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Evaluation of the antimicrobial activity of *Castanea sativa* against imipenem-resistant non-fermentative bacteria

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ABSTRACT

Aims: Carbapenems, particularly imipenem, are a class of antibiotics commonly used for treating infections caused by antibiotic-resistant gram-negative bacteria. However, the increasing prevalence of imipenem-resistant bacterial strains underscores the necessity of discovering alternative therapeutic options. The current study assessed the ethanol (EtOH) extract of *Castanea sativa (C. sativa)* Mill. flowers for its antimicrobial activity and potential as an adjuvant in antibiotic therapy against imipenem-resistant clinical strains of *Acinetobacter baumannii (A. baumannii)* and *Pseudomonas aeruginosa (P. aeruginosa)*.

Methods: A total of 40 non-duplicate clinical strains of *P. aeruginosa* (n=21) and *A. baumannii* (n=19), belonging to non-fermentative gram-negative bacteria, were included in the study. The antimicrobial activity of the extract and its potential to potentiate imipenem were evaluated using the disk diffusion method.

Results: The inhibition zone diameters for the extract alone ranged from 7 mm to 10 mm for *P. aeruginosa* strains and from 11 mm to 14 mm for *A. baumannii* strains. When combined with imipenem, these diameters increased slightly, ranging from 8 mm to 11 mm and 11 mm to 15 mm for *P. aeruginosa* and *A. baumannii* strains, respectively.

Conclusion: The findings suggest that the EtOH extract of *C. sativa* flowers possesses antimicrobial activity against imipenem-resistant *P. aeruginosa* and *A. baumannii* strains, albeit with a minimal imipenem-potentiating effect. Further studies are required to explore the full potential of this extract in combating resistant bacterial infections.

Keywords: *Acinetobacter baumannii*, *Castanea sativa* Mill., carbepenems, drug potentiation, *Pseudomonas aeruginosa*

INTRODUCTION

The escalating threat of antibiotic-resistant bacteria is one of the most pressing challenges in modern medicine.[1] In particular, multidrug-resistant (MDR) pathogens, such as *Acinetobacter baumannii (A. baumannii)* and *Pseudomonas aeruginosa (P. aeruginosa)*, are of particular concern.[2] *A. baumannii* and *P. aeruginosa* are clinically significant non-fermentative gram-negative bacteria frequently implicated in nosocomial infections. Their high prevalence in healthcare settings is attributed to their remarkable ability to colonize hospital environments, as well as their intrinsic and acquired resistance mechanisms against a broad spectrum of antibiotics, including those routinely employed in clinical settings.[3,4]

Carbapenems, including imipenem and meropenem, are broad-spectrum β-lactam antibiotics used to treat infections caused by antibiotic-resistant gram-negative and gram-positive bacteria.[5] However, the alarming global rise in carbapenem-resistant isolates, including *A. baumannii* and *P. aeruginosa*, underscores the pressing need for alternative antimicrobial strategies.[3,6] In this context, plant-derived natural products have attracted significant interest as potential sources of bioactive compounds with antimicrobial properties.[7]

Castanea sativa (C. sativa) Mill. (sweet chestnut), belonging to the *Fagaceae* family, has been traditionally utilized in ethnomedicine for diverse therapeutic applications, particularly in treating various ailments. Notably, its flowers are commonly used in teas and beverages, attributed to their purported health benefits.[9] Numerous studies have investigated the antimicrobial properties of *C. sativa* extracts against various microorganisms.[10-13] Nevertheless, research specifically evaluating its imipenem-potentiating activity and effectiveness against carbapenem-resistant clinical strains remains relatively limited. Addressing this gap, the present study aimed to evaluate the ethanol (EtOH) extract of *C. sativa* flowers for its antimicrobial activity and potential as an adjuvant in antibiotic therapy against imipenem-resistant clinical strains of *A. baumannii* and *P. aeruginosa*.

METHODS

Ethics

This study did not require ethical approval as it did not involve any human subjects or animal experiments. All procedures were carried out in accordance with the ethical rules and the principles.

