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Original Article

Antimicrobial activity and cytotoxicity of commercial rosemary essential oil (*Rosmarinus officinalis* L.)

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***Abstract*:**
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Rosemary is well known as a spice and widely used plant in ethnomedicine worldwide. In this paper, commercial essential oil of rosemary was tested for antimicrobial and cytotoxic activity together with its effect on germination. Antimicrobial activity testing showed moderate effect to both G-positive and G-negative bacteria. In order to determine its effect to the cell membrane, spectrophotometric analysis was performed. It was determined that rosemary affects the cell membrane of bacteria. Cytotoxic activity of *Rosmarinus officinalis* essential oil had been evaluated. As a plant object, germinative bulbs of *Allium cepa* were used. Cytotoxic activity that corresponded to the concentration of essential oil was determined. It had been noticed that rosemary essential oil affected mitotic phase i.e. it significantly slowed down the mitosis. Also, investigation of rosemary essential oil's activity to germination was performed. It was determined that it had high effect to the germination. Concentration of 5 mg/ml completely inhibited the germination of *Triticum vulgare.*

Key words: essential oil, cytotoxicity, antimicrobial activity, effect on germination

Introduction

Essential oils are natural, concentrated, volatile aromatic compounds isolated from plants. These compounds posses a wide spectrum of pharmacological activities. The main advantage of natural agents is that they do not enhance the "antibiotic resistance", a phenomenon encountered with the long term use of synthetic antibiotics.

Rosemary (*Rosmarinus officinalis* L.) is plant belonging to the family Labiateae. This plant is widely used for many purposes – it is well known as a culinary spice and frequently employed in the practice of aromatherapy (Davidson et al., 1989). Also, it is used in ethnomedicine as general stimulant, for improvement of circulation, treatment of rheumatic pains, hyperglycemia and skin care (Hamedo, 2009). Rosemary is known to have antioxidant and antibacterial properties (Shahidi et al. 1992). Putnam et al. (2006) reported that rosemary essential oil inhibit osteoclast activity and increase bone density *in vitro*. Also, cytotoxic activity of rosemary essential oil has been demonstrated by several authors (Khafagi et al. 2000; El-Meleigy et al. 2010).

The essential oil of rosemary contains several compounds in high percentage and many others in traces. These major compounds determine the biological properties of the essential oil and can act

in synergic manner or regulate one on another (Faixova & Faix, 2008). In the papers dealing with chemical composition of rosemary essential oil, α-pinene is reported as the major component, followed by 1,8 – cineole, camphene, β-myrcene, camphor and borneole (Jamshidi et al., 2009, Moghtader & Afzal, 2009). Some studies reported 1,8 – cineole as main component (Fu et al., 2007, Debersac et al., 2001).

Rosemary is confirmed as antimicrobial agent against both Gram-positive and Gram-negative bacteria, as well as against fungi (Valero & Salmeron, 2003, Prabuseenivasan et al., 2006, Fu et al. 2007a, Moghtader & Afzal, 2009).

In the present study, antimicrobial activity of commercially available rosemary essential oil was investigated against a panel of microorganisms for minimal inhibitory and bactericidal activity. MIC and MBC were determined using broth microdilution method. The most sensitive determined bacterial strain was used to investigate the mode of action of this oil. In order to evaluate if the oil exhibits membrane damage effect, testing of the 260 nm absorbing material leakage was employed. Also, cytotoxic activity of rosemary essential oil was determined.

Material and methods

Essential oil, bacterial strains and culture conditions

Essential oil of rosemary, produced by Sinefarm, Vršac, Srbija, was purchased from the local pharmacy.

Bacterial strains used in this study were *Staphylococcus aureus* ATCC 25923, *S. aureus* (clinical isolate), *Escherichia coli* (clinical isolate), *Bacillus subtilis* ATCC 6633 and *Klebsiella pneumoniae* (clinical isolate). Bacterial cultures were maintained on the nutrient agar (NA) at the Department of Biology and Ecology, Microbiological Laboratory, Faculty of Science and Mathematics, Niš.

Testing of antimicrobial activity

The antimicrobial activity was evaluated using broth microdilution method (NCCLS, 2003). Minimum inhibitory concentrations determination was performed by a serial dilution method in 96 well microtitre plates. Bacterial strains were cultivated in Mueller Hinton agar at 37 ºC for 18 h. Cultures were used for making bacterial suspensions, whose turbidity was adjusted to 0.5 McFarland and confirmed using a spectrophotometer (UV-VIS 1650 Shimatzu, Japan). Final density of inoculum was 5 x 10^5. Dilutions of stock rosemary essential oil solutions were made with MHB (Mueller Hinton Broth) and inoculums were added to all wells. One inoculated well was included to allow control of the broth suitability for organism growth. One non-inoculated well, free of antimicrobial agents, was also included to ensure medium sterility. Bacterial growth was determined by adding 20 µL of 0.5 % triphenyl tetrazolium chloride (TTC) aqueous solution (Sartoratto et al., 2004). Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the oil inhibiting visible growth (red colored pellet on the bottom of the wells after the addition of TTC), while minimal bactericidal concentration (MBC) was defined as the lowest oil concentration killing 99.9 % of bacterial cells. To determine MBC, the broth was taken from each well without visible growth and inoculated in MHA for 24 h at 37ºC. Experiments were done in triplicate and the mean values are presented.

