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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Direct and indirect antimicrobial activity of the root extract of *Onosma visianii* Clem

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SUMMARY

Introduction/Objective The escalating resistance of numerous pathogens to currently available therapeutic agents has sparked a renewed interest in the search of novel antimicrobial compounds. Plants have become a potentially valuable source of these compounds. This study aimed to assess the direct and indirect antimicrobial effects of *Onosma visianii* Clem root extract on reference and clinical bacterial strains using broth microdilution and a modified ethidium bromide/acridine orange (EB/AO) fluorescence assay.

Methods The ethanolic extract obtained from the dried root of *Onosma visianii* Clem was used to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) by broth microdilution, as well as the half maximal inhibitory concentration (IC_{50}) values for reference and clinical bacterial strains. The EB/AO fluorescence method was employed to assess the effect on efflux pumps in methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE), using 50% of the MIC value of the extract.

Results The MIC, MBC, and IC_{50} values for Gram-positive bacteria were all below 15 μg/mL. The extract demonstrated strong antibacterial activity against VRE and, in particularly, against MRSA isolates (MIC = 7.81 μg/mL and MBC = 7.81 μg/mL). Furthermore, treatment with 50% of the MIC concentration produced a significant inhibitory effect on efflux pumps in Gram-positive bacteria, ranging from 18% to 26% compared with untreated cells.

Conclusion The root extract of *Onosma visianii* Clem exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria, with particularly strong effects against VRE and MRSA. In addition, the observed inhibition of bacterial efflux pumps underscores its promise as a candidate for pharmacological evaluation of the antibiotic properties of the *Onosma visianii* Clem root extract.

Keywords: *Onosma visianii* Clem; antimicrobial effect; efflux pumps

INTRODUCTION

For decades, antibiotics have been widely used in human and veterinary medicine, as well as in agriculture, due to their high efficacy and low toxicity, and have proven highly successful in treating bacterial infections. The rising prevalence of antibiotic resistance, driven in part by indiscriminate drug use, poses a major threat that could return us to a pre-antibiotic era [1]. Among the mechanisms of multidrug resistance are bacterial efflux pumps, which expel antibiotics from the cell, thereby reducing their efficacy [2]. Inhibiting these pumps represents a potential strategy to enhance the susceptibility of resistant pathogens to existing antibiotics [3]. The escalating resistance of many pathogens to existing drugs has revitalized interest in plants as a rich yet underexplored source of novel antimicrobials, with only about 1% of traditionally used species having undergone phytochemical investigation [4]. Advances in modern research technologies now enable the detailed characterization of plant-derived compounds, spurring a growing number of microbiological studies on their antimicrobial properties as alternative therapies – an approach of particular relevance in developing regions, where up to 80% of people still rely on traditional medicines [5, 6]. *Onosma visianii* Clem (Boraginaceae) has long been employed in traditional medicine for treating a range of conditions, including wounds and burns, while other species in this family exhibit notable anti-inflammatory effects [7]. Building on our previous findings that naphthoquinone 1-7 (a shikonin derivative) isolated from *O. visianii* roots possesses cytotoxic and antibacterial activity [8], the present study aims to assess the direct and indirect antimicrobial effects of *O. visianii* root extract on reference and clinical bacterial strains using broth microdilution and a modified ethidium bromide/acridine orange (EB/AO) fluorescence assay.

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METHODS

The study was designed as an *in vitro* experimental study and was conducted at the Center for Microbiology, Institute of Public Health, Kragujevac, Serbia. The extract used in the experiments was obtained through ethanolic extraction of the dry root of *O. visianii* Clem at the University of Kragujevac, Faculty of Science, Kragujevac, Serbia.

Reagents and bacteria

The extract of *O.visianii* Clem was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Merck, St. Louis, MO, USA) and diluted with nutrient broth (Oxoid, Thermo Fisher Scientific, Basingstoke, United Kingdom) to achieve a concentration of 2000 μg/mL, ensuring that the concentration of DMSO in the stock did not exceed 3.5%. The initial volume of the stock used for examination was 100 μL [9].

The following reference strains [American Type Culture Collection (ATCC)] were included in the study: *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 10145), and *Escherichia coli* (ATCC 25922). Additionally, clinical bacterial strains, including vancomycin-resistant *Enterococcus faecalis* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and *Pseudomonas aeruginosa*, were also examined.