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Preparation of Plant Extracts

The flowers of *C. sativa* were collected from the İkizdere district of Rize Province, Turkiye, in July 2021. The extraction was performed using the maceration method.[10] Briefly, 20 g of freshly collected and dried flower parts were finely ground in a mortar, transferred to a flask, and then extracted with 70% EtOH at a ratio of 1:10 (w/v). The mixture was stirred at room temperature using a magnetic stirrer for 24 to 48 hours. Subsequently, the extracts were filtered through filter paper, and the solvent was evaporated under reduced pressure using a rotary evaporator at 40°C. The extract was then dissolved in pure dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and stored at -20°C.

Isolates and Antibiotic Susceptibility Test

Forty carbapenem-resistant *P. aeruginosa* (n=21) and *A. baumannii (n=19)* strains, isolated from various clinical samples submitted to the Microbiology Laboratory of Kastamonu Training and Research Hospital between April 2018 and June 2021, were included in the study. Duplicate strains isolated from the same patient were excluded. The identification of the isolates was carried out using the VITEK 2 Compact automated system (BioMérieux, France), along with conventional microbiological methods, including colony morphology, gram staining, oxidase testing, and pigment production. Additionally, the identification of *A. baumannii* strains was verified through the amplification of the *bla*OXA-51-like gene region. Imipenem susceptibility of the isolates was determined and evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines using the VITEK 2 Compact automated system.[14] The strains were named with KPA (Kastamonu *P. aeruginosa*) and KA (Kastamonu *A. baumannii*) codes and isolate numbers. The strains were stored in nutrient broth and Mueller-Hinton broth containing 20% glycerol (v/v) at -80°C until further use.

Disk Diffusion Method

The antimicrobial activity of the extract was assessed using the disk diffusion method, following EUCAST recommendations.[15] In brief, the inoculum was prepared from fresh cultures of the strains to a density of 0.5 McFarland (~1.0×10^8 CFU/ml) and subsequently seeded onto the surface of Mueller-Hinton agar (MHA). Three imipenem disks (10 µg/disk, Oxoid, UK) were then placed onto the medium as standard antibiotics, along with two blank disks. To investigate both the antimicrobial activity of the extract and its potential imipenem-potentiating activity, an imipenem disk, and a blank disk were each impregnated with 10 µL of a 10 mg/ml extract stock solution, resulting in a final concentration of 100 µg of extract per disk. For comparison, an imipenem disk and a blank disk were each impregnated with 10 µL of pure DMSO as a negative control, as DMSO was used as the solvent for the plant extract. The remaining imipenem disk was used to confirm imipenem resistance. The cultures were incubated at 37°C for 24 hours, and the diameters of the inhibition zones (IZ) around the disks were measured using a ruler (in millimeters) at the end of the incubation period. The imipenem-potentiating activity of the extract was categorized into three classes: indifferent/no potentiation (ΔIZ<4 mm), low potentiation/additive effect (4≤ΔIZ<6 mm), and potentiation effect (ΔIZ≥6 mm), based on the inhibition of growth of the strains ($\Delta IZ=IZ_{imipenem+extract}-IZ_{extract}$).[16]

Statistical Analysis

The data were assessed for normality using the Wilks-Shapiro test. The results were analyzed using the Mann-Whitney U test with SPSS version 23 (IBM Inc., Armonk, NY, USA) for Windows. Tha data were presented as the median and the interquartile range [Me (Q25-Q75)] and a p-value of less than 0.05 was considered statistically significant.

