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Inhibitory Effect of *Ranunculus kotschyi* Boiss. Extract on Multidrug Resistant *Acinetobacter baumanii* and Other Pathogenic Bacteria

Yalçın KARAGÖZ[1*], Kenan KARAGÖZ[2]

Highlights:

- *Ranunculus kotschyi* was identified using molecular techniques
- Extract fractions of *Ranunculus kotschyi* were found to be effective against pathogenic bacteria including multidrug resistant *Acinetobacter baumanii*
- The active fraction was analysed in LC-QTOF-MS system and syringic acid was found to be the main component

ABSTRACT:

Emergence of multi-drug-resistant bacteria poses an imminent and clear threat to human health. *Acinetobacter baumanii* is such an organism, which may cause up to 40% mortality due to bacteremia. *Ranunculus kotschyi*, a widespread herb, is utilized in Türkiye for treatment of rheumatism, leg pain and bruises in folk medicine, and also consumed as food. Molecular identification of *Ranunculus kotschyi* was performed using ITS1 partial sequence, 5.8S and ITS2 partial sequence. Phylogenetic analyses of the plant were conducted. The plant sample was extracted and fractioned using activity guided fractionation to yield an active fraction (RK4A). RK4A was analysed using LC-QTOF-MS and the presence of syringic acid in RK4A was revealed. The antimicrobial effects of RK4A and syringic acid against multi-drug-resistant *Acinetobacter baumanii*, as well as other pathogens, namely *Klebsiella pneumonia*, *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*, were screened in broth media. Minimum inhibitory concentration (MIC) values were determined using microdilution method. Our results revealed that RK4A and syringic inhibited growth of all tested bacteria in broth. The MIC values of RK4A against *Acinetobacter baumanii*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Enterococcus faecalis* were 500, 62.5, 31.25, 125 and 15.525 µg/ml, respectively. The MIC values of syringic acid against *Acinetobacter baumanii*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Enterococcus faecalis* were 1000, 62.5, 62.5, 125 and 31.25 µg/ml, respectively. Our results suggest that *Ranunculus kotschyi* and syringic acid may provide alternatives in the treatment of infections caused by *A. baumanii* and other multidrug-resistant bacteria. Further research is needed in order to discover action mechanisms of the reported antimicrobial effects and enhance the observed effects of RK4A and syringic acid.

Keywords:

- *Ranunculus kotschyi*
- Syringic acid
- Antimicrobial effect
- Multidrug-resistant bacteria
- *Acinetobacter baumanii*

[1*]Yalçın KARAGÖZ (**Orcid ID**: 0000-0002-4835-4508) Agri Ibrahim Cecen University Faculty of Pharmacy Department of Pharmaceutical Botany, Agri, Türkiye

[2] Kenan KARAGÖZ (**Orcid ID**: 0000-0003-1908-5836), Agri Ibrahim Cecen University Faculty of Science and Letters, Department of Molecular Biology and Genetics, Agri, Türkiye

***Corresponding Author:** Yalçın KARAGÖZ, e-mail: ykaragoz@agri.edu.tr

INTRODUCTION

The uncontrolled use of antibiotics in the latter half of the 20th century led to the development of antibiotic-resistant bacteria. Despite efforts to create new antibiotics to prevent resistance, bacteria continued to evolve and develop additional defence mechanisms against every new antibiotic. As a result, the emergence of "super bacteria" that can withstand multiple classes of antibiotics has become a serious threat to humanity (WHO, 2017). Meanwhile, the major pharmaceutical companies' contribution to antibiotic development is decreasing annually, with some closing their antibiotic development departments entirely (Spellberg et al., 2004). Given the current circumstances, smaller organizations and research teams must take on the task of exploring and discovering novel forms of antibiotics.