Antibacterial activity

The McFarland (Hemofarm a.d., Vršac, Serbia) turbidity standard was used to standardize the approximate number of bacteria to 0.5 McFarland. Bacterial strains were subcultured and incubated at 37°C in a thermostat for 24 hours prior to use. A bacterial suspension with a density of 0.5 McFarland was prepared using the direct colony method. From this suspension, 100 μL was mixed with 2000 μL of physiological saline in a test tube, resulting in a bacterial concentration of 5×10^6 CFU/mL. From this bacterial suspension, 10 μL (equivalent to 5×10^4 bacteria) was inoculated into the wells of a microtiter plate, resulting in a final concentration of 5×10^5 CFU/mL [10].

In a microtiter plate with rounded-bottom wells [60 wells in five horizontal rows of a 96-well microtiter plate, (Spektar, Čačak, Serbia)], 100 μL of nutrient broth was added to each well. In the first two rows, 100 μL of the extract stock was added to the initial wells, and then it was serially diluted using the double dilution method into the subsequent wells. The concentrations of the extract ranged from 1000 to 0.488 μg/mL. Subsequently, 10 μL of the prepared bacterial suspension was added to each well. Validity control and blanks for the extract and nutrient broth were included. The inoculated microtiter plates were incubated for 24 hours at 37°C, and the minimum inhibitory concentrations (MICs) were determined [11]. The MIC is defined as the lowest concentration of the test substance at which no visible increase in bacterial growth is observed, indicated by clear wells.

The minimum bactericidal concentration (MBC) was determined by subculturing 10 μL of suspension from each well where no turbidity was observed onto nutrient agar.

After 24 hours of incubation, the plates were examined for visible growth, and the MBCs were determined. MBC is defined as the concentration of the extract that does not result in visible growth on the plate [11].

The direct cytotoxicity of the extract was calculated using equation (1).

$$\text{Cytotoxicity} = ((\text{Acontrol-Atest}) / \text{Acontrol}) \times 100 \quad (1)$$

In this context, A_control refers to the absorbance measurement of the validity control, which is a bacterial suspension without the extract, whereas A_test denotes the absorbance measurement of the test sample (bacterial suspension containing the extract) [9].

Efflux pump activity assay

To determine the activity of efflux pumps in bacteria, the dyes AO/EB (Sigma-Aldrich,) were used. AO is a dye that binds to deoxyribonucleic or ribonucleic acids (DNA or RNA) in organisms and fluoresces in different colors, aiding in the differentiation of cellular organelles. EB, when bound to DNA, exhibits enhanced orange fluorescence when exposed to ultraviolet light. The fluorescence intensity of AO/EB was used to assess the efflux pump activity of certain bacteria [12].

For the efflux pump activity assay, bacterial suspensions of two untreated clinical strains, *Pseudomonas aeruginosa* and MRSA, were prepared. The fluorescence intensity of AO/EB in both untreated bacteria decreased over time as the efflux pumps eliminated the dyes.

Since Gram-positive bacteria eliminated EB/AO more efficiently, the efflux pump activity assay using the *Onosma visianii* Clem root extract was conducted only on Gram-positive bacteria. Two clinical strains, VRE and MRSA, were treated with the *Onosma visianii* Clem root extract. The experimental procedure for each tested isolate was the same as that for determining the MIC values [13]. A bacterial suspension at 50% MIC was used for the efflux pump activity assay [14].

A bacterial suspension with a density of 0.5 McFarland was prepared using the direct colony method, as outlined in European committee on antimicrobial susceptibility testing guidelines [15]. A suspension was prepared on a slide consisting of 10 μL of bacterial suspension and 1 μL of dye solution (100 μg/mL AO and 100 μg/mL EB) dissolved in distilled water [16]. The slides were examined under a fluorescence microscope at 400 × magnification and photographed in real-time at three time points: 0 minutes, 15 minutes, and 30 minutes. The fluorescence intensity was determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Comparison was made with untreated strains of VRE and MRSA, using bacterial suspensions with a concentration of 5×10^5 CFU/mL [17]. The accumulation rate of EB/AO, characterized by an increase

Table 1. Antibacterial activity of *Onosma visianii* Clem root extract on reference and clinical bacteria strains