RESULTS

It was determined that the 40 strains included in the study were isolated from respiratory secretions (n=21, 52.5%), blood (n=11, 27.5%), urine (n=5, 12.5%), and wound cultures (n=3, 7.5%). No inhibition zone diameter was observed for DMSO and imipenem disks. The median inhibition zone diameter for the extract against *P. aeruginosa* strains was 9 mm (IQR: 9 mm, range: 7-10 mm), while for the extract-imipenem combination, it was 10 mm (IQR: 9-11 mm, range: 8-11 mm). Similarly, for *A. baumannii* strains, the median inhibition zone diameter was 13 mm (IQR: 13-14 mm, range: 11-14 mm) with the extract and 14 mm (IQR: 14 mm, range: 11-15 mm) for the extract-imipenem combination (**Figure 1**). As determined by comparing the inhibition zone diameters, *A. baumannii* strains were significantly more susceptible to the extract than *P. aeruginosa* strains ($p<0.05$). The imipenem-potentiating activity of the extract was categorized as "no potentiation" for all strains, with the difference in inhibition zone diameters (ΔIZ) being less than 4 mm. The results are summarized in the **Table**. Furthermore, a representative result from the disk diffusion test is shown in the **Figure 2**.

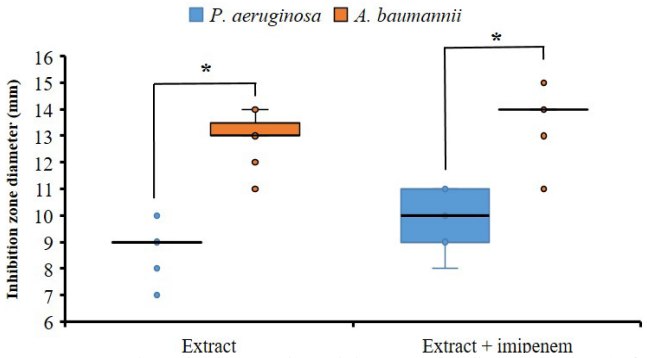

Figure 1. Boxplots displaying the inhibition zone diameters (mm) for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains treated with extract and its combination with imipenem. Median values are shown as horizontal lines within the boxes, which represent the interquartile range. Whiskers indicate data range, and dots outside the whiskers represent outliers. Statistically significant differences are marked with asterisks (*)

Figure 2. Representative image of antibacterial activity of the tested agents against imipenem-resistant strains (1: Extract, 2: Extract+imipenem, 3: DMSO, 4: Imipenem, 5: DMSO+imipenem)

DMSO: Dimethyl sulfoxide

Table. Antibacterial zone diameters for tested agents

Strain	Extract (mm)	Extract+imipenem (mm)	ΔIZ (mm)
KPA-1	9	11	2
KPA-2	10	10	0
KPA-3	10	11	1
KPA-4	9	11	2
KPA-7	9	9	0
KPA-9	9	11	2
KPA-10	9	9	0
KPA-12	9	9	0
KPA-15	9	10	1
KPA-16	9	10	1
KPA-17	9	10	1
KPA-18	9	11	2
KPA-19	8	8	0
KPA-20	9	11	2
KPA-21	9	9	0
KPA-28	10	11	1
KPA-37	8	9	1
KPA-43	10	10	0
KPA-51	9	11	2
KPA-54	7	9	2
KPA-67	9	11	2
KA-1	14	15	1
KA-2	12	13	1
KA-3	13	14	1
KA-4	13	14	1
KA-5	13	14	1
KA-6	13	14	1
KA-7	14	15	1
KA-8	13	14	1
KA-9	14	15	1
KA-10	14	15	1
KA-11	13	14	1
KA-12	13	14	1
KA-19	13	14	1
KA-20	12	13	1
KA-28	13	14	1
KA-34	13	14	1
KA-35	14	14	0
KA-40	11	11	0
KA-49	13	14	1

KPA: *Pseudomonas aeruginosa*, **KA:** *Acinetobacter baumannii*, **ΔIZ: Change in inhibition zone diameter**

DISCUSSION

The increasing prevalence of antibiotic resistance, particularly to carbapenems, among common nosocomial pathogens such as *P. aeruginosa* and *A. baumannii* poses a significant threat to public health.[17] This rising resistance underscores the urgent need to identify or develop novel antibacterial agents or compounds that can enhance the effectiveness of carbapenems in combating resistant pathogens. In this regard, natural products, particularly those derived from plants and their bioactive compounds, are expected to provide a viable solution to this challenge.[18]

This study evaluated the antimicrobial efficacy of EtOH extracts from *C. sativa* flowers against imipenem-resistant *P. aeruginosa* and *A. baumannii* strains. The antimicrobial activity of various parts of *C. sativa* has been reported in numerous studies. For example, Živković et al.[19] investigated the effects of shell and catkin extracts, whereas Üreyen Esertas et al.[20] focused on extracts from the flower parts. Similarly, Rodrigues et al.[21] evaluated aqueous extracts from burs and leaves, along with hydroalcoholic extracts from shells. Agarwal et al.[22] examined extracts from tree bark, and Genovese et al.[12] highlighted the antimicrobial properties of extracts from the pellicle.

