modified crystal violet assay (Khan et al., 2017). *C. albicans* 475/15 cells were harvested at 6708 g, RT for 5 min (Heraeus biofuge stratos centrifuge, Thermo electron corporation, MA, USA). The cells were washed twice and

suspended in 50 mM PBS (pH 7.4). Rosmarinic acid at MIC was added to the cell suspension and incubated at 37°C for 30 min. Control samples were prepared similarly without treatment. The cells were harvested at 6708 g, RT for 5 min and suspended in PBS containing 10 µg/mL of crystal violet. The cell suspension was then incubated for 10 min at 37°C and centrifuged at 6708 g, RT for 5 min, after which the OD_{590} of the supernatant was measured using a Multiskan FC Microplate Photometer (Thermo Scientific). The optical density (OD) value of crystal violet solution, which was originally used in the assay, was considered as 100% excluded. The percentage of crystal violet uptake for all samples was calculated using the following formula:

%Dye uptake = 100-[(OD of the sample/OD value of crystal violet solution) × 100].

2.10. Mitochondrial activity

MTT assay was used for the determination of *C. albicans* 475/15 mitochondrial activity upon treatment with rosmarinic acid (MIC) according to Ansari et al. (2016) with some modifications. Overnight culture of *C. albicans* was diluted (OD_{600} 0.1) and incubated at 37°C for 5 h. Upon incubation, *C. albicans* was treated with MIC of rosmarinic acid for 3 h. Fungal cells (500 µL) were centrifuged and washed twice with medium. MTT (500 µL, 100 µg/mL) was added to the samples and incubation continued for further 2 h. Cells were again harvested and washed twice. The remaining pellets were dissolved in DMSO by shaking for 5 min. The suspensions were centrifuged and OD_{570} of the supernatants was measured using a Multiskan FC Microplate Photometer (Thermo Scientific). The obtained results are presented as mitochondrial activity of treated sample compared to the activity of untreated control set as 100%.

2.11. Statistical analysis

The experiments were performed in three replicates. The data were calculated as a mean ± standard error, and statistically analyzed using GraphPad PRISM 6 software by Student's *t*-test, with *, $p \leq 0.05$; **, $p \leq 0.01$; **** $p \leq 0.0001$.

3. Results

3.1. Anticandidal potential of rosmarinic acid

Rosmarinic acid showed promising antifungal capacity indicated with MIC in range 0.1-0.2 mg/mL (**Table 1**). Strains *C. albicans* ATCC 10231, *C. krusei* H1/16 and *C. tropicalis* ATCC 750 were slightly more resistant to the rosmarinic acid application (MIC 0.2 mg/mL) compared to other yeasts examined. Rosmarinic acid had significantly lower antifungal potential then the commercial drug ketoconazole.

3.2. Rosmarinic acid antibacterial spectrum

Rosmarinic acid had antibacterial properties ranging from excellent (MIC 0.002 mg/mL towards *Streptococcus salivarius*) to not active (*Staphylococcus aureus* MIC>0.8 mg/mL) (**Table 2**). Along with *S. salivarius*, the rest of *Streptococcus* strains tested were highly susceptible to rosmarinic acid (MIC 0.05 mg/mL towards *S. parasanquinis*, *S. oralis*, *S. angiosus, S. agalactiae*

and *S. dysgalactiae*). *S. aureus*, *Stenotrophomonas maltophilia* and *Staphylococcus hominis* were the most resistant strains (MIC 0.4 mg/mL) (**Table 2**). Rosmarinic acid had lower antibacterial potency compared to commercial drug (**Table 2**).

3.3. Rosmarinic acid moderatly inhibits *Candida* spp. attachment

Rosmarinic acid has variously affected different *C. albicans* strains attachment ability in a dose-dependent manner (**Figure 1**). Strain *C. albicans* 475/15 was efficiently prevented to attach and form biofilm biomass with MIC of rosmarinic acid (>50% inhibition). Its effect on *C. albicans* ATCC 10231 was moderate.

