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Antimicrobial activities of some *Verbascum* L. species against clinical isolates of *Klebsiella pneumoniae* causing lower respiratory tract infections

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ABSTRACT: Increasing antibiotic resistance in *Klebsiella pneumoniae* has necessitated the development of novel antimicrobial therapies. In recent years, many studies have been conducted on using plants as antimicrobial agents, and many natural compounds have been found to be effective sources of antibacterial agents. In the present study, the antimicrobial activity of *K. pneumoniae* strains isolated from patients with lower respiratory tract infections (LRTIs) was investigated against some *Verbascum* L. species. Five *K. pneumoniae* strains, isolated from bronchoalveolar lavage (BAL) samples of patients with LRTIs, were identified by VITEK MS MALDI-TOF (BioMérieux, France). Then, the antimicrobial activity of 85% ethanol and 2% aqueous extracts of nine *Verbascum* species (*V. ancyritanum* Bornm., *V. cheiranthifolium* Boiss., *V. georgicum* Benth., *V. kastamunicum* Murb., *V. lasianthum* Boiss. ex Benth., *V. mucronatum* Lam., *V. sinuatum* L. subsp. *sinuatum* var. *adenosepalum* Murb., *V. speciosum* Schrad., *V. uschakense* Hub.-Mor.), three of which are endemic to Turkish flora, was investigated by using the microdilution method. Both 85% ethanol and 2% aqueous extracts of *Verbascum* species showed MIC values between 128 - 256 µg/mL and MBC in the range of 256 - 1024 µg/mL. The endemic species, *V. ancyritanum* presented the most effective antimicrobial activity. In the present study, the antimicrobial activity of endemic species, *V. ancyritanum, V. kastamunicum*, and *V. uschakense*, was investigated for the first time. All extracts were found to have moderate antimicrobial activity against the studied bacteria.

KEYWORDS: Lower respiratory tract infections; *Verbascum* L.; *Klebsiella pneumoniae;* antimicrobial activity.

1. INTRODUCTION

Lower respiratory tract infections (LRTIs) are among the most prevalent infectious diseases affecting individuals all over the world, with a substantial morbidity and mortality rate impacting people of all ages [1]. LRTIs account for 4.4% of admissions to hospitals and 6% of all physician consultations [2]. The most frequent LRTIs are pneumonia, acute trachea bronchitis, acute bronchitis, and chronic bronchitis [3]. The most common bacterial agents of LRTIs are bacteria such as *Pseudomonas* spp., *Acinetobacter* spp., *Klebsiella pneumoniae, Streptococcus pneumoniae*, and *Haemophilus influenzae* [3-5]. The incidence of LRTIs, common etiologic agents, and antimicrobial resistance patterns vary both geographically and over time [6, 7].

Klebsiella pneumoniae is the second most common Gram (-) bacterium in hospitals, accounting for 10% of all hospital-acquired infections, and is a significant contributor to hospital-acquired pneumonia in recent years [8]. Additionally, although *K. pneumoniae* is a significant pathogen responsible for LRTIs in the elderly population [9], there is insufficient research on its role in LRTIs [10, 11]. Over the past two decades, multiple drug resistance (MDR) and an increasing antibiotic resistance profile have complicated the treatment of LRTIs caused by *K. pneumoniae* [12-14]. The World Health Organization (WHO) has identified *K. pneumoniae* as a highly important species and is encouraging the investigation and development of novel antibiotics in response to the increasing problem of antimicrobial resistance worldwide [15]. Thus, further research is

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required to determine the optimal antimicrobial agents and combination regimens for treating LRTIs caused by bacteria such as *K. pneumoniae*.

The genus *Verbascum* L., also known as"mullein," belongs to the Scrophulariaceae family and is widely distributed worldwide, comprising approximately 360 species. There are 256 species in the Turkish flora belonging to this taxon, 201 of which are endemic [16, 17]. In traditional medicine, *Verbascum* species play an important role in the treatment of various diseases such as lung infections, asthma, tuberculosis, and wound healing [18-20]. As described in traditional medicine, additional research is needed to determine the pharmacological applicability of the data on *Verbascum* sp. [21].