Testing the integrity of the cell membrane

Very important indication of disturbed membrane integrity is the release of intracellular components, such as large molecules DNA and RNA. Since these nucleotides have strong UV absorption at 260 nm, they are described as 260 nm absorbing materials (Liu et al., 2009). Leakage of 260 nm absorbing material was determined by measuring the absorbance of the culture supernatants according to the modified method of Carson et al. (2002). Briefly, the cultures of *S. aureus* were harvested and twice washed with PBS. After resuspension in PBS, cells were adjusted to absorbance of ~ 0.2. At time t = 0 min, essential oil was added to cell suspensions to give a final concentrations of MIC and 2 x MIC. Aliquots of the treated cell suspensions were removed at regular time intervals, centrifuged at 10 000 x g for 5 min and absorbance of supernatant was measured at 260 nm and plotted against time. The experiments were done in triplicate and the mean values are presented.

Allium cepa test

Cytotoxicity was determined by calculating the mitotic index of *Allium cepa* L. root cells after the 24 h treatment with rosemary essential oil. Small onion bulbs were placed in 10 ml distilled water in Petri dishes in an incubator at 25±1ºC for 24 h. After initiating growth, water had been replaced two more times (after every 24h). After 72 h, water had

been replaced with appropriate solution (appropriate concentration of essential oil of rosemary). Distilled water and 5 % ethyl alcohol were used as controls.

After the growth, root tips were collected and fixed. The fixative solution was glacial acetic acid / absolute alcohol (1/3 v/v). The root tips were kept in acetic-alcohol solution for 72 h. After fixation, the root tips were washed with water and then placed into a 70 % ethanol and stored in a refrigerator until use.

For examination the root tips were hydrolyzed in 1N HCl, placed on microscopic slide in 1% aceto-orcein and after a few minutes squashed.

For each root tip the number of mitotic and total meristemic cells was counted in 9 fields using power (100 x) light microscope. Cells manifesting different stages of mitosis i.e. interphase and prophase (P), metaphase (M), anaphase (A) and telophase (T) were recorded. The mitotic index (MI) was calculated using following formula:

Mitotic Index = (P+M+A+T) / total cells

Germination test

Three different concentrations of essential oil were used to determine whether the oil has effect to germination of *Triticum vulgare* seeds. Twenty wheat grains were placed in small Petri dishes and in an appropriate medium. Distilled water and 5 % ethanol were used as controls. Concentrations of rosemary essential oil were 5 mg/ml, 12.5 mg/ml, 25 mg/ml and 50 mg/ml. After 24 h of the treatment, seeds which germinated were counted and percentage of germination, compared to positive control was presented.

Results and disscusion

The results of minimal inhibitory (MIC) and minimal bactericidal concentrations (MBC) are presented in Table 1. The testing showed significant antibacterial activity of rosemary with MIC values ranging from 1.562 – 3.125 µl/ml, while MBC ranged from 1.562 to >25.00 µl/ml. The most sensitive strain was *S. aureus* with both MIC and MBC values of 1.562 µl/ml, while the most resistant one was *Escherichia coli*, whose bactericidal concentration exceeded the tested values. Determined resistance of *E. coli* can be attributed to the outer cell envelope, the feature of all Gram-negative bacteria, which makes them generally more resistant to all external agents. This is confirmed by higher MBC values of *K. pneumoniae* (Gram negative strain). Fu et al. (2007a) investigated antibacterial activity of rosemary and the results obtained in our study showed very good agreement with activity against *E. coli*, *B. subtilis* and *S. aureus* (1.250 µl/ml against all three bacteria) determined in that paper. Moghtader & Afzali (2009) reported activity of rosemary against both Gram positive and negative strains. Previous investigation of Fu et al. (2007) reported activity of rosemary essential oil against *Propionibacterium acnes* in the concentration of 0.56 mg/ml.

In order to determine the mode of action of rosemary essential oil against the most sensitive of the tested bacteria, *Staphylococcus aureus* ATCC 25923, spectrophotometric method was employed. Since damaged cell membrane increases its permeability, small molecular weight molecules such as phosphates and potassium ions first leach out from the cell, followed by larger molecules, such as DNA and RNA (Chen et al, 2002). Since these nucleotides have strong UV absorption at 260 nm, they are described as "260 nm absorbing materials". The UV-VIS study on the release of materials absorbing at 260 nm showed significant leakage proportional to the oil concentration (Fig.1). On contrary, control did not showed any releasing of this material during 120 min period. This indicates membrane damage related to the addition of the rosemary essential oil.