Acinetobacter baumanii (*A. baumanii*) is a Gram-negative bacterium that can cause hospital-acquired infections (Durante-Mangoni & Zarrilli, 2011; Nowak & Paluchowska, 2016). It is an important opportunistic pathogen that can colonize many parts of the body and occur on body parts as a transient or a regular flora member (Neethu et al., 2018). *A. baumanii* can cause many types of infections like bacteremia, pneumonia, meningitis, and wound infections (Sebeny et al., 2008). The mortality rate can be up to 40% in bacteremia due to *A. baumanii* (Esterly et al., 2011). It is also one of the most significant multi-drug-resistant bacteria (Manchanda et al., 2010). The pathogen was considered one of the focus organisms implicated in antimicrobial resistance by the World Health Organisation (WHO) (Kaka & Senbadejo, 2020; WHO, 2017). *A. baumanii* is capable of thriving in different low-nutrient environments. Furthermore, it has developed resistance against several antibiotics, such as beta-lactams, lincosamides, glycopeptides, and macrolides (Nowak & Paluchowska, 2016). It can use various mechanisms like the production of beta-lactamase and other antibiotic-modifying enzymes, efflux pump, and target site modifications of antibiotics (Lee et al., 2017).

Plants are a remarkable resource for discovering novel active compounds (Asadi-Samani et al., 2017; Kalantari et al., 2007). Due to their richness in bioactive content, fewer side effects and less toxicity to tissues, plants have always been of interest to researchers, and discovering new active compounds from plants has always been a fundamental strategy for treating infectious diseases (LisBalchin & Deans, 1997).

Ranunculaceae plants are likewise promising in terms of bioactive substances. Ranunculaceae is a big family. It is known to include around 2500 species (Fostok et al., 2009). There are 94 taxa belonging to this family in Türkiye, and 19 of them are endemic (Davis et al., 1988). Fresh Ranunculaceae plants are poisonous, but heat or drying removes the poison (Terzioglu et al., 2008). Previous studies reported some *Ranunculus* species to possess antimicrobial activity (Atcı & Karagöz, 2018; Bazzaz & Haririzadeh, 2003; Bhatti et al., 2015; Rasool et al., 2014; Terzioglu et al., 2008).

As far as we know, however, there is no record of the antimicrobial activity of *Ranunculus kotschyi*, the annual wild plant. The plant can cause dermatitis in some cases (Kadı et al., 2021; Omer et al., 2011) and is used in the treatment of rheumatism, leg pain and bruises in folk medicine (Karakaya et al., 2019). *Ranunculus kotschyi* is also consumed as food in Türkiye (Kaval et al., 2015).

This study aimed to investigate the inhibitory effects of *Ranunculus kotschyi* extracts on multidrug-resistant *Acinetobacter baumanii* along with other important pathogens, namely *Klebsiella pneumonia*, *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*.

MATERIALS AND METHODS

Plant Material

Ranunculus kotschyi samples were collected from Ağrı province Eleşkirt district Aydıntepe village (39 ° 49'57.4 "N 42 ° 25'13.6" E) in May 2020. Two individual samples were put aside for identification and the herbarium. The remaining samples were cut into small pieces and used for extraction. The plant was identified according to Davis et al (1975, 1988). A voucher specimen is kept in the personal collection of YK with accession number YK-2020-01.

Solvents and Media

All solvents used in our study were of analytical purity (> 99%) and purchased from TEKKİM (Istanbul, Türkiye). Complex media and other chemicals were purchased from the Merck.

Bacteria

Multidrug resistant *Acinetobacter baumanii* was isolated from soil. The strain was identified by 16S rDNA sequence analysis and deposited to GenBank with OP115876 accession number. The strain was fully resistant to tetracycline, kanamycin, penicillin G and cefotaxime additionally resistant to meropenem according to CLSI recommendation. *Klebsiella pneumonia* ATCC 700603, *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were also used for antimicrobial susceptibility tests.

Molecular Identification of Plant

ITS region was used for molecular identification. The ITS region (ITS1 partial sequence, 5.8S and ITS2 partial sequence) was amplified by 17SE and 26SE primers (Sun et al., 1994). Genomic DNA was extracted from 30 mg dried plant tissue by DNeasy Plant Mini Kit (Qiagen, Germany). PCR reactions were made according to previously established procedures (Cires et al., 2012). Briefly; 50 µL of reaction mixture, containing 50 ng of template DNA, 5 µL of 10X reaction buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 1 U Taq DNA polymerase (Sigma) and 0.08 µM each of primers, was run in thermocycler. DNA was amplified following conditions; initial denaturing at 94°C for 5 min, 40 cycles of denaturing at 94°C for 1 min, 56°C for 1 min and extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. After the amplification, PCR products were run in 1,5 % agarose gel to check amplification and quality. Then products sequenced by BM Labosis (Ankara / Turkey). Assembling and editing of sequence data were performed via BioEdit program version 7.2. Then the sequence data were compared with the relevant data in the NBCI database (National Centre for Biotechnology Information) using the blast algorithm.

