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Original article

In Vitro Antimicrobial Activity of Different *Verbascum Niveum* Extracts

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SUMMARY

Introduction. Medicinal plants are the main ingredient of numerous medicines and pharmaceutical products, and antimicrobial activity has been demonstrated in a large number of medicinal plant extracts and essential oils. Considering that *Verbascum L.* species (mullein), officially approved by the European Medicines Agency, show antimicrobial properties, but also that there are species of this genus that have not been sufficiently studied, the aim of this study was to evaluate the antimicrobial activity of six different extracts of *Verbascum niveum* Ten.
Material and methods. Antimicrobial bioassays were performed with one fungus (*Candida albicans*) and four bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis*) by broth microdilution method according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing. The results were expressed as the minimal inhibitory concentration (MIC) of an extract (mg/ml) that prevents the visible growth of bacteria/fungi.
Results. The MIC values ranged from 0.39 to 1.25 mg/ml. The strongest effect was obtained with the ethanolic extract of leaves against *Staphylococcus aureus* (0.39 mg/ml).
Conclusion. Although Verbascum species are considered to be an excellent antimicrobial agent, according to our result, the tested extracts had modest antimicrobial activity.

Keywords: ***Verbascum niveum*, broth microdilution method, ethanolic, water, and propylene glycol extracts**

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INTRODUCTION

The emergence of infections resistant to multiple drugs and new types of infectious diseases require the study and development of new antimicrobial drugs (1). That is why the interest in using plants as potential antimicrobial agents has been growing recently (2). Medicinal plants are the main ingredient of numerous drugs and pharmaceutical products (3), and their antimicrobial effects have been proven in a large number of medicinal plant extracts and essential oils (1).

Genus *Verbascum L.* belongs to the *Scrophulariaceae* family and contains about 360 species (4). For centuries, *Verbascum* species (mullein) have been used due to their beneficial effects on human health (5). Based on very prevalent traditional use, in the documents of the European Medicines Agency (EMA) for drug *Verbasci flos*, three species of this genus are mentioned: *Verbascum thapsus L.*, *V. densiflorum* Bertol., and *V. phlomoides* L. (6).

Medicinal uses of *Verbascum* species are the treatment of respiratory problems such as bronchitis, asthma, coughs (dry, whooping, and chronic), and tuberculosis. However they have also been used to treat skin, abdomen, and bowel diseases, inflammation, and other health complaints (1).

The presence of saponins is the reason why *Verbascum* species seeds are poisonous to fish and are called 'fishplant' in northern Anatolia (5). Also, in the northern and northwest regions of Iran, *Verbascum* speciosum Schrad. is used to kill fish (2). Similarly, people in the southeast part of Serbia use mullein for fishing.

According to EMA official documents, *V. thapsus* possesses antibacterial activity against *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*, while *V. densiflorum* possesses antiviral activity against several influenza A strains, an influenza B strain and fowl plague virus, and virucidal activity against *Herpes simplex* virus type 1 (6). Mullein is especially valued as an antimicrobial and anti-inflammatory agent (7).

Taking into account that *Verbascum* species officially approved by EMA have shown antimicrobial properties, but also that there are species of this genus that have not yet been sufficiently studied, we wanted to test the antimicrobial activity of different extracts of *Verbascum niveum* Ten (a species for which we did not find available data).

MATERIAL AND METHODS

Plant material

Flowers and leaves of *V. niveum* were collected during the summer of 2021 in the vicinity of Bosilegrad. Botanical identification was performed by prof. Bojan Zlatković (Faculty of Science and Mathematics, University of Niš) and the voucher specimens are stored in the herbarium of the Faculty of Science and Mathematics, University of Niš (Herbarium code: 14615).

Extracts preparation

The plant material (flowers and leaves) was dried at room temperature in a dark place with constant airflow. After grounding using a laboratory mill, the material was stored in glass bottles until examination. Extracts (VNE – *V. niveum* ethanol extract, VNA - *V. niveum* aqueous extract, VNP – *V. niveum* propylene glycol extract) were made with three different solvents (50% ethanol, distilled water, and 80% propylene glycol) by percolation according to European Pharmacopoeia 10.0 (2019) (8). After filtration, ethanolic and water extracts were evaporated to dryness in a rotary vacuum evaporator at 40°C. Propylene glycol extracts were collected as liquids and directly used in testing. Yields after evaporation are expressed as DER (drug:extract ratio) – the initial amount of drug used for the preparation of a certain amount of extract. The DER values for ethanolic and aqueous extracts were: VNE *flos* – 2.48:1; VNE folium – 7.33:1; VNA *flos* – 7.06:1; VNA *folium* – 9.90:1, respectfully. The extracts were stored for one month in the refrigerator until examinations. For dry extracts (ethanol and distilled water extracts), analyses were performed by dissolving the samples in dimethyl sulfoxide (DMSO). Propylene glycol extracts were used as such.