Consequently, the extract exhibited inhibition zones ranging from 7 mm to 10 mm for *P. aeruginosa* strains and 11 mm to 14 mm for *A. baumannii* strains, with considerable antibacterial activity against both pathogens in this study. Moreover, the extract showed significantly larger inhibition zones against *A. baumannii* strains than *P. aeruginosa*, suggesting potentially inherent differences in susceptibility mechanisms between these species. These differences may stem from variations in outer membrane properties, such as permeability or differential efflux pump activity between these species, which could influence their susceptibility to the extract.[23]

The findings underscore the therapeutic potential of the *C. sativa* flower extract as a novel approach to combating drug-resistant pathogens. Its antimicrobial properties may be attributed to phenolic compounds, such as trigalloyl-hexahydroxydiphenoyl-glucoside and pentagalloyl-glucoside, previously identified as major components of *C. sativa* flower extracts.[24,25] Since phytochemicals are known to exhibit antimicrobial activity through mechanisms such as inhibition of bacterial cell-wall biosynthesis, disruption of cell membranes, inhibition of bacterial protein biosynthesis, DNA replication and repair, and interference with metabolic pathways, it is likely that the bioactive compounds in the extract exert antimicrobial effects through similar mechanisms.[26] Although the specific bioactive components responsible for these effects were not investigated in this study, the findings suggest potential applications of *C. sativa* flower extract in developing advanced antimicrobial solutions, including topical formulations or combination therapies to combat multidrug-resistant infections.

In addition to examining the antimicrobial activity, the potential potentiating effect of the extract on imipenem was also assessed in this study. When combined with imipenem, the extract resulted in a slight increase in the inhibition zones compared to when used alone. For *P. aeruginosa* strains, the inhibition zones increased from a range of 7-10 mm to 8-11 mm. Similarly, for *A. baumannii*, the zones expanded from 11-14 mm to 11-15 mm. Although the potentiation of imipenem was relatively modest, these findings highlight the potential for further optimization through precise combination ratios or the identification of synergistic bioactive compounds. Existing literature suggests that plant-derived bioactive compounds, mainly phenolic compounds, may potentiate antibiotic efficacy by inhibiting efflux pumps, a mechanism that enhances drug retention within bacterial cells and counteracts resistance mechanisms.[26,27] However, further in-depth studies are required to fully elucidate the specific mechanisms by which the extract potentiates imipenem's effectiveness.

Limitations

Despite the promising results observed with the extract in the disk diffusion assay, its limitations-especially the inability to assess the activity of nonpolar compounds due to poor diffusion in agar-necessitate further research employing more advanced methodologies such as checkerboard assays or time-kill curves. These additional studies will help elucidate how *C. sativa* extracts can enhance the effectiveness of carbapenems, especially against multidrug-resistant strains.

CONCLUSION

The results of this study demonstrate that the EtOH extract of *C. sativa* flowers exhibits antibacterial activity against imipenem-resistant clinical non-fermentative bacterial strains, underscoring its potential as a promising candidate for the development of novel antibacterial agents. Future synergy testing will be pivotal in confirming the therapeutic potential of combining *C. sativa* extract with carbapenems. Moreover, isolating and characterizing the bioactive compounds responsible for these effects could facilitate the development of novel antimicrobial formulations.

ETHICAL DECLARATIONS

Ethics Committee Approval

This study did not require ethical approval as it did not involve any human subjects or animal experiments.

Informed Consent

Because the study has no study with human participants, no written informed consent form was obtained.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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