The effectiveness of *Verbascum* species, which are used in traditional medicine, especially for respiratory tract infections due to their mucolytic effect, against the increasing antibiotic resistance is not fully known [22, 23]. The literature contains insufficient data to assess the effectiveness of *Verbascum* species in this regard. This study was intended to investigate the antibacterial activity of nine *Verbascum* species (*V. ancyritanum* Bornm., *V. cheiranthifolium* Boiss., *V. georgicum* Benth., *V. kastamunicum* Murb., *V. lasianthum* Boiss. ex Benth., *V. mucronatum* Lam., *V. sinuatum* L. subsp. *sinuatum* var. *adenosepalum* Murb., V. *speciosum* Schrad., and V. *uschakense* Hub.-Mor.) against *K. pneumoniae* strains isolated from LRTI samples in order to confirm the traditional knowledge of the genus regarding respiratory tract infections. Thus, the aim is to highlight the potential of these *Verbascum* species as new-generation antimicrobial agents in tackling the issue of multidrug resistance in *K. pneumoniae* strains.

2. RESULTS

In the study, samples (n=5) isolated from BAL samples obtained from patients and stored in the hospital culture collection were analyzed at the species level. All isolates were identified as *K. pneumoniae* using the VITEK MS MALDI-TOF.

The antimicrobial susceptibilities of *K. pneumoniae* strains isolated from individuals with LRTIs were evaluated against the reference drug ciprofloxacin. Two of the bacterial strains (KP4 and KP5) were resistant to ciprofloxacin, while the others were sensitive.

The antimicrobial and bactericidal properties of 85% ethanol and 2% aqueous extracts of nine *Verbascum* species on clinical *K. pneumoniae* strains were investigated. MIC and MBC results are presented in Table 1. The 2% aqueous extracts of *Verbascum* species demonstrated MIC values between 128 - 256 µg/mL, and MBC values between 512 - 1024 µg/mL. Similarly, the MBC findings ranged from 256 to 1024 µg/mL, while the MIC results for the 85% ethanol extracts were between 128 and 256 µg/mL. All extracts were found to have a moderate antimicrobial activity on the studied bacteria. The 2% aqueous extracts *of V. ancyritanum, V. kastamunicum* and *V. uschakense* exhibited the lowest MIC value on the KP1 strain (128 µg/mL), while 85% ethanol extracts of *V. ancyritanum* and *V. cheiranthifolium* showed the highest antimicrobial activity against the ciprofloxacin-resistant strain KP4 (MIC 128 µg/mL, MBC 256 µg/mL). Overall, among the plant extracts tested, *V. ancyritanum* exhibited the highest antimicrobial activity compared to the other species.

3. DISCUSSION

Lower respiratory tract infections are prevalent bacterial infections that result in significant utilization of antibiotics and hospitalization [28]. *K. pneumoniae*, a member of the *Enterobacteriaceae* family, is a frequent cause of LRTIs and is commonly isolated as a hospital-acquired pathogen [29]. Although *K. pneumoniae* is an important pathogen in LRTIs, research on it as a causative agent of LRTIs is limited. Therefore, our study was conducted with *K. pneumoniae* strains isolated from BAL samples collected from hospitalized patients.

In the present study, the antimicrobial activity of nine *Verbascum* species, including three endemic species collected from various regions of Türkiye, was investigated against clinical *K. pneumoniae* isolates. *V. ancyritanum* was determined to be the most effective species on the *K. pneumoniae* strains due to the MIC values of both 85% ethanol and 2% aqueous extracts. The MIC values (128 µg/mL) of 2% aqueous extracts of the three species (*V. ancyritanum, V. kastamunicum,* and *V. uschakense*) used in the study were found to be more effective than 85% ethanol extracts. The antimicrobial activity of the endemic species *V. ancyritanum, V. kastamonicum*, and *V. uschakense* was examined for the first time in this study. In addition, the antimicrobial activity potential of 2% aqueous extracts of endemic *Verbascum* species against *K. pneumoniae* was in parallel with their use in traditional medicine [18].

Table 1. Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of *Verbascum* extracts against *K. pneumonia* Strains