Phylogenetic Analysis

The ITS region was used for phylogenetic analysis. The analysis was performed with *Ranunculus kotschyi* (RK) and some other *Ranunculus* spp. *Berberis montana* and *Berberis polyodonta* were used as out group. Sequence data of out group and the other *Ranunculus* spp. were obtained from GenBank (NBCI). All the sequence data were aligned using Clustal W algorithm. Then Phylogenetic analysis were performed. The evolutionary history was deduced using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the

units of the number of base substitutions per site. This analysis involved 60 nucleotide sequences. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Extraction and Fractionation

Fresh *Ranunculus kotschyi* sample (leaves and flowers, 300 g) was extracted with boiling water in a reflux cooling system for 12 hours with the occasional water addition. The extract was filtered through a Buchner funnel (porosity 3) and evaporated in a rotary evaporator under reduced pressure to give a dark green-brown paste. The resulting paste was extracted with methanol and filtered. The methanol-soluble filtrate was labelled as RKM and the methanol-insoluble residue as RKW. The RKM was rotary evaporator dried and dissolved in minimal methanol. This solution was treated with excess acetone, which formed a brown precipitate. This precipitate was labelled as RKMP. The acetone-rich solution obtained after filtration, labelled as RKMA, was treated with excess ethyl acetate to give a yellow-brown oily phase, which was labelled RKO. The filtrate was dried and marked as RK2A. RK2A was dissolved in minimal acetone, and a few drops of ethyl acetate were added. The precipitated part was marked as RK3A, the solution as RK3E. After repeating the process once more, the resulting residue was labelled RK4A and the solution as RK4E. After each separation, obtained fractions underwent antimicrobial activity tests, and the active part the subsequent separation process. Figure 1 presents the general summary of the study design and antimicrobial activity test results.

Antimicrobial Susceptibility Tests

Broth test

Antimicrobial effects of the extract and antibiotics were directly tested in broth culture. The extract and antibiotics including penicillin G, tetracycline, kanamycin, cefotaxime and meropenem were dissolved in 25% DMSO and inoculated to Mueller Hinton broth (Merck 110293) medium at the concentration of 2 mg/ml. Bacteria were presently inoculated to broth medium and zero-point bacteria number were counted (at ~10^5 CFU /ml concentration) by serial dilutions. Then tubes incubated at 37 °C for 16 h. After the incubation alive bacteria number were counted via serial dilutions in three replicates and recorded as average. Non-inoculated medium, sterile distilled water and 25% DMSO (final concentration %1,25) were used as control. After effective compound determination, syringic acid was also tested in same way.

MIC test

Microdilution method (Sahin et al., 2003) was used for determination the MIC (Minimal inhibitory concentration) value of the components effective against to multi drug resistant *A. baumanii*. Consecutive wells containing extract, syringic acid and meropenem at the concentration range of 7,8 to 1000 µg/ml was prepared in Mueller Hinton broth. Then, bacterial suspensions at the final concentration of ~10^5 CFU / ml were added to each well and total volume reached 200 µl. The plate was covered with a sterile plate sealer. The contents of each well were mixed on a microtiter plate shaker at 150 rpm for 20 s then incubated at 37 °C for 16 h. %25 DMSO (final concentration %1,25), sterile distilled water and non-inoculated medium were used as control All tests were repeated three times. MIC was defined as the lowest concentration of the extract or antibiotics to inhibit the growth of microorganisms.

LC-QTOF-MS Analysis

The active fractions (RK4A and RK4E) were further analysed with Agilent 6530 Accurate-Mass Q-TOF LC/MS Mass spectrometer equipped with ZORBAX Eclipse Plus C-18 (2.1x50mm, 1.8 Micron)

column. Conditions were Solvent A 100% methanol, Solvent B 0.1% trifluoro acid (TFA) in water; flow rate 1 ml/min. Gradient was 30% A %70 B for 2 minutes, 40% A 60% B for 5 minutes, 50% A 50% B for 5 minutes, 60% A 40% B for 5 minutes and 70% A 30% B for 15 minutes. Column temperature was 40 °C. Samples were analysed with both positive and negative ionisations.