Species	MIC ± SD (μg/ml)	MBC ± SD (μg/ml)	IC_{50} ± SD (μg/ml)
Enterococcus faecalis (ATCC 29212)	**7.81 ± 4.50**	**7.81 ± 0**	9.74 ± 6.80
Staphylococcus aureus (ATCC 25923)	**0.48 ± 0**	**0.48 ± 0**	**< 0.48**
Pseudomonas aeruginosa (ATCC 10145)	500 ± 288.67	> 1000	185.59 ± 1.18
Escherichia coli (ATCC 25922)	250 ± 0	500 ± 0	244.08 ± 76.56
Enterococcus faecalis (VRE)	15.62 ± 0	15.62 ± 0	5.98 ± 0.76
Staphylococcus aureus (MRSA)	7.81± 4.50	7.81 ± 0	2.64 ± 1.03
Pseudomonas aeruginosa	1000 ± 0	> 1000	265.03 ± 72.84
Escherichia coli	1000 ± 0	> 1000	765.5 ± 0

MIC – minimum inhibitory concentration; SD – standard deviation; MBC – minimum bactericidal concentration; IC_{50} – half of the maximum inhibitory concentration; VRE – Vancomycin-resistant *Enterococci*; MRSA – Methicillin-resistant *Staphylococcus aureus*

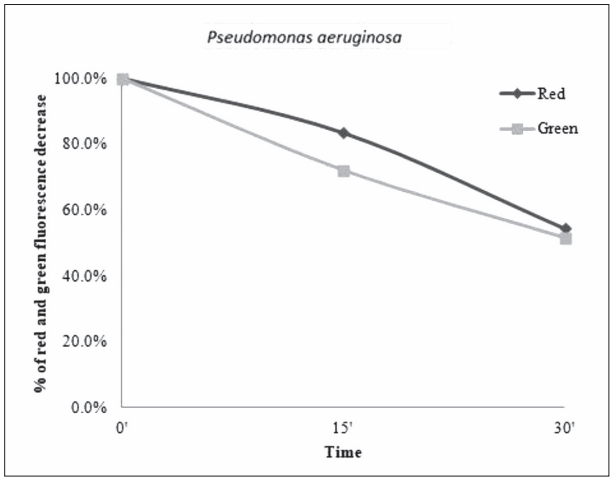

Figure 1. Fluorescence intensity ethidium bromide / acridine orange of untreated *Pseudomonas aeruginosa* at three time points: 0', 15', and 30'

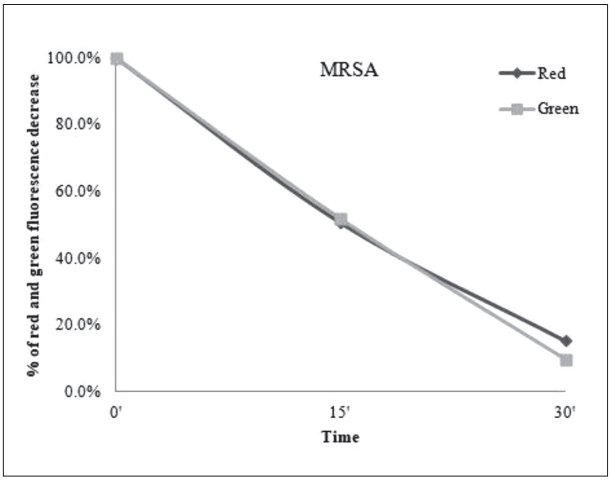

Figure 2. Fluorescence intensity ethidium bromide / acridine orange of untreated methicillin-resistant *Staphylococcus aureus* at three time points: 0', 15', and 30'

in red and green fluorescence intensity, was determined as the fluorescence index relative to the baseline (0 minutes) for both treated and untreated samples at the three time points. Statistical data processing was performed using standard deviation, and the IC_{50} value was calculated by measuring absorbance at 450 nm and using GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA).

RESULTS

Antibacterial activity

O. visianii Clem root extract displayed potent antibacterial activity, with markedly higher efficacy against Gram-positive than Gram-negative bacteria (Table 1). Among reference strains, *Staphylococcus aureus* (ATCC 25923) was the most susceptible (MIC, MBC, and IC_{50} = 0.48 μg/mL), whereas *Enterococcus faecalis* (ATCC 29212) showed reduced sensitivity (MIC/MBC = 7.81/7.81 μg/mL; IC_{50} = 9.74 μg/mL). In contrast, Gram-negative reference strains were far less susceptible, with MIC/MBC values of 250/500 μg/mL for *Escherichia coli* (ATCC 25922) and 500/1000 μg/mL for *Pseudomonas aeruginosa* (ATCC 10145).

