BIOLOGICA NYSSANA
10 (2) December 2019: 169-173
DOI: 10.5281/zenodo.3600197
13th Symposium on the Flora of Southeastern Serbia and Neighboring Regions

Antibiofilm activity of *Verbascum pinnatifidum* Vahl. ethanolic extract

Original Article

Mehmet Göse
Department of Plant Physiology, Institute of Biology, Graduate School of Natural and Applied Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
m_gose@hotmail.com

Nurcihan Hacıoğlu Doğru
Department of Biology, Faculty of Arts and Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
nurcihan.n@gmail.com (corresponding author)

Received: July 1, 2019
Revised: September 9, 2019
Accepted: September 13, 2019

Abstract:

Microbial biofilms pose health risks in clinical environments, food industry and drinking water systems. Microorganisms within biofilms are more resistant to antibiotics and chemical agents than planktonic cells in suspension. New alternatives for controlling infections have been proposed focusing on the therapeutic properties of medicinal plants and their antibiofilm activities. Here, we investigated in vitro antibiofilm activity of ethanol extract of *Verbascum pinnatifidum* Vahl. against *Escherichia coli* NRRL B-3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *S. haemolyticus* ATCC 43252 and *Candida albicans* ATCC 10231 test microorganisms based on crystal violet binding assay. The highest antibiofilm activity was shown against biofilm formed by *B. subtilis* ATCC 6633 and *S. haemolyticus* ATCC 43252 at the lowest MIC values of 2.5 and 10 µg/mL, respectively. The current findings indicated that bacterial biofilm formation can be potentially managed using *V. pinnatifidum* plant extracts.

Key words:
antibiofilm activity, Verbascum pinnatifidum

Apstract:

Antibiofilm aktivnost etanolnog ekstrakta *Verbascum pinnatifidum* Vahl.

Biofilmovi mikroorganizama predstavljaju zdravstveni rizik u kliničkim uslovima, prehrambenoj industriji i sistemima pijaće vode. Unutar biofilmova, mikroorganizmi su rezistentniji na antibiotike i hemijske agense od planktonskih ćelija u suspenziji. Predložene su nove alternative za kontrolisanje infekcija, koje su fokusirane na terapeutske osobine medicinskih biljaka i njihovu antibiofilm aktivnost. Ovde je, korišćenjem kristal violet testa, vršeno istraživanje *in vitro* antibiofilm aktivnosti etanolnog ekstrakta *Verbascum pinnatifidum* Vahl. u odnosu na vrste *Escherichia coli* NRRL B-3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *S. haemolyticus* ATCC 43252 i *Candida albicans* ATCC 10231. Najveća antibiofilm aktivnost, sa najnižim MIK vrednostima od 2,5 i 10 µg/mL, utvrđena je za biofilmove formirane od strane vrsta *B. subtilis* ATCC 6633 i *S. haemolyticus* ATCC 43252, respektivno. Ovi nalazi ukazuju na to da stvaranje bakterijskih biofilmova potencijalno može biti sprečeno korišćenjem ekstrakta *V. pinnatifidum.*

Ključne reči:
antibiofilm aktivnost, Verbacum pinnatifidum

Introduction

Microbial biofilms are communities of bacteria, embedded in a self-producing matrix, forming on living and nonliving solid surfaces (Vasudevan, 2014). They are considered as an important virulence factor that causes persistent chronic and recurrent infections; they are highly resistant to antibiotics and host immune defenses (Grant and Hung, 2013). Biofilm resistance is due to several reasons, like restricted diffusion of antibiotics into biofilm matrix, expression of multidrug efflux pumps, type IV secretion systems, decreased permeability, and the action of antibiotic-modifying enzymes (Alekshun and Levy, 2007). The increased biofilm resistance

to conventional treatments enhances the need to develop new control strategies (Simões et al., 2007; Sanchez et al., 2016).

Verbascum plants have been used medicinally since ancient times in folk medicine as a remedy for respiratory problems such as bronchitis, dry coughs, whooping cough, tuberculosis, and asthma. The leaves, roots and the flowers have been used also as anodyne, sedative, diuretic, sudorific, expectorant and antidiarrheal agents in traditional World and Turkish medicine (Baytop, 1999; Georgiev et al., 2011). Therefore *Verbascum* plant species have been also previously reviewed for their antiviral, antimicrobial, antimalarial, antioxidant, anti-inflammatory, antinociceptive, antitumor, anticancer, cytotoxic, immunomodulatory, anticholinesterase, antiulcerogenic, antihepatotoxic, antihyperlipidemic, anthelminthic, antitussive and antigermination activities (Tatli and Akdemir, 2006; Dulger and Hacioglu, 2009; Kahraman et al., 2010; Kahraman et al., 2011; Kozan et al., 2011; Ozcan et al., 2011; Boğa et al., 2016). However, there is no any literature about *Verbascum* plants antibiofilm activity, except Moghaddam et al. (2015), which evaluated the antibiofilm activity of *Verbascum pinnatifidum* Vahl. ethanol extract.