For testing of cytotoxicity, onion meristems (*Allium cepa* L.) were used as the study material. They are considered as one of the best biological models for the study of cytotoxicity, genotoxicity and environmental pollutants (Fiskesjo, 1985). *Allium cepa* root meristems have been widely used

Table 1. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of rosemary essential oil

Bacterial strain	strain type	MIC (µl/ml)	MBC (µl/ml)	Tetracycline (µg/ml)
S. aureus	ATCC 25923	1.562	1.562	0.090
S. aureus	clinical isolate	3.125	6.250	0.045
E. coli	clinical isolate	3.125	>25.00	0.045
B. subtilis	ATCC 6633	1.562	6.250	0.090
K. pneumoniae	clinical isolate	1.562	12.50	0.045

for the evaluation of cytotoxicity and anti-mitotic activity of various compounds (Shehab, 1980; Williams & Omoh, 1996; Al-Meshal, 1987). In the present paper, cytotoxicity and anti-mitotic activity of essential oil of *R. officinalis* L. was tested using *Allium cepa* test. The inhibitory effect of essential oil of rosemary on the mitotic activity of root meristems was evaluated and the effect was compared with distilled water and 5% ethyl alcohol. The results are presented in Tab. 2.

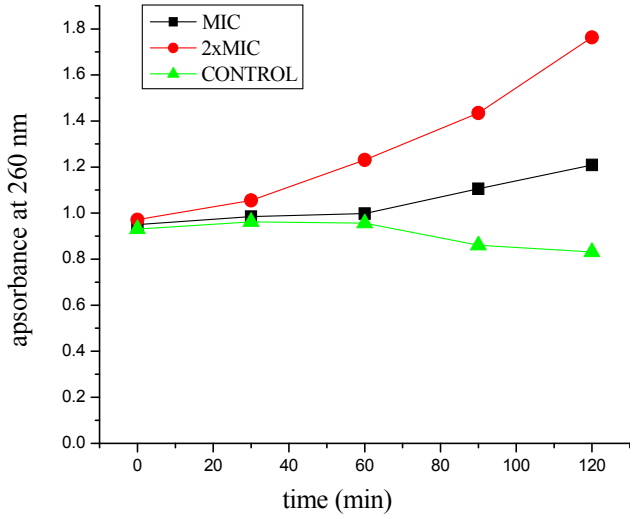

Figure 1. Release of 260 nm absorbing material from *S. aureus* ATCC 25923 cells during the treatment with MIC and 2 x MIC concentrations of rosemary essential oil (OD – optical density)

Table 2. Mitotic index in *Allium cepa* (L.) meristem following incubation with various concentrations of essential oil of *Rosmarinus officinalis* (L.)

concentration	unit	MI	MI in %
d H_2O	-	0.424	42.40
EtOH	%	0.172	17.20
5.00	mg/ml	0.257	25.70
12.5	mg/ml	0.235	23.50
25.0	mg/ml	0.218	21.80
50.0	mg/ml	0.191	19.10

The results showed that different concentrations of essential oil have different percentage of activity, but all of them affect root growth, length and number. Also, it was demonstrated that essential oil has inhibitory effect on proliferation of cells. Higher concentrations of essential oil exhibit stronger inhibitory effect on mitosis (Tab. 2). Previous studies revealed that rosemary essential oil exhibits strong cytotoxicity against brine shrimp nauplii (Khafagi et al. 2000). The test which was taken is not only used for predicting cytotoxicity, but is also used as a predictor of antitumor and pesticide activity. Data showed that rosemary essential oil except high cytotoxicity, might posses antitumor and pesticide potential. It is supposed that oxygenated monoterpenes of essential oil exhibit a variable degree of cytotoxicity. As a typical lipophilic substance, it passes through the cell wall and cytoplasm membranes and disrupts their structure (Faixova & Faix, 2008).

Kivanc & Akgül (1988) reported that the most active components of the essential oils were phenols, followed by aldehydes and ketons. One of the major compound of rosemary essential oil is 1, 8-cineole. Asanova et al. (2003) demonstrated that this component has very strong cytotoxicity and pronounced antitumor action. It is possible that this component has the major role in cytotoxicity of this essential oil. 1, 8 - cineole exhibits a strong toxic effect on eukaryotic cells (Obeng-Ofori et al., 1997, Santos et al., 2004).

Another potent compound is ß-caryophyllene. It was demonstrated that this substance may not have direct influence on cytotoxicity of essential oil, but may impact accumulation of some substances by increasing permeability of the plasma-membrane. In that way, it affects and increases cytotoxic effect of compounds with which it interacts (Legaule & Pichette, 2007).

According to all this facts, it is not surprising that concentration of only 5 mg/ml of rosemary essential oil totally inhibited germination of wheat. This was the lowest concentration tested. Other concentrations also totally inhibited germination of *Triticum vulgare* seeds. Limonene, one of the components of rosemary essential oil, has ability to moderate proliferation of cells (Manuele et al., 2008), and in that manner it affects cell mitosis.

Conclusion

Essential oil of rosemary was previously reported as an important antimicrobial agent with activity at very low concentrations. Our study confirmed the previous results regarding MIC and MBC values of this essential oil. For the first time, it was determined that rosemary essential oil exhibits activity by damaging the cell membrane of gram positive bacteria and further tests are necessary to explore the exact mechanism of this activity. The determined cytotoxic and antimicrobial activity of rosemary essential oil is of great importance in preservation of food products and also very important information for its pharmacological application.

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