Test organisms

The antimicrobial bioassays were done with one fungal and four bacterial strains: *Candida albicans* CNM-CL F8555a, Gram-negative strains: *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853, and Gram-positive strains: *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212.

Experimental procedure

The antimicrobial activity was carried out by a broth microdilution method, according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (9, 10). The broth microdilution method is the reference method for antimicrobial susceptibility testing of rapidly growing aerobic bacteria. EUCAST recommends testing according to the International Standard ISO 20776-1 (11).

Ten serial doubling dilutions of each sample were prepared in the 96-well microtiter plates (0.20 mg/ml – 100 mg/ml). The bacterial and fungal inoculates were prepared by making a direct broth suspension of isolated colonies selected from an 18- to 24-hour blood agar plate. The suspension was adjusted to achieve turbidity equivalent to a 0.5 McFarland standard. Within 15 minutes of preparation, the adjusted inoculum suspension was diluted in broth (1:20 to yield 5×10^6 CFU (colony-forming unit)/ml). The volume of the dilutions in the well was 0.1 ml and the inoculum volume was 0.01 ml; when 0.01 ml of this suspension is inoculated into each well, the final test concentration of bacteria is approximately 5×10^5 CFU/ml. The plates were incubated for 24 h at 37°C for bacteria and 48 h at 25°C for fungi. The results were expressed as minimal inhibitory concentrations (MIC) - the lowest concentration of a chemical, usually a drug (in our case extract – mg/ml), which prevents visible growth of bacteria. As positive controls for bacterial and fungal strains were used colistin (*Escherichia coli* and *Pseudomonas aeruginosa*), vancomycin (*Staphylococcus aureus* and *Enterococcus faecalis*), and fluconazole (*Candida albicans*), whereas DMSO (the solvent used for the dissolution of the dry extracts), and propylene glycol (80%) were used as the negative controls. All test experiments were replicated at least three times on separate plates.

RESULTS

Table 1 shows the results of the antimicrobial activity of the tested samples. DMSO and 80% propylene glycol also showed some antimicrobial activity. Therefore, the MIC values lower than the solvents' values are considered positive results. As referent antimicrobial drugs, colistin, vancomycin, and fluconazole exhibited higher antimicrobial activity than the tested extracts. The MIC values were in the range of 0.39 to 12.5 mg/ml. The most prominent effect was achieved for leaf ethanolic extract against *Staphylococcus aureus* (the MIC value was 0.39 mg/ml).

Table 1. *Results of the antimicrobial assay presented as mg/ml*

Microorganism	VNE flos	VNE folium	VNA flos	VNA folium	VNP flos	VNP folium	DMSO	Propylene glycol	Antibiotic
Escherichia coli ATCC 25922	12.50	6.25	12.50	6.25	12.50	6.25	25	25	0.001
Pseudomonas aeruginosa ATCC 27853	6.25	3.12	6.25	3.12	6.25	6.25	12.5	12.5	0.002
Staphylococcus aureus ATCC 29213	12.50	0.39	12.50	3.12	1.56	1.56	25	25	0.001
Enterococcus faecalis ATCC 29212	12.50	0.78	12.50	1.56	6.25	1.56	25	12.5	0.002
Candida albicans CNM-CL F8555a	6.25	6.25	12.50	12.50	12.50	12.50	12.50	12.50	0.128

DISCUSSION

As some *Verbascum* species have already shown to possess antimicrobial activity (2, 12, 13), we aimed our work at *Verbascum niveum* extracts testing. A broth microdilution method was used to test the antimicrobial activity of six extracts against one fungal and four bacterial strains.