Plant Species	Solvent	*Klebsiella pneumoniae* Strains											
		KP1		KP2		KP3		KP4		KP5		*K. pneumoniae* ATCC 13883	
		MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL	MIC µg/mL	MBC µg/mL
V. ancyritanum	85% EtOH*	256	512	256	1024	256	1024	**128**	256	256	512	256	512
	2% Aq**	**128**	512	256	1024	256	1024	256	1024	256	512	256	512
V. cheiranthifolium	85% EtOH	256	512	256	1024	256	1024	**128**	256	256	1024	256	512
	2% Aq	256	512	256	1024	256	1024	256	1024	256	512	256	512
V. georgicum	85% EtOH	256	512	256	1024	256	1024	256	512	256	1024	256	512
	2% Aq	256	512	256	1024	256	1024	256	512	256	512	256	512
V. kastamunicum	85% EtOH	256	512	256	1024	256	1024	256	512	256	512	256	512
	2% Aq	**128**	512	256	1024	256	1024	256	1024	256	512	256	512
V. lasianthum	85% EtOH	256	512	256	1024	256	1024	256	512	256	1024	256	512
	2% Aq	256	512	256	1024	256	1024	256	512	256	512	256	512
V. mucronatum	85% EtOH	256	1024	256	1024	256	1024	256	512	256	1024	256	512
	2% Aq	256	1024	256	1024	256	1024	256	512	256	512	256	512
V. sinuatum	85% EtOH	256	1024	256	1024	256	1024	256	512	256	1024	256	512
	2% Aq	256	1024	256	1024	256	1024	256	512	256	512	256	512
V. speciosum	85% EtOH	256	1024	256	1024	256	1024	256	512	256	1024	256	512
	2% Aq	256	512	256	1024	256	1024	256	512	256	1024	256	512
V. uschakense	85% EtOH	256	1024	256	1024	256	1024	256	512	256	1024	256	512
	2% Aq	**128**	512	256	1024	256	1024	256	1024	256	512	256	512
Ciprofloxacin	-	0,25(S)[a]	0,5	0,06 (S)	0,125	0,06(S)	0,125	4 (R)[b]	8	16 (R)	32	0,06	0,125

* 85% EtOH: 85% Ethanol Extract, ** 2% Aq: 2% Aqueous Extract
[a] S: Sensitive, [b] R: Resistance

The MIC value for both the 2% aqueous extracts and 85% ethanol extracts of *V. georgicum, V. lasianthum, V. mucronatum, V. sinuatum,* and *V. speciosum* was determined to be 256 µg/mL. Previous studies in the literature reported that antimicrobial activity was determined mostly by the disk diffusion method. In a study with *V. georgicum* Bentham methanol extract (300 mg/disc), no activity on *K. pneumoniae* was reported [30]. The ethanolic extract of *V. mucronatum* was revealed to have strong antimicrobial activity against some Gram (-) bacteria by Hacioğlu et al. [31]. Senatore et al. [32] determined that the methanol extract of *V. sinuatum* showed inhibition against *K. pneumoniae* ATCC 27736 (256 µg/mL). In another report, the ethanol extract of *V. sinuatum* showed a similar MIC value (250 µg/mL) against *K. pneumoniae* [33]. The mucoid structure and cell wall properties of clinical strains of *K. pneumoniae* used in the current study may have contributed to their ability to withstand *Verbascum* extracts. Due to the lipopolysaccharide in their cell walls, Gram (-) bacteria tend to be more resistant to plant extracts [33].

We determined the MIC value of the 85% ethanol extract of *V. cheiranthifolium* against the ciprofloxacin-resistant KP4 strain was determined to be 128 µg/mL. Birgül et al. examined the antimicrobial activities of hexane, chloroform, and methanol extracts of *V. cheiranthifolium* var. *asperum* using the disc diffusion method. Only the methanol extract induced an inhibition zone (8.7 mm) against *K. pneumoniae* ATCC 13883, while the other extracts had no activity [34].

4. CONCLUSION

Today, new-generation antimicrobial therapies are required because of the developing antibiotic resistance against *K. pneumoniae*. Recent research shows that various plants serve as powerful antimicrobial agents, with numerous natural chemicals identified as potent antibacterial sources.

To conclude, the antimicrobial activity of *K. pneumoniae* strains isolated from BAL samples of patients with LRTIs was investigated against nine *Verbascum* species collected from Türkiye. Our results suggest that *Verbascum* species examined in our research demonstrated potential antibacteriostatic activity against *K. pneumoniae*. *V. ancyritanum* presented the most effective antimicrobial activity. However, further studies are required to clarify *Verbascum* species' potential in treating LRTI-associated *K. pneumoniae* infections.

5. MATERIALS AND METHODS

5.1. Plant materials

Plant materials were collected from different regions of Türkiye during the flowering time. *V. ancyritanum* and *V. kastamunicum* were identified by Prof. Dr. Hayri Duman, Gazi University, Faculty of Science, Department of Botany, Ankara, Türkiye. Assoc. Prof. M. Ufuk Özbek identified the rest of the plant samples. The voucher specimens were deposited in the Herbarium of Hacettepe University, Faculty of Pharmacy, Ankara, Türkiye (Table 2).