Material and methods

Plant materials

Verbascum pinnatifidum was collected from Canakkale, Kumkale near the lantern d.s. sandy area, (40.008101 N, 26.203127 E) in 2018 and identified with the aid of Flora of Turkey (Davis et al., 1988) by Dr. Ersin Karabacak. Voucher specimens were deposited in the Biology Department at Çanakkale Onsekiz Mart University, Çanakkale, Turkey.

Preparation of plant extracts

The plant parts were air-dried. Dry powdered plant material (10 g) was extracted with 300 mL of 80% ethanol (Merck, Darmstadt, Germany) for 24 h by using Soxhlet equipment (Khan et al., 1988). The extracts were filtered using Whatman filter no.1, and the filtrates were then evaporated under reduced pressure and dried using a rotary evaporator at 55 °C. Dried extracts were stored in labelled sterile screw-capped bottles at + 4 °C.

Test Microorganisms

Gram negative bacteria - *Escherichia coli* NRRLB 3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606 and Gram positive bacteria – *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *S. haemolyticus* ATCC 43252 and yeast culture - *Candida albicans* ATCC 10231 were used as test microorganisms.

Minimum inhibitory concentration assay

To determine the plant extract doses to be used in biofilm inhibition study, Minimum inhibitory concentration (MIC) values of all samples were determined. MIC was investigated as recommended instruction of the Clinical and Laboratory Standards Institute (CLSI, 2006). The lowest concentration of extract inhibiting the visible growth of each test microorganisms was taken as the MIC. The medium, 0.1% (w/v) Streptomycin (ST), Nystatin (NYS100) and 10% DMSO were used as the non-treated, positive and negative controls, respectively (Teanpasian et al., 2017).

Biofilm inhibition assay

Microplate biofilm method (Merrit et al., 2005) was used to evaluate the inhibition of biofilm formation by *V. pinnatifidum* plant ethanol extract against test microorganisms. Cultures were incubated in 5 mL Tryptic Soy Broth (TSB) medium containing 5% glucose. Cultures were diluted 1:100 in TSB and loaded into each well in 4 sterile microplates. Different concentrations of plant extract (MIC and sub-MIC concentrations: 50, 25, 12.5% of MIC) were prepared and transferred to each microplate well. After incubation at 37 ± 0.1°C for 48 h planktonic bacteria were removed from the wells and wells were washed twice with distilled water. 200 µL of 0.1% crystal violet solution was added to each well (20 minutes). The crystal violet bounded extracts were poured and washed until the crystal violet removed. The microtiter plates were inverted, and the remaining liquid was drained and dried in room heat. Finally, the adhered biofilm bounded crystal violet was eluted in ethanol (95%), and the absorbance was measured at 550 nm by using an automated ELISA reader. All experiments were repeated thrice in triplicate. The measurement of the antibiofilm effect of the extract was made by the percentage reduction formulation.

$$\% \text{ Inhibition} = (A_{control} - A_{sample} / A_{control}) \times 100$$

$A_{control}$: Absorbance of the control (containing 100 µL TBS instead of plant extract) reaction

A_{sample}: Absorbance of the test compound

Results and discussion

Ethanol extract of *V. pinnatifidum* was tested against eight clinical bacterial and fungal strains (**Tab. 1**). Ethanol was observed as the best solvent for extracting antimicrobial substances in a previous study (Jonathan and Fasidi, 2003). Results of MIC were quite variable between each test microorganisms ranging from 2.5 to 20.0 µg/

Table 1. The percentage of inhibition of biofilm structure of test microorganisms