Attachment of non-albicans *Candida* strains, with the exception of *C. tropicalis*, was more significantly affected by rosmarinic acid compared to *Candida albicans* (**Figure 1**) Application of rosmarinic acid in 0.5 MIC (0.05 mg/mL) prevented *C. glabrata* 4/6/15 attachment for more than 50%. Both *C. krusei* H1/16 and *C. parapsilosis* ATCC 22019 attachments has been inhibited for more than 50% when rosmarinic acid was applied.

3.4. Rosmarinic acid eradicates *Candida* sp. preformed biofilms

Established *C. krusei* H1/16 biofilms were the most resistant to the treatment with rosmarinic acid (minimal biofilm eradication concentration, MBEC>1.6 mg/mL). Biofilms formed by *C. albicans* 475/15 and *C. albicans* ATCC 10231 were obstructed with 0.4 mg/mL of rosmarinic acid. Unlike cell attachment, formed biofilms were more profoundly affected in *Candida albicans* strains compared to non-albicans *Candida* (**Table 3**).

3.5. Rosmarinic acid moderate impact on exopolysaccharide production

C. albicans 475/15 was the one most significantly affected by rosmarinic acid in attachment inhibition and biofilm eradication assay and was chosen as a model strain for the further experiments.

Exopolysaccharide production in *C. albicans* 475/15 biofilm was moderately affected by application of rosmarinic acid (>20% inhibition with MIC of rosmarinic acid), while the effect of ketoconazole was negligible (<10% inhibition) (**Figure 2a**).

3.6. Production of proteases is not significantly altered by rosmarinic acid

With MIC of rosmarinic acid production of proteases in *C. albicans* decreased for less than 20%, suggesting that its antivirulence effects are mediated through other mechanisms (**Figure 2b**).

3.7. Rosmarinic acid affects *C. albicans* 475/15 cell integrity and mitochondrial function

Uptake of crystal violet in the absence of rosmarinic acid was 15.8% while after 0.1 mg/mL rosmarinic acid treatment it has more than doubled (39.6%) suggesting that membrane permeability was altered ($p \leq 0.01$) (**Figure 3a**).

Rosmarinic acid treatment significantly reduced the activity of *C. albicans* mitochondria (**Figure 3b**). MTT assay suggested that with 0.1 mg/mL (MIC) of rosmarinic acid mitochondria activity could be lowered for more than 50%.

3.8. Influence on membrane integrity is not mediated through ergosterol binding

Binding to essential membrane lipid, ergosterol, was tested as a potential mechanism of antifungal activity. Rosmarinic acid MIC did not change in the presence of ergosterol, unlike the positive control amphotericin B (MIC increased 3 times, **Table 4**) suggesting that antifungal action of rosmarinic acid is accomplished by some other mechanism.

4. Discussion

Due to the emerging incidence of drug resistance among pathogens, any novel compound that might have antimicrobial activity is of great importance. More than 17000 deaths annually are caused by drug resistant *Candida* species. Azoles, CYP51-targeting drugs with ketoconazole and fluconazole as members, are just one of the drug classes with increasing resistance rates due to the ability of *Candida* spp. to overexpress membrane transporters, modify different steps in ergosterol biosynthesis pathway and other mechanisms (Bhattacharya et al., 2020). Ketoconazole, the positive control used in our assays manifests side effects in patients including: fatal drug induced hepatitis and gastrointestinal disorders (Maertens, 2004). Despite lower antibacterial potency compared to the commercial antibacterial drug (**Table 2**) rosmarinic acid is a potential alternative to amoxicillin with clavulanic acid, medicine inducing gastrointestinal side effects (Huttner et al., 2020). Due to the aforementioned side effects and data regarding the worldwide increasing antibiotic resistance we are witnessing the rising need for the development

of novel therapeutics. Different microbial targets along with novel approaches including anti-virulence therapeutics are currently extensively explored (Belete, 2019; Wang et al., 2020).