Table 2. Collection provinces and herbarium numbers of the plant taxa

Taxa	Collection province and date	Herbarium number
V. ancyritanum Bornm.	B4 Ankara, 18.05.2018	HUEF18390
V. cheiranthifolium Boiss.	B7 Sivas, 31.07.2020	HUEF20017
V. georgicum Benth.	B3 Afyonkarahisar, 09.06.2020	HUEF20020
V. kastamunicum Murb.	B3 Afyonkarahisar, 02.06.2018	HUEF18388
V. lasianthum Boiss. ex Benth.	B3 Eskişehir, 08.07.2020	HUEF20024
V. mucronatum Lam.	B3 Afyonkarahisar, 08.07.2020	HUEF20021
V. sinuatum L. subsp. *sinuatum* var. *adenosepalum* Murb.	B8 Diyarbakır, 20.06.2020	HUEF20025
V. speciosum Schrad.	B4 Kırıkkale, 25.07.2020	HUEF20022
V. uschakense Hub.-Mor.	B3 Afyonkarahisar, 09.06.2020	HUEF20019

5.2. Preparation of plant materials

The air-dried and powdered aerial parts (5 g) of the plants were extracted with 85% ethanol (2 x 50 mL) in a water bath at 40 °C using a rotary extractor without vacuum. The extraction solvent was evaporated, and the combined extracts were lyophilized. The crude extracts were kept at -20 °C until use.

2 g of each air-dried and powdered plant material was extracted with 100 mL of distilled water, which was boiled and cooled to 80 °C with a magnetic stirrer to prepare 2% aqueous extracts. Following the filtration and evaporation of the water, the crude extracts were lyophilized and stored at -20 °C until use.

Verbascum sp. extract was dissolved individually in dimethyl sulfoxide (DMSO, Sigma, USA) and then filter-sterilized through 0.45 µm syringe filter (Millipore, UK) before microbiological tests.

5.3. Bacterial isolates and identification

Randomized five (n=5) *K. pneumoniae* clinical isolates examined in this study were obtained from the Microbiology Culture Collection of the Gazi University Faculty of Medicine Department of Medical Microbiology, Ankara, Türkiye. These bacterial strains were isolated from bronchoalveolar lavage (BAL) samples collected from patients suffering from lower respiratory tract infections. The reference strain *K. pneumoniae* ATCC 13883 was also included in the study. Two of the bacterial isolates were resistant to ciprofloxacin, while the others were sensitive.

Isolates were identified using the VITEK MS MALDI-TOF (BioMérieux, France) system. The isolates removed from the deep freezer were cultured twice in 5% sheep blood agar (Becton Dickinson, Germany) and incubated for 24 hours at 37°C in an aerobic environment. A small amount of bacteria was obtained from the isolated colonies using a disposable 1 µL loop and inoculated as a thin layer onto the designated areas of single-use target slides. Following inoculation, 1 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution was added, and the bacteria were allowed to air dry. The reference strain *Escherichia coli* ATCC 8739 was used as a control. The prepared slides were run through the VITEK MS device, and the MYLA software v3.2 (BioMérieux, France) was used to automatically evaluate the acquired spectra. The results were evaluated and identified by comparison with bacteria in the device library [24, 25]. Identified strains were stored at −80 °C in brain heart infusion broth (BHI) with 15% v/v glycerol.

5.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility assays were performed according to CLSI M7-A10 (The Clinical and Laboratory Standards Institute, 2018) standard [26]. To determine the minimum inhibitory concentration

(MIC) values, 1024-2 µg/mL concentrations of 85% ethanol and 2% aqueous extracts were tested. One hundred microliters (100 µL) of dissolved plant extract and ciprofloxacin (32–0.06 µg/mL) (J&K Scientific, China), used as a control, were serially diluted with Cationic Muller Hinton Broth (CAMHB, Merck, Germany) in a microplate. Strains were subcultured in freshly prepared BHI agar and incubated for 24 hours at 37°C before use in experiments. Strains were adjusted to 0.5 McFarland turbidity standard and then diluted 1:10 in saline. A volume of 5 µL from the bacterial suspension was inoculated into wells, resulting in a final concentration of 5 x 10^5 CFU/mL. The final volume in each well was 100 µl. The microplates were incubated 24 hours at 37°C and then the MIC of each sample was determined. The MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after 24 hours incubation.

In order to determine the minimum bactericidal concentration (MBC) values, 10 µL were subcultured from non-turbid wells, and then spot inoculated onto Muller Hinton Agar (MHA, Merck, Germany). After the plates were incubated at 37 °C for 24 hours, the growth of the cultures was recorded. The lowest concentration of extracts required to kill the bacterial population completely was defined as MBC [27]. All experiments were repeated twice.

5.5. Ethical considerations

Before starting the study, ethical approval from Gazi University Gazi University Non-Interventional Clinical Research Ethics Committee (Decision No: 112, Date: 06/02/2023) was obtained.

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