	MIC (µg/mL)		% Inhibition of biofilms formation			
Test Microorganisms	Plant extract	Control ST/ NY100	MIC	MIC/2	MIC/4	MIC/8
E.coli NRRLB 3704	20.0	4.0	7.27±0.33	3.34±0.08	-	-
P. aeruginosa ATCC 27853	10.0	1.0	74.65±6.23	65.33±5.67	43.56±4.22	34.76±1.76
P. vulgaris ATCC 13315	10.0	4.0	70.50±0.54	60.03±1.55	45.54±1.00	40.01±0.23
A. baumanii ATCC 19606	10.0	2.0	57.42±0.12	43.12±0.10	-	-
B. subtilis ATCC 6633	2.5	4.0	90.02±0.01	70.01±0.10	55.78±1.23	43.26±1.43
S. aureus ATCC 6538P	10.0	4.0	70.54±0.60	-	-	-
S. haemolyticus ATCC 43252	10.0	5.0	76.12±3.11	58.41±3.12	44.45±4.11	23.56±1.56
C.albicans ATCC 10231	2.5	2.5	10.45±1.14	5.45±0.99	-	-

-: No inhibition of biofilm formation was observed due to the lack of antibiofilm effect.

mL (**Tab. 1**). It was observed that almost all tested microorganisms were sensitive towards the ethanol extract of *V. pinnatifidum*. There is no data about *V. pinnatifidum* antimicrobial activity in the literature. Our findings about MIC ranges confirmed the observations of some other researchers, which stated that some *Verbascum* species have antimicrobial activity against Gram positive and negative bacteria and yeast and mold cultures (Dulger and Hacioglu, 2008;2009; Ozcan et al., 2010; Morteza-Semnani et al., 2012; Noori et al., 2012; Anil et al., 2016; Dulger and Dulger, 2018). However, we found that *V. pinnatifidum* extract was also effective against *E. coli* NRRLB 3704 and *C. albicans* ATCC 10231, contrary to some studies with the other *Verbascum* species in the literature (Dulger and Hacioglu, 2008; Morteza-Semnani et al., 2012; Amin et al., 2015).

The results of potential inhibition of test microorganism's biofilm formation by ethanol extract of *V. pinnatifidum* were shown in **Tab. 1**. The results indicated that *V. pinnatifidum* extract in 2.5 to 10.0 µg/mL concentrations could inhibit the biofilm formation of all the tested microorganisms except *E. coli* NRRLB 3704 and *C. albicans* ATCC 10231. Throughputs also showed that antibiofilm function of extract was in a dose-dependent manner. The highest antibiofilm activity was noticed against biofilm formed by *B. subtilis* ATCC 6633 and *S. haemolyticus* ATCC 43252 at the lowest MIC values of 2.5 µg/mL and 10 µg/mL, respectively.

Verbascum pinnatifidum has not been investigated in terms of antibiofilm activity. In a study conducted by Moghaddam et al. (2015), ethanol extract of *V. thapsus* had inhibitory effect on biofilm formation of *Streptococcus mutans*, *S. sanguinis*, and *S. salivarius*. The current findings indicated that biofilm forming of bacteria could be potentially managed using *V. pinnatifidum* plant extracts.

Biofilm is considered to be one of the essential virulence factors that cause persistent chronic and recurrent infections, because of their high resistance to antibiotics and host immune defensive system (Grant and Hung, 2013). Bacteria are more resistant to antibiotics than planktonic cells because they are preserved by exopolysaccharide. This necessitated the screening of new and natural antibiotic sources in the fight against biofilm. New alternatives for controlling infections have been proposed focusing on the therapeutic properties of medicinal plants and their antibiofilm activities. Many studies showed that the biofilm formation by pathogens leads to an increase in their virulence (Vuong et al., 2004; Antunes et al., 2010). Therefore, if the biofilm formation is inhibited, the bacterial infection can be prevented (Erdönmez et al., 2018).

Biofilm formation can be controlled by quorum sensing, a bacterial communication system which causes a rapid and coordinated change of expression pattern in the bacterial population in response to population density (Pratiwi et al., 2015). The fact that at MIC and sub-MIC concentrations, *V. pinnatifidum* extract is capable of disturbing biofilm formation and causes biofilm breakdown, suggests that this disturbance may have been caused by the presence of compounds that inhibit quorum sensing. Other plant compounds could attenuate biofilm development by inhibiting bacterial peptidoglycan synthesis (Ogunlana et al., 1987), disrupting the permeability barrier of microbial membrane structures, causing the cell to leak out (Cox et al., 2000), modify bacterial membrane structure hydrophobicity (Türi et al., 1997; Das, 2014), or disturbing the extracellular polymeric matrix in the biofilm to release biofilm from the surface of the solid substratum (Traba and Liang, 2011; Pratiwi et al., 2015). Further studies need to be performed

to confirm the actual mode of action of anti-biofilm activity from these extracts.