A similar pattern was observed for clinical isolates (Table 1). The extract exhibited the greatest activity against MRSA (MIC/MBC = 7.81 μg/mL; IC_{50} = 2.64 μg/mL), followed by VRE (MIC/MBC = 15.62 μg/mL; IC_{50} = 5.98 μg/mL). In contrast, clinical *P. aeruginosa* and *E. coli* isolates were highly resistant, with MIC/MBC values of 1000 μg/mL and IC_{50} values of 265.03 and 765.5 μg/mL, respectively.

Efflux pump activity assay

To investigate the potential of *O. visianii* Clem root extract to interfere with bacterial drug-resistance mechanisms, we assessed efflux pump activity in reference and clinical strains using AO and EB fluorescence assays. Fluorescence intensity measurements revealed that, after 30 minutes, *P. aeruginosa* eliminated approximately 50% of the dyes, whereas MRSA exhibited markedly stronger efflux pump activity, expelling about 90% within the same time frame (Figures 1 and 2).

Because the extract displayed stronger inhibitory effects on Gram-positive bacteria, which also showed higher baseline efflux activity, subsequent assays focused on MRSA and VRE. In MRSA treated with *Onosma visianii* Clem root extract at 50% of its MIC, 72.9% (EB) and 64.8% (AO) of the dyes were retained after 30 min compared with the initial 100%, whereas untreated MRSA retained only 55.6% and 44%, respectively, indicating extract-mediated inhibition of efflux pumps (Figure 3).

Similarly, in VRE treated with *O. visianii* Clem root extract at 50% MIC, 80.9% (EB) and 74.2% (AO) of the

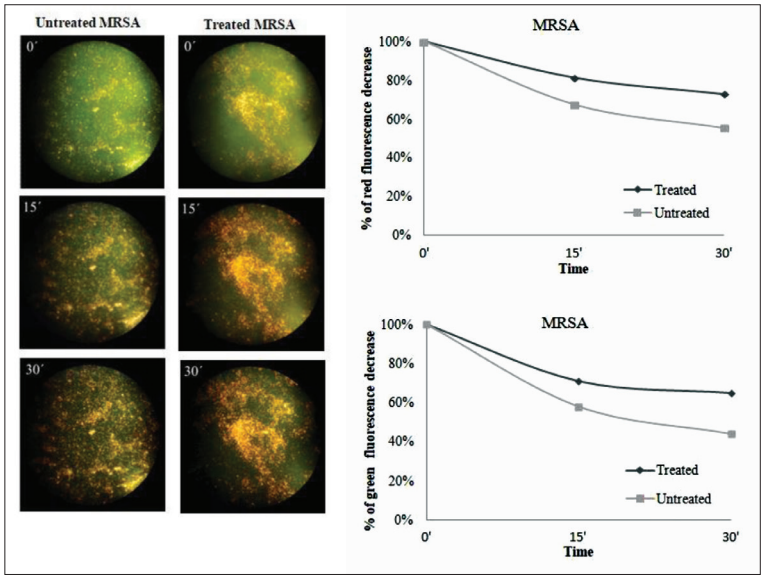

Figure 3. Fluorescence intensity ethidium bromide / acridine orange of treated and untreated methicillin-resistant *Staphylococcus aureus* (MRSA) with *Onosma visianii* Clem root extract at the concentration 50% of the minimum inhibitory concentrations value at three time points: 0', 15', and 30'

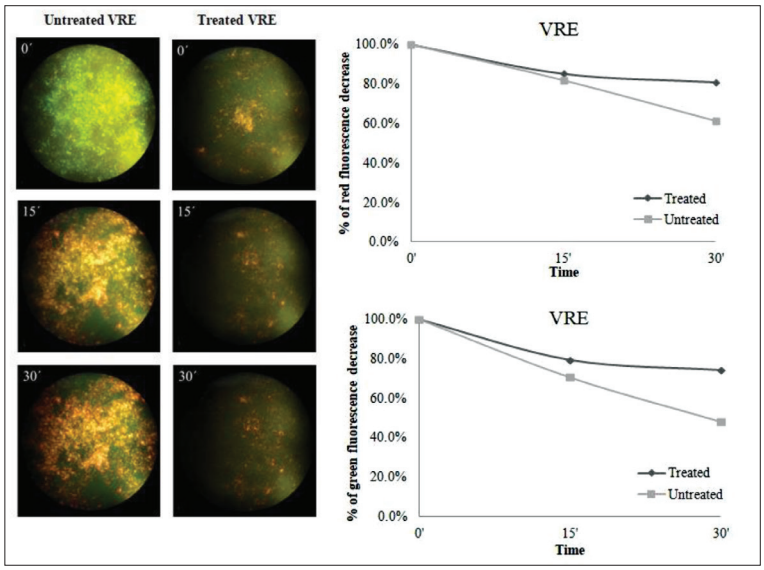

Figure 4. Fluorescence intensity ethidium bromide / acridine orange of treated and untreated vancomycin-resistant *Enterococcus faecalis* (VRE) with *Onosma visianii* Clem root extract at a concentration of 50% minimum inhibitory concentrations value at three time points: 0', 15', and 30'

