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Antimicrobial potential of the leaves of common mullein (*Verbascum thapsus* L., Scrophulariaceae) on microorganisms isolated from urinary tract infections

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Abstract
Objective: Nowadays, Urinary tract infections (UTIs) have become very common. Scientists have come up with new treatment for UTIs that avoids the use of antibiotics. Therefore, studies for new alternative remedies are necessary. The aim of this study was to investigate the antibacterial potential of the leaves of *Verbascum thapsus* L. (Scrophulariaceae) (known as Common Mullein) against the pathogens causing complicated urine tract infections.
Methods: Air-dried leaves of the plant were extracted using 95% ethanol. The extract was screened against urinary tract pathogens (*Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Candida albicans*) by disc diffusion method and microdilution method. Some antibacterial and antifungal antibiotics were used as a positive reference standard to determine the sensitivity of the strains.
Results: The extracts showed strong antimicrobial activity against *Escherichia coli*, *Enterococcus faecalis* and *Candida albicans* with inhibition zones of 19.2, 16.8 and 16.2 mm, with MIC's and MBC's or MFC's of 32(64), 64(128) and 64(128) µg/mL, respectively. Also, the extracts exhibited moderate activity against the other test microorganisms.
Conclusion: Our findings support the use of *Verbascum thapsus* L. in traditional medicine for the treatment against the urine tract pathogens. Hence, it is suggested to isolate and identify the active compounds of the plant for novel antimicrobial agents in future.

Keywords: Urinary tract infection (UTI), Antimicrobial activity, *Verbascum thapsus*

1. Introduction
Common mullein, also known as Wooly Mullein (*Verbascum thapsus* L., Scrophulariaceae) has been used as a medicinal herb since ancient times. The leaves and flowers are reported to have expectorant and demulcent features which are used to treat respiratory problems such as bronchitis, dry coughs, whooping cough, tuberculosis, asthma and hoarseness. The plant is reported to be mildly diuretic and to have a soothing and anti-inflammatory effect on the urinary tract, and to act as a mild sedative. It has also been used as a domestic remedy for pneumonia, fever, congestion, allergies, migraine, catarrhs and colic [1, 2].
During our routine field excursions, it was found that this plant is used to treat respiratory tract infections and externally boils and abscesses. Also, it has also been as traditional remedy to treat various ailments such as spasmodic, digestive disorders and menstrual problems. Therefore, the aim was to determine *V. thapsus* extracts that have been shown earlier to have biological activity against the urinary tract pathogens.

2. Materials and methods
2.1. Plant material
The plant were collected from Ikizdere-Rize, Turkey in September, 2011 and identified by Dr. Tulay Tutenocakli. A voucher specimen (voucher number GD70-4) of the plant was deposited in Department of Medical Biology of Duzce University in the author's personal collection.

2.2. Preparation of extract
The leaves of the plant were dried in an oven at 40 ˚C (12h) and powdered. Each dry powdered plant material (20 g) was extracted with 150 mL of 95% ethanol (Merck, Darmstadt, Germany) for 24h by using Soxhlet equipment [3]. The extract was filtered using Whatman filter No. 1, and the filtrate solvent was evaporated under vacuum using a rotary evaporator at 55 ˚C (yield: 11.8% for ethanol). The resulting dried extract was stored in labeled sterile screw-capped

bottled at -20 ˚C. The extract (in the form of sticky black substances) amounting to around 2 g was dissolved in 0.1 mL of DMSO (5 mg/g) (dimethyl sulfoxide) before testing.

2.3. Microorganisms

Urinary tract pathogens (*Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Proteus mirabilis* and *Candida albicans*) were isolated from the urine of patients diagnosed with urinary infections in Faculty of medical at Canakkale Onsekiz Mart University, and Canakkale Anadolu Hospital, Canakkale, Turkey. VITEC 2 (bioMerieux, France) system was used for identification.