RESULTS AND DISCUSSION

Extraction and fractionation

Figure 1 summarizes extraction and fractionation results

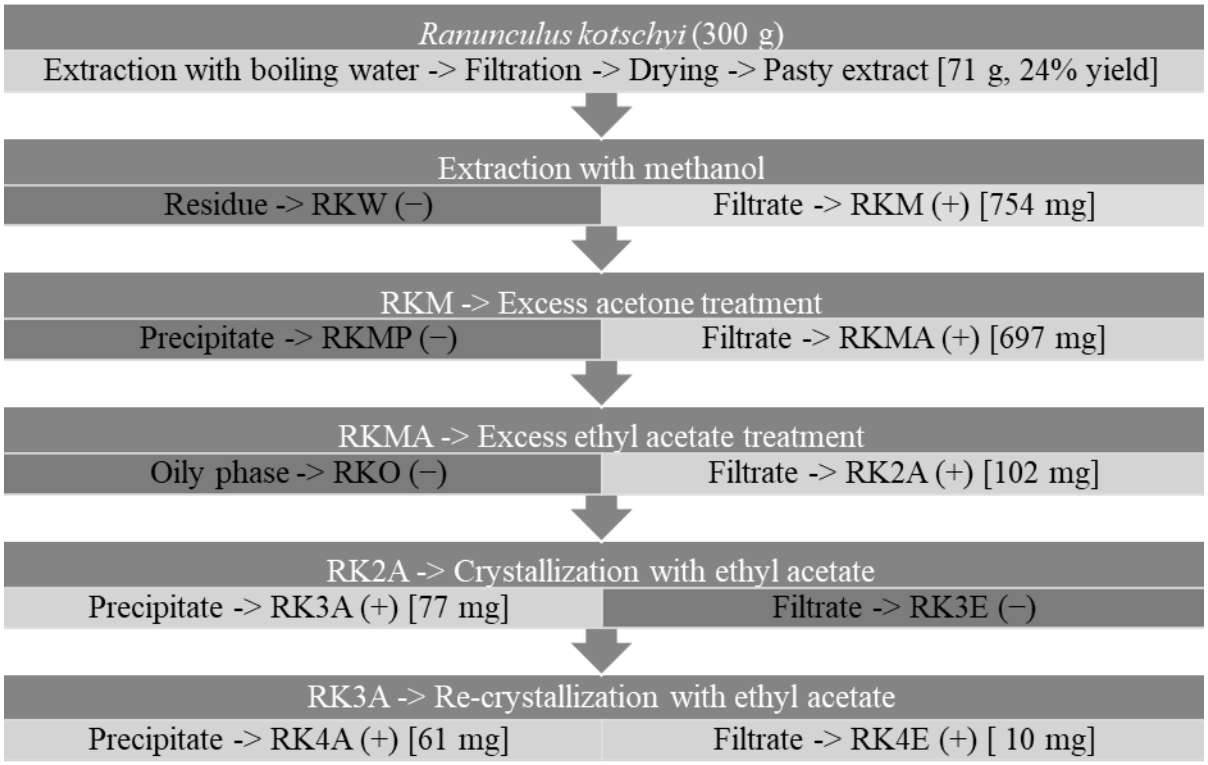

Figure 1. General study design, extraction and fractionation and antimicrobial test results. (−): antimicrobial activity absent, (+) antimicrobial activity present

Molecular Identification of Plant

In length of 596 bp ITS region (ITS1 partial sequence, 5.8S and ITS2 partial sequence) sequence data of RK were analysed with NCBI blast algorithm. According to the result our sequence data showed 98.83% similarity with *Ranunculus kotschyi.* Sequence data were deposited to GenBank with MW989738 accession number.

Phylogenetic Analysis

Totally 60 sequence data belong to genus *Ranunculus*, were analysed. According to phylogenetic analyses our sample was located on the same branch with two other *Ranunculus kotschyi*. Phylogenetic three were presented in Figure 2. Accession numbers of the sequences were given in brackets.