Conclusion

Our investigation was the first report on the antibiofilm property of the *V. pinnatifidum* ethanol extract. In this research, we found that forming of bacterial biofilm could be potentially being managed using *V. pinnatifidum* plant extract, especially against Gram (+) bacteria. Detailed phytochemical investigations are required to determine the types of substances responsible for the biological activities of *V. pinnatifidum* plant species. We hope that our results will provide useful data for discovering new compounds with better activity than agents currently available.

Acknowledgments. This investigation is a part of Master thesis of Mehmet GÖSE. This study was financially supported by the Çanakkale Onsekiz Mart University Scientific Research Projects Coordination Unit, Turkey (FYL-2018-2693). This paper was presented at the Symposium on The Flora of Southeastern Serbia and Neighboring Regions, June, 20-23 2019, Serbia. Authors would like to thank Assoc. Prof. Dr. Ersin KARABACAK for defining plant species.

References

Alekshun, M.N., Levy, S.B. 2007: Molecular mechanisms of antibacterial multidrug resistance. *Cell*, 128(6): 1037-1050.

Amin, J.N, Batool, M., Abu-hadid, M.M. 2015: Screening antibacterial and antifungal activities and evalution of exhaustive extractions yields for *Verbascum sinuatum* L. *International Research Ayurveda Pharmacy*, 6 : 105-110.

Anil, S., Dosler, S., Mericli, A. H. 2016: Chemical composition and antimicrobial activity of *Verbascum caesareum*. *Chemistry of Natural Compounds*, 52(1): 125-126.

Antunes, L.C.M., Ferreira, R.B., Buckner, M.M., Finlay, B.B. 2010: Quorum sensing in bacterial virulence. *Microbiology*, 156(8): 2271-2282.

Baytop, T. 1999: *Türkiye'de Bitkiler ile Tedavi*. Nobel Tıp Kitabevleri, İstanbul. 480 p.

Boğa, M., Ertaş, A., Yılmaz, M.A., Kızıl, M., Çeken, B., Haşimi, N., Özden T.Y., Demirci, S., Yener, İ., Deveci, Ö. 2016: UHPLC-ESI-MS/MS and GC-MS Analyses on Phenolic, Fatty Acid and Essential Oil of *Verbascum pinetorum* with Antioxidant, Anticholinesterase, Antimicrobial and DNA Damage Protection Effects. *Iranian Journal of Pharmaceutical Research,* 15(3): 393-405.

CLSI, 2006. Clinical and Laboratory Standarts Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Seventh Edition*. M07- A7, Villanova, PA, USA.

Cox, S., Mann, C., Markham, L., Bell, H., Gustafson, J. 2000: The mode of action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Applied Microbiology*, 88(1): 170-175.

Das, M.P. 2014: Effect of cell surface hydrophobicity in microbial biofilm formation. *European Journal of Experimental Biology*, 4(2): 254-266.

Davis, P.H., Mill, R.R., Tan, K. (eds.) 1988: *Flora of Turkey and the East Aegean Islands*. 10: 191-193. Edinburgh University Press, Edinburgh.

Dülger, B., Hacioğlu, N. 2008: Antimicrobial activity of some endemic *Verbascum* and *Scrophularia* species from Turkey. *Asian Journal of Chemistry*, 20: 3779-3785.

Dülger, B., Hacioğlu, N. 2009: Activity of three endemic *Verbascum* species against hospital isolates methicillin-resistant *Staphylococcus aureus*. *Biotechnology & Biotechnological Equipment*, 23: 760-762.

Dülger, B., Dülger, B. 2018: Antibacterial activity of *Verbascum antinori*. *Konuralp Medical Journal*, 10(3): 395-398.

Erdönmez, D., Kenar, N., Türkmen, K.E. 2018: Screening for anti-quorum sensing and antibiofilm activity in *Viscum album* L. extracts and its biochemical composition. *Trakya University Journal of Natural Sciences*, 19(2): 175-186.

Georgiev, M., Alipieva, K., Orhan, I., Abrashev, R., Denev, P., Angelova, M. 2011: Antioxidant and cholinesterases inhibitory activities of *Verbascum xanthophoeniceum* Griseb. and its phenylethanoid glycosides. *Food Chemistry,* 128: 100-105.

Grant, S.S., Hung, D.T. 2013: Persistent bacterial infections, antibiotic tolerance, and the oxidative stress response. *Virulence*, 4(4): 273–283.

Jonathan, S.G., Fasidi I.O. 2003: Antimicrobial activities of two Nigerian edible macro fungi – *Lycoperdon pusilum* (Bat. Ex) and *Lycoperdon giganteus* (Pers). *African Journal of Biomedical Research*, 6: 85 –90.