This is the first study of rosmarinic acid antimicrobial action towards the selected bacterial and fungal clinical isolates, obtained from tonsillar tissues, and oral cavities of patients. Rosmarinic acid was previously tested as an inhibitor of *C. albicans* ATCC 10231 growth at 1000 µg/mL (Cheah et al., 2014), unlike the present study where it inhibited the same strain with MIC 200 µg/mL (**Table 1**). The discrepancy between the results of our study and previous investigation (Cheah et al., 2014) might be due to the different observation of results and/or different growth conditions. Rosmarinic acid also had an antibacterial effect (MIC 0.3 mg/mL) towards *Stenotrophomonas maltophilia* (Abedini et al., 2013) which is in accordance with 0.4 mg/mL determined in the present study. *Staphylococcus aureus* was susceptible to rosmarinic acid with MIC 625-1250 µg/mL (Slobodníková et al., 2013) while in the present study MIC> 800 µg/mL was determined. Similar MIC values (MIC 0.12-2 mg/mL) were recorded for different strains (Mencherini et al., 2007). According to the author best knowledge, this is the first study of rosmarinic acid anticandidal/antibacterial spectrum observed with its inhibitory and fungicidal/bactericidal concentration determined parallel towards more than 20 different *Candida* and bacterial strains in total.

This study is the first to examine the wideness of rosmarinic acid capacity to interfere with *Candida* biofilms as highlighted by the recording of its inhibitory activity towards six yeast species biofilms. Antibiofilm potential of rosmarinic acid has been previously described (Slobodníková et al., 2013) towards *S. aureus*. Further investigation is needed in order to enlighten rosmarinic acid role both in inhibition of biofilm formation (attachment inhibition) and

in eradication of pre-formed biofilms – the antibiofilm modes determined in the present study. This study investigated for the first time antibiofilm/antivirulence mechanisms of rosmarinic acid by observing slight decrease in biofilm exopolysaccharide production after treatment along with negligible impact on protease production. Since biofilms are seen as a risk factor for mortality in *C. albicans* patients (Rajendran et al., 2016) this activity is of high importance in order to develop novel therapeutics able to fight biofilm establishment and existence and should be further investigated.

The antimicrobial studies of natural products mainly do not reveal the mechanisms these constituents use in order to fulfill the antimicrobial effect. However, in order to make full use of biological potential of some product it is compulsory to gain insight into the mechanisms it employs. Antifungal mechanism of rosmarinic acid in the present study was associated with altered membrane permeability and not ergosterol binding. Rosmarinic acid has the ability to penetrate into preformed membranes, while not altering their structure (Fadel et al., 2011). According to the authors best knowledge none of the previous studies have indicated impact of rosmarinic acid on microbial cell integrity. Likewise, this is the first study to highlight the impact of rosmarinic acid on *C. albicans* mitochondria activity. It was shown previously that it causes mitochondrial dysfunction in *Arabidopsis* seedlings (Araniti et al., 2018). Mitochondria are indispensable for fungal growth and survival and though can be explored as novel targets for antifungal leads (Li and Calderone, 2017). Mitochondrial function positively correlates with the fungal virulence (Shingu-Vazquez and Traven, 2011) hence, its inhibition is one of the antivirulence activities along with the antibiofilm and protease inhibiting traits of rosmarinic acid

determined in the present study. This study provided initial evidences for the possible development of rosmarinic acid based mitochondria targeting antifungal leads.

Rosmarinic acid, a natural polyphenolic compound found in many dietary products used on daily basis, can be seen as a promising candidate for the development of novel antimicrobial therapeutics. Despite its higher MIC values compared to the commercial drugs it employs different pathways in order to induce the antimicrobial effects and though might be associated with lower resistance rates and less side effects, the traits that have to be studied more thoroughly. Also, the structure of rosmarinic acid can be used as the basis for the design of novel antimicrobial rosmarinic acid-based structures that might have increased capacity to fight microbial infections and are associated with unique mechanisms of antimicrobial action.

5. Conclusion

In this work rosmarinic acid was shown to be a prominent antimicrobial agent with promising inhibitory activity towards range of fungal and bacterial pathogens. There was evidence of its ability to reduce biofilm formation/formed biofilms in range of *Candida* species. Also, disruptions in membrane integrity and mitochondrial activity were observed as potential antifungal mechanisms. In the search for novel antibiotics rosmarinic acid should be further explored due to its broad antimicrobial spectrum and wide presence in natural bioactive products.