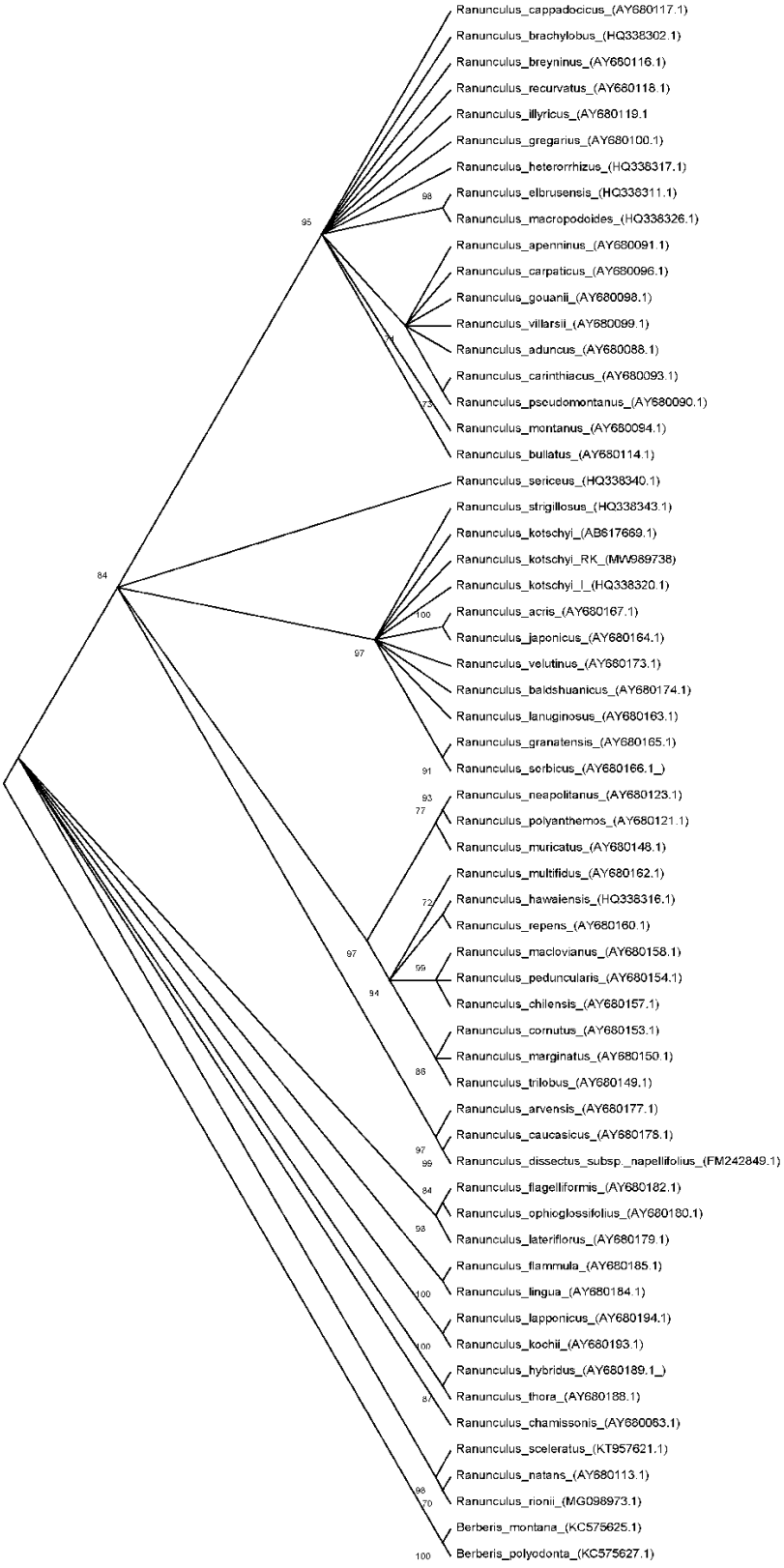

Figure 2. Phylogenetic tree of the Ranunculus species

LC-QTOF-MS Analysis

LC-QTOF-MS results are presented in Table 3. RK4A and RK4E both contained a major compound with m/z 197.04549 in negative ionisation mode. It was the most abundant compound in both fractions. The instrument library gave a putative formula of $C_9H_{10}O_5$ for this compound. This corresponds to a widespread plant metabolite syringic acid, which was purchased and utilized for antimicrobial susceptibility tests. Positive ionisation mode gave no significant results (i.e. database score [similarity per cent] over 85) for both fractions.