dyes were retained after 30 min compared with 61.1% and 48% in untreated controls, again demonstrating reduced efflux activity in the presence of the extract (Figure 4).

DISCUSSION

The use of antibiotics in healthcare has been widespread for many years; however, their overuse and unregulated application have led to the emergence of antibiotic resistance, which has become a global public health concern. Consequently, increasing attention has been directed toward investigating the effects and mechanisms of plant extracts, as numerous plant species have been employed for centuries in traditional medicine.

Different mechanisms of action have been identified for the antibacterial activity of medicinal herbs. Some plant extracts inhibit cell wall synthesis, while others accumulate in the bacterial membrane and disrupt its structure and function, leading to cell damage and death [18]. Moreover, certain plant extracts have shown effectiveness against bacterial efflux pumps, which play a key role in mediating multidrug resistance in both Gram-positive and Gram-negative bacteria [19]. Efflux pumps perform an essential function in safeguarding bacteria from toxic materials by actively expelling drugs and toxins. Inhibiting or weakening these efflux pumps can improve the efficacy of antibiotics against resistant bacterial strains [20, 21]. The present study examined the antibacterial properties and the efficacy of the dried root extract of *Onosma visianii* Clem in inhibiting efflux pumps.

Previous results obtained from studies conducted at our university which demonstrated the antibacterial and cytotoxic effects of *Onosma visianii* Clem represent the basis for our research. Vukić et al. [8] conducted a study examining the antibacterial activity of seven distinct naphthoquinones extracted from the dried root of *Onosma visianii* Clem.

Each of the individual naphthoquinones demonstrated efficacy against clinical Gram-positive and Gram negative bacteria with MIC50 and MIC90 values on Gram-positive varying from 6.40 μg/mL to 12.79 μg/mL and 6.82 μg/mL to 13.60 μg/mL, respectively [8]. Conversely, our research assessed the activity of the full ethanolic extract, which encompasses all secondary metabolites to determine whether it is more effective, and compared it against a variety of clinical and reference bacteria. Additionally, our MIC values exhibited enhanced antimicrobial effects against *Staphylococcus aureus* (MIC 0.48 μg/mL) when compared to all isolates evaluated in the prior study. On the other hand, the activity against our Gram-negative bacteria was lower. Our findings also indicated the bactericidal properties of the extract and included IC_{50} values.

Moreover, we advanced our research by evaluating the extract's efficacy against strains of MRSA and VRE. A related study on the extract of *Consolida orientalis* reported MIC values for MRSA between 0.15 and >5 mg/mL, while VRE exhibited MIC values from 0.625 to 2.5 mg/mL. In comparison, our results demonstrate much stronger activity [22]. In another investigation, Amri et al. [23] analyzed the ethanolic extract of *Eupatorium odoratum* against Gram-positive and Gram-negative bacteria, finding no effectiveness against Gram-negative strains. Takongmo Matsuete et al. [24] also noted weaker activity, with a reported MIC value for *Pseudomonas aeruginosa* of > 1000 μg/mL.