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Conflicts of Interest The authors declare no conflict of interest.

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Table 1. Minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations of rosmarinic acid, mg/mL.

Yeasts	**Rosmarinic acid**		**Ketoconazole**	
	MIC	**MFC**	**MIC**	**MFC**
***C. albicans* 475/15**	0.1	0.2	0.003	0.006
***C. albicans* 13/15**	0.1	0.2	0.0016	0.05

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***C. albicans* 17/15**	0.1	0.2	0.0016	0.05
***C. albicans* 527/14**	0.15	0.3	0.0031	0.0062
***C. albicans* 10/15**	0.15	0.3	0.0031	0.05
***C. albicans* 532/15**	0.1	0.2	0.0031	0.0062
***C. albicans* ATCC 10231**	0.2	0.4	0.0016	0.006
***C. krusei* H1/16**	0.2	0.4	0.0016	0.003
***C. glabrata* 4/6/15**	0.1	0.2	0.0016	0.006
***C. tropicalis* ATCC 750**	0.2	0.4	0.0016	0.006
***C. parapsilosis* ATCC 22019**	0.1	0.2	0.003	0.006

Table 2. Minimal inhibitory (MIC) and bactericidal (MBC) concentrations of rosmarinic acid, results are in mg/mL.

Bacteria	**Rosmarinic acid**		**Amoxicillin + Clavulanic Acid**	
	MIC	**MBC**	**MIC**	**MBC**

Micrococcus luteus	0.1	0.2	0.0002	0.0004
Rothia mucilaginosa	0.1	0.2	0.007	0.014
Streptococcus agalactiae	0.05	0.1	0.007	0.014
	Rosmarinic acid		**Ketoconazole**	
Streptococcus angiosus	0.05	0.1	0.028	0.056
Streptococcus dysgalactiae	0.05	0.1	0.007	0.014
Streptococcus oralis	0.05	0.1	0.0004	0.001
Streptococcus parasanquinis	0.05	0.1	0.004	0.01
Streptococcus pyogenes	0.1	0.2	0.0004	0.001
Streptococcus salivarius	0.002	0.004	0.01	0.014
Staphylococcus aureus	>0.8	>0.8	0.001	0.002
Staphylococcus hominis	0.4	0.8	0.004	0.007
Enterobacter cloacae	0.1	0.2	0.028	0.056
Stenotrophomonas maltophilia	0.4	0.8	0.003	0.007

Table 3. Minimal biofilm eradication concentrations of rosmarinic acid towards different *Candida* strains.

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***C. albicans* 475/15**	0.4	0.012
***C. albicans* ATCC 10231**	0.4	0.012
***C. krusei* H1/16**	>1.6	0.1
***C. glabrata* 4/6/15**	0.8	0.012
***C. tropicalis* ATCC 750**	1.6	0.025
***C. parapsilosis* ATCC 22019**	0.8	0.006

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Table 4. Minimal inhibitory concentrations (μg/mL) of rosmarinic acid and amphotericin B (positive control) towards *C. albicans* in the absence and presence of ergosterol.

Compound	MIC	MIC in the presence of ergosterol
Rosmarinic acid	100	100
Amphotericin B	0.63	1.89

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Figures:

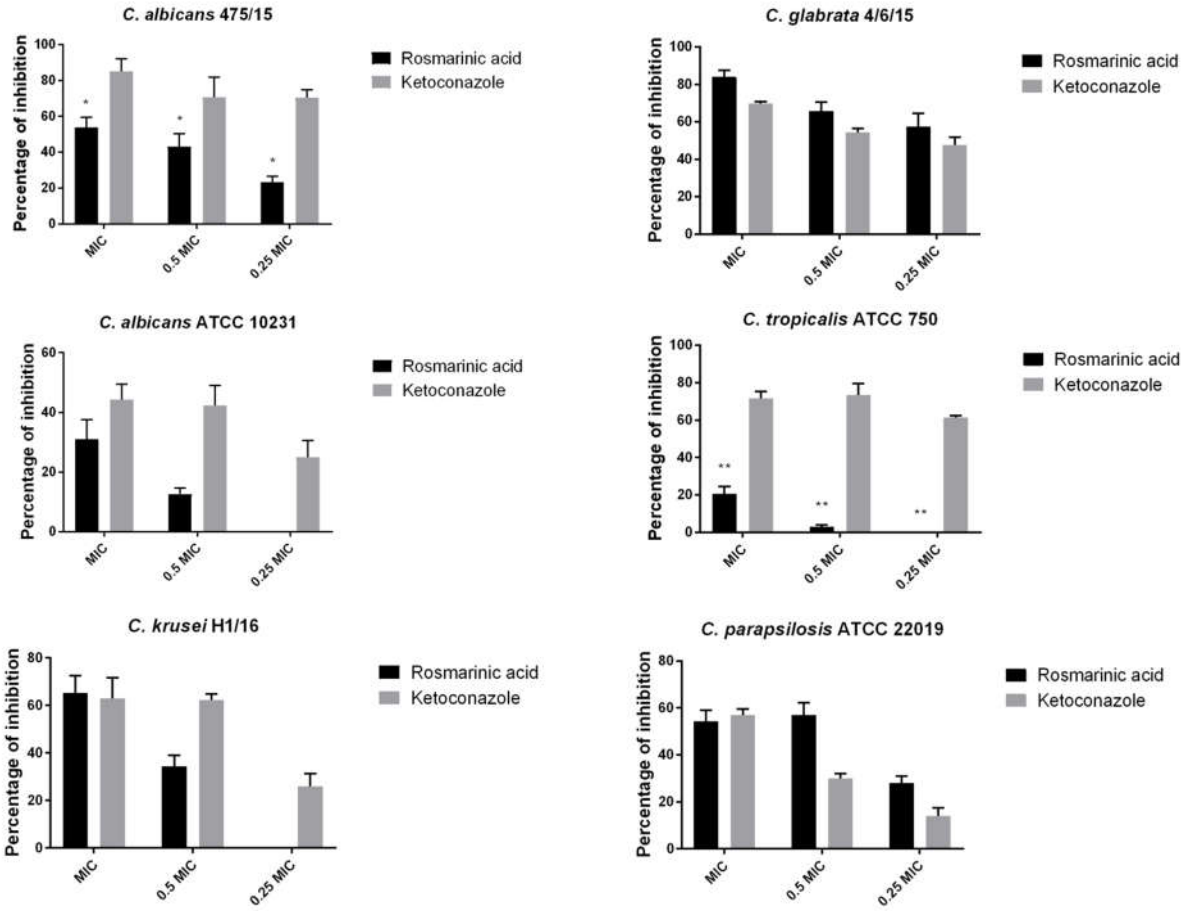

Figure 1. Percentage of inhibition of *Candida spp.* cell attachment after treatment with rosmarinic acid (0.25 MIC – MIC). The results are presented as mean ± SD (n=3). The asterisks represent statistical significance, *, $p \leq 0.05$.