Table 3. LC/MS analysis results. (+): present, (—): absent

Retention time (min)	m/z	Ion	Suggested Formula	RK4A	RK4E
1.43	197.04549	(M-H)-	$C_9H_{10}O_5$	+	+
1.82	311.07754	(M+CH3COO)-	$C_{12}H_{12}O_6$	+	—
1.88	507.11638	(M-H)-	$C_{23}H_{24}O_{13}$	—	+
3.55	115.00374	(M-H)-	$C_4H_4O_4$	+	+

Antimicrobial Susceptibility Tests

According to broth test results; in the presence of kanamycin, ceftriaxone and meropenem at 2 mg/ml concentrations, *E. coli*, *K. pneumoniae*, *S. aureus* and *E. faecalis* strains completely lost their viability. While *E. coli*, *S. aureus* and *E. faecalis* completely lost their viability, *K. pneumoniae* partially survived in the presence of tetracycline at the same concentration. Penicillin G couldn't inhibit growth of *E. coli* and *K. pneumoniae* strains. Additionally, *S. aureus* and *E. faecalis* partially survived in the presence of Penicillin G.

Growth of multidrug resistant *A. baumani* was not inhibited by common antibiotics except meropenem. In the presence of the other antibiotics *A. baumani* growth reached substantial amounts. Extract of *Ranunculus kotschyi* and syringic acid also fully inhibited growth of the bacteria. Additionally, all the bacteria including multi drug resistant *A. baumanii* completely lost their viability in the presence of extract and syringic acid at 2 mg/ml concentrations. Results were summarized in Table 1.

Table 1. Antimicrobial effect of the antibiotics, *Ranunculus kotschyi* extract and syringic acid on bacteria

		Penicillin G	**Tetracycline**	**Kanamycin**	**Ceftriaxone**	**Meropenem**	**RK4A**	**Syringic acid**	**Control**	**DMSO**
	Zero point	**16 Hours Later**								
A. baumanii	1.40E+05	1.55E+08	5.60E+07	3.25E+07	2.00E+06	NVB	NBV	NBV	4.6E+09	4.25E+09
E. coli	5.40E+05	3.40E+07	NVB	NVB	NVB	NVB	NVB	NVB	4.8E+09	3.90E+09
K. pneumoniae	4.60E+05	1.40E+08	2.15E+03	NVB	NVB	NVB	NVB	NVB	1.15E+09	1.05E+09
S. aureus	3.20E+05	5.00E+04	NVB	NVB	NVB	NVB	NVB	NVB	7.50E+08	5.10E+08
E. faecalis	4.00E+05	5.50E+04	NVB	NVB	NVB	NVB	NVB	NVB	2.8E+09	2.33E+09

NVB: no visible colony in the 10^0 dilution

According to MIC test results; meropenem inhibited tested bacteria in the range of < 7.8 to 62.50 µg/ml concentrations. While RK4A inhibited the microbial growth in the range of 15.625 to 500 µg/ml concentrations, syringic acid inhibited growth of the microorganisms in the range of 31.25 to 1000 µg/ml

concentrations. DMSO did not affect microbial growth and no growth was observed in sterile control. Results were summarized in Table 2.

Table 2. MIC (µg/ml) value of the meropenem, *Ranunculus kotschyi* extract and syringic acid

	Meropenem	RK4A	Syringic acid
A. baumanii	62.50	500	1000
E. coli	< 7.80	62.50	62.50
K. pneumoniae	< 7.80	31.25	62.50
S. aureus	15.625	125	125
E. faecalis	< 7.80	15.525	31.25

Resistance to antibiotics is one of the most important health problems. Microorganisms are constantly developing resistance to different antibiotics owing to their adaptation skills and resistance mechanisms. Horizontal gene transfer also facilitates the rapid spread of this resistance among bacteria. Because of this, multi-drug-resistant bacteria emerge. Researchers are constantly working to discover or develop new antimicrobial compounds to deal with this problem.