Kahraman, Ç., Tatlı, I.I., Orhan, I.E., Akdemir, Z.S. 2010: Cholinesterase inhibitory and antioxidant properties of *Verbascum mucronatum* Lam. and its Secondary Metabolites. *Zeitschrift für Naturforschung*, 65c: 667– 674.

Kahraman, Ç., Ekizoğlu, M., Kart, D., Akdemir,

Z.S., Tatlı, I.I. 2011: Antimicrobial activity of some *Verbascum* species growing in Turkey. FABAD *Journal of Pharmaceutical Sciences*, 36: 11-15.

Khan, N.H., Kamal, M.S.A., Rahman, M. 1988: Antibacterial activity of *Euphorbia thymifolia* Linn. *Indian Journal of Medical Research*, 87: 395-397.

Kozan, E., Çankaya, I.T., Kahraman, Ç., Akkol, E.K., Akdemir, Z. 2011: The *in vivo* anthelmintic efficacy of some *Verbascum* species growing in Turkey. *Experimental Parasitology*, 129: 211-214.

Merritt, J.H., Kadouri, D.E., O'Toole, G.A. 2005: Growing and analyzing static biofilms. *Current Protocols in Microbiology*, 1(1B).1-17.

Moghaddam, H.K., Mirzaii, M., Khaksari, M., Fazli, M., Rahimi, F., Behzadi, A.A. 2015: Antibacterial and anti-adherent activity of great mullein (*Verbascum thapsus* L.) ethanolic extract on *in vitro* biofilm formation of three oral *Streptococci*. *International Journal of Health Studies*, 1(2): 34-37.

Morteza-Semnani, K., Saeedi, M., Akbarzadeh, M. 2012: Chemical composition and antimicrobial activity of the essential oil of *Verbascum thapsus* L. *Journal of Essential Oil Bearing Plants*, 15(3): 373 – 379.

Noori, M., Malayeri, B., Moosaei, M., Pakzad, R., Piriye, M.H. 2012: Effects of heavy metals on the antibacterial properties of *Verbascum speciosum* Schard. *Revista Científica UDO Agrícola,* 12(2): 463-471.

Ogunlana, E., Hoeglund, G., Onawunmi. O. 1987: Effects of lemongrass oil on the morphological characteristics and peptidoglycan synthesis of *Escherichia coli* cells. *Microbios,* 50(202): 43-59.

Ozcan, B., Esen, M., Calışkan, M., Mothana, R.A., Cihan, A.C., Yolcu, H. 2011: Antimicrobial and antioxidant activities of the various extracts of *Verbascum pinetorum* Boiss. O. Kuntze (Scrophulariaceae). *European Review for Medical and Pharmacological Sciences*, 15: 900-905.

Pratiwi, S.U.T., Lagendijk, E.L., Hertiani, T., Weert, S., Cornellius, A.M.J., Vand Den Hondel, J. 2015: Antimicrobial effect of Indonesian medicinal plants extracts on planktonic and biofilm growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus. International Journal of Pharmacy and Pharmaceutical Sciences,* 7(4): 183-191.

Sánchez, E., Morales, C.R., Castillo, S., Leos-Rivas, C., García-Becerra, L., Martínez, D.M.O. 2016: Antibacterial and antibiofilm activity of methanolic plant extracts against nosocomial microorganisms. *Evidence-Based Complementary and Alternative Medicine,* 2016: 1-8.

Simões, L.C., Simões, M., Vieira, M.J. 2007: Biofilm interactions between distinct bacterial genera isolated from drinking water. *Applied and Environmental Microbiology*, 73(19): 6192–6200.

Tatlı, I.I, Akdemir, Z.S. 2006: Traditional uses and biological activities of *Verbascum* Species. *FABAD Journal of Pharmaceutical Sciences*, 31: 85-96.

Teanpaisan, R., Kawsud, P., Pahumunto, N., Puripattanavong, J. 2017: Screening for antibacterial and antibiofilm activity in Thai medicinal plant extracts against oral microorganisms. *Journal of Traditional and Complementary Medicine*, 7: 172-177.

Türi, M., Türi, S., Koljalg, R. 1997: Influence of aqueous extracts of medicinal plants on surface hydrophobicity of *Escherichia coli* strains of different origin. *APMIS*, 105(12): 956-962.

Vasudevan, R. 2014: Biofilms: microbial cities of scientific significance. *Journal of Microbiology & Experimentation*, 1(3): 1–16.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R., Otto, M. 2004: A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion and virulence. *Journal of Biological Chemistry*, 279(52): 54881-54886.