2.4. Disc diffusion method

The paper disc diffusion method was employed [4]. Sterile 6 mm disc filter paper disc (Schleicher & Schul, No. 2668, Dassel, Germany) were impregnated with 50 µL of the plant extract. The bacterial cultures were inoculated on Nutrient Broth (Oxoid) and incubated for 24 h at 37±0.1 °C, while the yeast cultures were inoculated on Malt Extract Broth (Oxoid) and incubated for 48 h at 28.0±0.1 °C. Adequate amounts of Mueller Hilton Agar (Oxoid) were dispensed into sterile plates and allowed to solidify under aseptic conditions. The counts of bacterial and yeast cultures were adjusted to yield 10^7-10^8/mL and 10^5-10^6/mL, respectively, using the standard McFarland counting method. The test microorganisms (0.1 mL) were inoculated with a sterile swab on the surface of appropriate solid medium in plates. The agar plates inoculated with the test microorganisms were incubated for 1 h before placing the extract impregnated paper disc on the plates. The bacterial plates were incubated at 37±0.1 °C for 24 h while yeast plates were incubated at 28±0.1 °C for 48 h. After incubation, all plates were observed for zones of growth inhibition and the diameter of these zones was measured in millimetres. All tests were performed under sterile conditions in duplicate and repeated three times. Penicillin (10 µg/disc) (Oxoid), tobramycin discs (10 µg/disc) (Oxoid), ampicillin/sulbactam 1:1 (20 µg/disc) (Oxoid), nystatin (30 µg/disc) (Hi-Media), clotrimazole (30 µg/disc) (Abtek biologicals) and ketoconazole (20 µg/disc) (Liofilchem) discs were used as positive controls.

2.5. Microdilution Method

Determination of the minimum inhibitory concentration (MIC) was carried out according to the method described by Zgoda and Porter (2001), with some modifications [5]. A dilution series of the extract, ranging from 10 to 0.5 mg/mL, were prepared and then transferred to the broth in 96–well microtitre plates. The final concentrations were in the range 1000 to 50 µg/mL in the medium. Before inoculation of the test organisms, the bacterial and yeast strains were adjusted to 0.5 McFarland and diluted 1:1000 in Mueller Hinton Broth (Oxoid) and Malt Extract Broth (Oxoid), respectively. The plates were incubated at 35 °C for 18-24 h for bacteria and 30 °C for 48 h for the yeast cultures. All the tests were performed in broth and repeated twice. While the MIC values of the extracts were defined as the lowest concentration that showed no growth, minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by plating samples from clear wells onto Mueller Hinton Agar and Malt Extract Agar, respectively. MBC and MFC were defined as the lowest concentration yielding negative subculture. Reference antibacterial agents of ampicillin, streptomycin as well as reference antifungal agent of nystatin were obtained from their respective manufacturers and dissolved in phosphate buffer solution (ampicillin, pH: 8.0; 0.1 mol/mL), DMSO (nystatin), or in water (streptomycin). The stock solutions of the agents were prepared in medium according to the Clinical and Laboratory Standards Institute [6].

3. Results and Discussion

The antimicrobial activities of *V. thapsus* L. extracts against the pathogens causing complicated urinary tract infections examined in this study were qualitatively and quantitatively assessed by presence of inhibition zoned, MIC, MBC, and MFC (Table 1 and Table 2).

The ethanolic extracts obtained from the leaves of *V. thapsus* were strong antimicrobial activities against the pathogens, with inhibition zones at 11.0 – 19.2 mm. *Escherichia coli* is more susceptible to the extract of *V. thapsus* as compared to all standard antibacterial antibiotics such as Penicillin, Ampicillin and Tobramycin (inhibition zone is 19.2 mm). *Enterococcus faecalis* and *Candida albicans* are more resistant to the extract. The extract showed higher antibacterial activity on *Proteus mirabilis* than those of standard antibacterial antibiotics except for Tobramycin. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are more susceptible to some antibacterial antibiotics.

The ethanolic extracts were further tested by microdilution to determine the MICs and MBCs. The lowest MICs and MFCs of the extract were 32(64) µg/mL against *Escherichia coli*, followed *Enterococcus faecalis* and *Candida albicans* (MIC values is 64(128) µg/mL). The extracts have weak antimicrobial effect against the other pathogens, with MICs and MBCs ranged from 1000(1000) - 500(1000) µg/mL. These values are far below than the standard antibiotics.

Some studies concerning the effectiveness of extraction methods highlight that ethanol extraction yields higher antimicrobial activity than the other solvents [7]. According to present results, ethanol extract has stronger and broader spectrum of antibacterial activity. This information confirmed that the ethanol has higher effective solvent for extraction of antimicrobial substances in *V. thapsus*.