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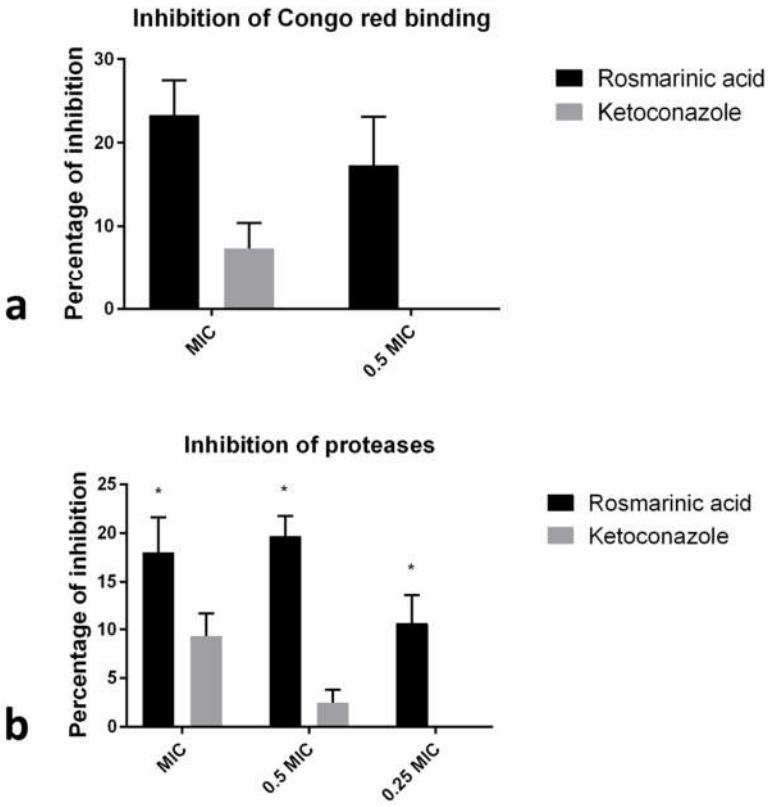

Figure 2. Inhibition of *C. albicans* virulence factors a) Inhibition of congo red binding (%) to *C. albicans* 475/15 biofilm. Concentration equal to 0.25 MIC did not induce any inhibition; b) Inhibition of *C. albicans* 475/15 proteases after application of rosmarinic acid (0.025-0.1 mg/mL) (%).The results are presented as mean ± SD (n=3).. The asterisks represent statistical significance, *, $p \leq 0.05$.

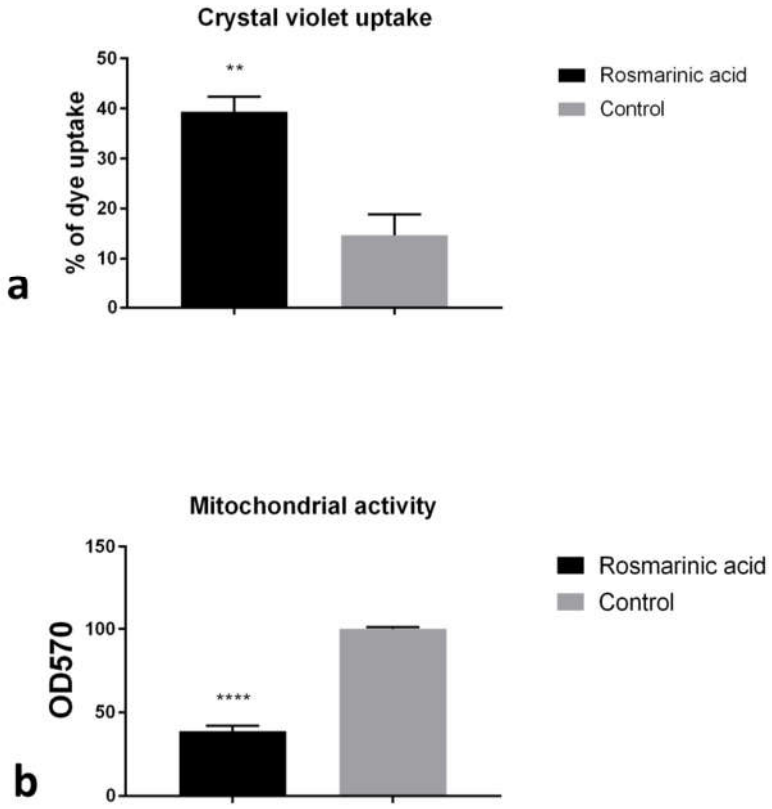

Figure 3. Mechanisms of rosmarinic acid antifungal activity a) Crystal violet uptake of rosmarinic acid (MIC, 0.1 mg/mL) treated *C. albicans* 475/15 (%); b) relative mitochondrial activity after treatment as determined by MTT assay compared to untreated control that was set as 100 %. The results are presented as mean ± SD (n=3). The asterisks represent statistical significance, **, $p \leq 0.01$, **** $p \leq 0.0001$.