The antimicrobial activity of a plant extract depends on the organ of the plant used, the type of solvent used for extraction, the extraction method, and the type of microorganisms tested (14). Therefore, we tested six extracts, obtained from flowers and leaves with different solvents. Since propylene glycol (PG) and dimethyl sulfoxide (DMSO) have shown certain antimicrobial activity, as an indication of a positive result we considered only the MIC values lower than the ones for PG and DMSO solvents. In general, the MIC values we obtained for all the tested extracts against bacterial strains were lower than the MIC values for the PG and DMSO solvents. In the case of fungi, however, the results for extracts and for the solvents were mostly similar. Only ethanolic extracts showed some activity against *Candida albicans*, and we can say that our extracts are effective against bacteria but their effectiveness against the tested fungal strain is poor. The results of our study confirmed the findings of other researchers that some *Verbascum* species have substances with antimicrobial properties (15 - 18).

Escherichia coli and *Pseudomonas aeruginosa* were Gram-negative bacteria that we used. Leaf and flower extracts showed stronger antimicrobial activity than pure solvents. It was also observed that the activity of leaf extracts was higher than that of flower extracts. In our experiment, *Pseudomonas aeruginosa* was detected as the most susceptible strain, with leaf extracts exhibiting the strongest effect (Table 1).

Verbascum L. species owe their antimicrobial activities to a wide range of compounds such as glycosides, alkaloids, and saponins. Many reports suggest that phenolic compounds have antimicrobial activity (12), and in general, phenolics are the predominant active chemicals in plants, with Gram-positive bacteria being the most sensible germs (19). That is exactly what we obtained in this study. *Staphylococcus aureus*, and *Enterococcus faecalis* were used as Gram-positive bacterial strains, and most of our extracts were more effective against them. The strongest activity was determined for leaf ethanolic and aqueous extracts against the tested Gram-positive bacterial strains. In fact, the lowest MIC value was detected for leaf ethanolic extract against *Staphylococcus aureus* (Table 1). *Staphylococcus aureus* is a bacterium that colonizes the skin and mucosal surfaces of healthy individuals, and it is also one of the most common causes of community-acquired and hospital infections (20). The antibacterial activity against this bacterium was also recorded by Dulger et al. (5) for the extracts of three different *Verbascum* species (*V. olympicum* Boiss., *V. prusianum* Boiss., and *V. bombyciferum* Boiss.). They investigated 80% methanol extracts by agar-disk diffusion method and found that investigated species revealed antimicrobial activity to some Gram-positive bacteria and yeasts. *Staphylococcus aureus* and *Micrococcus luteus* were found to be the most sensitive microorganisms (5).

Our study shows that *V. niveum* extracts possess certain effectiveness against the tested bacterial strains, but their antimicrobial activity is generally weak.

CONCLUSION

Based on our results, we can conclude that the antimicrobial activity of *V. niveum* extracts is weak. Although the results that we obtained are weak, given that this work is the first report on the antimicrobial activity on *V. niveum*, they are of certain value. We will continue our *V. niveum* investigation in order to analyze its chemical composition and biological activities.

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In vitro antimikrobna aktivnost različitih ekstrakata *Verbascum niveum*

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SAŽETAK

Uvod. Lekovite biljke su glavni sastojak brojnih lekova i farmaceutskih proizvoda, a antimikrobno dejstvo je dokazano za veliki broj biljnih ekstrakata i etarskih ulja. S obzirom na to da vrste roda *Verbascum* (divizme), koje su oficinalne prema Evropskoj agenciji za lekove, ispoljavaju antimikrobna svojstva, ali i da postoje još uvek nedovoljno proučene vrste ovog roda, cilj našeg rada bila je procena antimikrobne aktivnosti šest različitih ekstrakata vrste *Verbascum niveum* Ten.

Materijal i metode. Ispitivanja su rađena sa jednim gljivičnim sojem (*Candida albicans*) i četiri soja bakterija (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis*) mikrodilucionom metodom, prema preporukama Evropskog komiteta za ispitivanje antimikrobne osetljivosti. Rezultati su izraženi kao minimalna inhibitorna koncentracija (MIC) ekstrakta (mg/ml), koja sprečava vidljiv rast bakterija/gljivice.

Rezultati. MIC vrednosti bile su u opsegu od 0,39 do 1,25 mg/ml. Najizraženiji efekat postignut je sa etanolnim ekstraktom lista na soj *Staphilococcus aureus* (0,39 mg/ml).

Zaključak. Uprkos tvrdnjama da su *Verbascum* vrste odlični antimikrobni agensi, prema našim rezultatima, utvrđeno je da ekstrakti ove biljne vrste ispoljavaju skromnu antimikrobnu aktivnost.

***Ključne reči: Verbascum niveum*, bujon mikrodilucioni metod, etanolni, vodeni i propilenglikolni ekstrakti**