Verbascum L. species contain a wide range of compounds, such as glycosides [8-11], alkaloids [12], and saponins [13]. Members of the family Scrophulariaceae have been reported to contain a group of unusual macrocyclic spermine alkaloids [14, 15]. Constituents of *V. thapsus* include polysaccharides; iridoid glycosides including harpagoside, harpagide and aucubin (especially in the leaf); flavonoids, including 3'-methylquercitin, hesperidin and verbascoside; saponins and volatile oils [16-20]. Flavonoids and saponins may be responsible for their antimicrobial activity. Activity of flavonoids is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. Lipophilic flavonoids may also disrupt microbial membranes [21].

It is reported that *V. thapsus* has antiviral activity against influenza in chicken embryos [19]. In previous study, leaf extracts of this plant have been shown to be active against bovine herpes virus type 1, and showed slight antibacterial and antifungal activity [22-24]. Methanol extract has been shown to be effective against mosquito larvae [25]. In another study, biological activity of common mullein extracts and commercial mullein products using selected bench top bioassays, including antibacterial, antitumor and two toxicity assays (brine shrimp and radish seed) was assessed. Antibacterial activity was tested with *Klebsiella pneumoniae*, *Staphylococcus aureus*, *S. epidermidis* and *Escherichia coli* [1]. In that study, aqueous extracts were found that the most effective. Only *K. pneumoniae* and *S. aureus* showed sensitivity to the mullein samples tested.

The results obtained from in the present study, ethanolic extracts of the plant have high antimicrobial activity against tested microorganisms, but *K. pneumoniae* is more resistant to the extract. The differences between this study and the mentioned study may be due to species variation. Also, there may be differences in the extraction protocols to recover the active metabolites and differences in the assay methods.

4. Conclusion

The ethanolic extracts of *V. thapsus* have antimicrobial activity against the urine tract pathogens; however only *Escherichia coli*, *Enterococcus faecalis* and *Candida albicans* showed more sensitivity to the extracts, which may explain why the common mullein is used in folk medicine to treat urinary tract infections.

Table 1: Summary of antimicrobial activity of *V. thapsus* and some standard antibiotics

Microorganisms	Inhibition zones (mm)[a]						
	Plant Extract	Standard antibiotics					
		P	AMP	TOB	NYS	KETO	CLT
Enterococcus faecalis	16.8	14.0	16.0	18.0	Nt	Nt	Nt
Escherichia coli	19.2	16.0	14.0	10.0	Nt	Nt	Nt
Klebsiella pneumoniae	14.4	18.0	14.0	15.0	Nt	Nt	Nt
Pseudomonas aeruginosa	11.0	8.0	10.0	12.0	Nt	Nt	Nt
Proteus mirabilis	13.8	13.0	16.0	14.0	Nt	Nt	Nt
Candida albicans	16.2	Nt	Nt	Nt	18.0	22.0	16.0

[a] includes diameter of disc (6 mm); mean value of three independent experiments; Nt: not tested; P: penicillin (10 µg/disc); TOB: tobramycin discs (10 µg/disc); AMP: ampicillin (20 µg/disc); NYS: nystatin discs (30 µg/disc); KETO: ketoconazole (20 µg/disc); CLT: clotrimazole (30 µg/disc)

Table 2: Minimum inhibitory concentration (MIC) of the extracts of *V. thapsus*

Microorganisms	MIC (MBC or MFC)			
	Extract (µg/mL)	Standards		
		ST	AMP	NYS
Enterococcus faecalis	64 (128)	2.0 (4.0)	1.0 (4.0)	Nt
Escherichia coli	32 (64)	4.0 (4.0)	32 (64)	Nt
Klebsiella pneumoniae	500 (1000)	8.0 (16.0)	8.0 (8.0)	Nt
Pseudomonas aeruginosa	500 (1000)	1.0 (1.0)	16 (32)	Nt
Proteus mirabilis	1000 (1000)	4.0 (8.0)	0.5 (1.0)	Nt
Candida albicans	64 (128)	Nt	Nt	8.0 (16)

Nt: not tested; ST: Streptomycin, AMP: Ampicillin, NYS: Nystatin

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