Plants are one of the most important sources for the detection of new antimicrobial compounds. Much interest is focused on this subject (Chen et al., 2022; Maric et al., 2023; Vijay et al., 2023). Members of the genus *Ranunculus* are also a source for the development of new antimicrobial components. The antimicrobial activities of Ranunculus members have been demonstrated in different studies. In a former study, researchers tested the effects of *R. constantinapolitanus* and *R. arvensis* essential oils against various bacteria. The MIC of *R. constantinapolitanus* essential oil was 10 µg/ml for *S. aureus* and *E. faecalis*, and MIC of *R. arvensis* was 8 µg/ml for the same bacteria (Terzioglu et al., 2008). The same study also revealed that essential oils did not inhibit *E. coli* and *K. pneumoniae* at 300 and 400 µg/ml concentrations. In another study, *R. sericeus* extracts were tested against various bacteria. Methanol extract MICs were recorded as 1250 and 625 µg/ml and acetone extract MICs were recorded as 1250 and 312.5 µg/ml for *S. aureus* and *E. faecalis*, respectively (Atcı & Karagöz, 2018). In the present study, lower MIC values were obtained from *Ranunculus kotschyi* RK4A extract and syringic acid against *S. aureus* and *E. faecalis*. Additionally, *E. coli* and *K. pneumoniae* were inhibited by both RK4A extract and syringic acid in the range of 31.25 to 62.50 µg/ml concentrations.

A. baumanii is a problematic pathogen because of its multi-drug-resistant strains. As stated earlier, it is on the WHO list of dangerous pathogens and WHO encourages the development of new antimicrobial components against this pathogen. In a former study MICs of carbapenems against standard and carbapenem-resistant *A. baumanii* were determined. MIC of meropenem for standard strain ATCC27853 was recorded as 0,25 µg/ml and 64 µg/ml for carbapenem-resistant *A. baumanii* strains (Ju et al., 2022). These findings reveal the dimensions of the danger. Moreover, considering that these strains can have multi-drug resistance, it will be realized that the danger is growing. Therefore; the discovery and development of new antimicrobial compounds play a key role in the treatment of infectious diseases.

Bagheri et al. (2019) tested *Bunium persicum* and *Rheum ribes* extracts against standard strain of *A. baumanii* ATCC747 and reported that extracts' MICs were 128 and 256 µg/ml, respectively. In another study, Miyasaki et al. (2013) tested some active substances from *Terminalia chebula* against multidrug resistant *A. baumanii*. MICs of the active components terchebulin, chebulagic acid, chebulinic

acid and corilagin were recorded as 500, 1000, 62.5 and 1000 µg/ml, respectively. In our study, RK4A and syringic acid inhibited the multi drug resistance *A. baumanii* in a hopeful value. MIC of RK4A was recorded as 500 µg/ml and MIC of syringic acid was recorded as 1000 µg/ml. The differences between MIC levels may be due to other ingredients in the crude extract. In a former study syringic acid was tested against methicillin resistant *S. aureus* and syringic acid inhibited the growth of methicillin resistant *S. aureus* (Manuja et al., 2013). In another study, researchers have stated that syringic acid was able to inhibit the growth of opportunistic pathogen *Cronobacter sakazakii* and they revealed that the inhibitory effect was stronger than thymol and eugenol (Frankova et al., 2014). Zaldivar & Ingram (1999) reported that syringic acid inhibited the growth of the *E. coli* LY01 strain.

CONCLUSION

Although there is some research on the antimicrobial effect of syringic acid, we believe more research is needed about this matter. As far as we know, there is no research on the antimicrobial effect of *Ranunculus kotschyi* and the antimicrobial effect of syringic acid against multidrug-resistant *A. baumanii*. This study screened the antimicrobial effect of *Ranunculus kotschyi* extract on different bacteria including multi-drug resistant *A. baumanii*. This study is important in terms of pioneering further research on developing new antimicrobial components against multi-drug-resistant *A. baumanii*. We believe future studies should aim to modify syringic acid or combine it with different compounds and make it more effective. This will offer an alternative in the treatment of infections caused by *A. baumanii* and other multidrug-resistant bacteria.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

YK: Study design; manuscript preparation; collection, identification, ectraction and fractionation of plant material, interpretation of LC-QTOF-MS results. KK: Isolation and identification of *Acinetobacter baumanii*; molecular identification of plant; conduction of all antimicrobial tests, preparation of manuscript.

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