The variation in vulnerability can be explained by the distinct structural characteristics of the cell walls found in Gram-positive and Gram-negative bacteria [25]. Gram-negative bacteria typically have a more intricate mechanism for multidrug resistance because of their double membrane structure, which allows for the function of tripartite efflux pump systems [26]. The efflux pump activity assay revealed that the extract of *Onosma visianii* Clem inhibited the efflux pump activity in both MRSA and VRE, as demonstrated by the higher retention of fluorescent dyes in treated bacteria compared to untreated bacteria. Efflux pumps are crucial in contributing to multidrug resistance, and blocking their function can improve the potency of antibiotics [27]. In a comparable study, the bioflavonoid *Scutellaria baicalensis* was shown to affect the efflux pumps of *Staphylococcus aureus*, with EB dye retention at 73% after 30 minutes [28]. Similarly, Anokwah et al. [29] investigated the ethanolic extract of *Loeseneriella Africana*. When *S. aureus* was treated with 50% MIC, it retained 50% EB after 60 minutes, a value significantly lower than that observed in our study. Previous reports have also suggested that polyphenols rich plants can inhibit the efflux pumps Multidrug Resistance 1 and LmrP found in *E. faecalis*, which are critical contributors to multidrug resistance [30]. Our results are consistent with recent findings showing that bioactive compounds in plant extracts may combat multidrug-resistant bacteria by targeting efflux pump mechanisms [31]. The effectiveness of the extract is influenced by its chemical composition, the extraction method, and the timing of plant collection [32]. Despite the long history of this plant in traditional medicine, standardization of the extract is necessary before practical application. In addition, it is essential to evaluate its potential toxicity and the stability of its active components. Although the extract demonstrates promising *in vitro* activity, further standardization, toxicity testing, and stability assessment are required before practical application.

CONCLUSION

The root extract of *Onosma visianii* Clem exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria, with particularly strong effects against VRE and MRSA. In addition, the observed inhibition of bacterial efflux pumps underscores its promise as a candidate for pharmacological evaluation of the antibiotic properties of the *Onosma visianii* Clem root extract.

AUTHOR CONTRIBUTIONS

SH played a key role in conceptualizing and designing the study, acquiring and processing data, and drafting the manuscript. SM contributed to the study's conceptual framework, analyzed and interpreted the data, and revised the manuscript. SP participated in the conception and design of the study and was responsible for data analysis and interpretation. AT was involved in study design and contributed to data analysis and interpretation. SR took part in data analysis and interpretation and assisted with manuscript revisions. IP contributed to the study's conceptualization and the acquisition, analysis, and interpretation of data. AH was engaged in data analysis and interpretation. MM was involved in the conception and design of the study, supervised data acquisition, analysis, and interpretation, and revised the manuscript.

Conflict of interest: None declared.

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Директна и индиректна антимикробна активност екстракта корена *Onosma visianii* Clem

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САЖЕТАК

Увод/Циљ Растућа резистенција бројних патогена на тренутно доступне терапијске агенсе изазвала је поновно интересовање за истраживање нових антимикробних једињења. Биљке су постале потенцијално вредан извор ових једињења. Циљ ове студије био је да се процени директна и индиректна антимикробна активност екстракта корена *Onosma visianii* Clem на референтне и клиничке бактеријске сојеве, користећи методу микродилуције у бујону и модификовани етидијум бромид / акридин оранџ (*EB*/*AO*) тест флуоресцентне детекције.

Методе Етанолни екстракт припремљен из сувог корена *Onosma visianii* Clem коришћен је за одређивање минималних инхибиторних концентрација (*MIC*) и минималних бактерицидних концентрација (*MBC*) методом микродилуције у бујону, као и за одређивање вредности половине максималне инхибиторне концентрације (IC_{50}) за референтне и клиничке бактеријске сојеве. Метода *EB*/*AO* флуоресцентног бојења коришћена је за процену утицаја на ефлукс пумпе код сојева *Staphylococcus aureus* резистентног на метицилин (*MRSA*) и *Enterococcus faecalis* резистентног на ванкомицин (*VRE*), користећи концентрацију од 50% *MIC* вредности екстракта.

Резултати Вредности *MIC*, *MBC* и IC_{50} за Грам-позитивне бактерије биле су испод 15 *μg/mL*. Екстракт је показао јако антимикробно дејство према *VRE* и посебно према *MRSA* сојевима (*MIC* = 7,81 *μg/mL* и *MBC* = 7,81 *μg/mL*). Поред тога, третман са 50% *MIC* концентрације изазвао је значајну инхибицију ефлукс пумпи код Грам-позитивних бактерија, у распону од 18% до 26% у односу на нетретиране ћелије.

Закључак Екстракт корена *Onosma visianii* Clem показао је антимикробно дејство према Грам-позитивним и према Грам-негативним бактеријама, са нарочито израженим ефектом према *VRE* и *MRSA*. Осим тога, уочена инхибиција бактеријских ефлукс пумпи истиче обећавајући потенцијал екстракта корена биљке *Onosma visianii* Clem као кандидата за фармаколошка испитивања његових могућих антибиотских својстава.

Кључне речи: *Onosma visianii* Clem; антимикробно дејство; ефлукс пумпе