Advances in Pharmacology and Therapeutics Journal
Shahid Sadoughi University of Medical Sciences, Yazd, Iran

SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES

Original Article

Synergistic Activity of *Cressa cretica* Extract Combination with Ampicillin against *Klebsiella pneumoniae*

Running Title: *Cressa cretica* and Ampicillin on *Klebsiella pneumoniae*

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A R T I C L E I N F O

Received: 04/03/2023
Accepted: 07/10/2023

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Abstract

Aims: This study was conducted to evaluate the synergistic effect of ethanolic extract of *Cressa cretica* and ampicillin against the standard and multidrug-resistant (MDR) strains of *Klebsiella pneumoniae*.

Method: The chemical composition of the ethanolic extract of *C. cretica* was determined by high-performance liquid chromatography (HPLC). The minimum inhibitory concentration (MIC) of the extract was used to evaluate the antimicrobial performance of the extract. The checkboard broth method was used to investigate the synergistic effect of the extract with ampicillin.

Results: The ethanolic extract of *C. cretica* inhibited the growth of the standard strain of *K. pneumoniae* with MIC 100 mg/mL and did not affect the MDR strains of *K. pneumoniae*. The Fractional inhibitory concentration (FIC) showed an additive effect of ampicillin in combination with *C. cretica* on the standard strain of *K. pneumoniae*. The MIC of ampicillin in combination with *C. cretica* was reduced eightfold.

Conclusion: *C. cretica* ethanolic extract is containing chlorogenic acid, rutin, and catechin. The extract in combination with ampicillin can reduce the minimum inhibitory concentration of ampicillin. *C. cretica* ethanolic extract can reduce the ampicillin dosage in the treatment of Klebsiella-induced infections.

Keywords: *Klebsiella. pneumoniae*, Ampicillin, *Cressa cretica*

Citation: Bameri Z, Fattahi-Bafghie A, Zareshahi R, Zabihi M. Synergistic Activity of *Cressa cretica* Extract Combination with Ampicillin against *Klebsiella pneumonia*. Adv Pharmacol Ther J. 2023;3(2): 65-73.

Introduction

The growth of antibiotic-resistant bacteria has sounded an alarm in the world. In recent decades, due to the high prevalence of nosocomial infections and bacterial resistance in most parts of the world, traditional medicine, as one of the oldest treatments, has become popular. Therefore, the interest in the development of plant products to treat infectious diseases has increased. Researchers have tested a variety of plants to combat bacterial resistance to antibiotics, which are mainly used to treat most infections.

This ability of plant extracts along with antibiotics can be interpreted in two ways: Using these compounds in combination with antibiotics modifies or blocks the mechanism of resistance in the bacterium and turns the bacterium from a resistant to a susceptible state, or lower the dosages of the antibiotics.

Cressa cretica belongs to the Convolvulaceae family, which has 40 genera and 1,200 species. This plant is perennial and its life cycle continues in summer with the drying of swampy and salty areas.

This plant is spread in different parts of the world such as India, Pakistan, Iran, Australia, and Wales. One of the important points about this plant is that its fruit is a valuable source of edible oil. This oil is free of undesirable compounds and is safe for human consumption. Also, its fatty acid composition is similar to commercial oils (1-5).

According to Priyashree et al., several flavonoid compounds were identified from the aerial parts of *C. cretica*, including scopoletin, quercetin, kaempferol, and rutin. *C. cretica* is the source of many chemical compounds, including glucosides, aluminum, calcium, copper, iron, manganese, sulfur, and zinc. It has various uses in traditional medicine including as an expectorant in Bahrain, as an agent to induce nausea in Sudan, and as an anti-intestinal worm and anti-tuberculosis in some parts of the world. *Klebsiella pneumoniae* is a gram-negative opportunistic pathogen and one of the most common causes of nosocomial infections. It causes pneumonia, sepsis, meningitis, and diarrhea, especially in infants. Therefore, the present study aimed to investigate the antimicrobial properties of the ethanolic extract of *C. cretica* and also examined the synergistic effect of this extract with ampicillin. against the standard and multidrug-resistant (MDR) strains of *K. pneumoniae*.

Material and Methods

Chemicals

Dimethyl sulfoxide (DMSO), was provided by Merck Chemical Company. ampicillin was supplied by Daana Pharmaceutical Company (Tehran, Iran). MTT(tetrazolium salt,) Trypticase Soy Broth (TSB), and Agar nutrient medium were provided by Sigma-Aldrich Chemical Company.

Plant material

Aerial parts of *C. cretica* were prepared by Ardakan Traditional Medicine Medicinal Herbs Research Institute, Iran, and were approved by the head of the Medicinal Herbs Center of Shahid Sadoughi University of Medical Sciences in Yazd, Iran. It received herbarium code SSU 0072. Then, the plant was powdered by an electric mill and the ethanolic extract was prepared through the maceration method. According to this method, 70

 DOI: https://doi.org/10.18502/aptj.v3i2.13458

grams of *C. cretica* powder was poured into a 500 ml beaker, and then about five times the volume of powder, i.e. 350 ml, of 80% ethanol was added and the beaker was placed on an electric shaker for 72 hours. The solution was filtered through filter paper and the resulting liquid was poured into a tray lined with aluminum foil and placed in the laboratory for one week to dry and yield a dry extract without solvent (5,6).

The dry extract (0.3 g) was dissolved in distilled water (1 ml). After dissolving and centrifuging, 2 µl of it was injected into liquid chromatography (HPLC) to identify its compounds.

Identification of compounds in *C. cretica* ethanolic extract by HPLC

0.3 grams of *C. cretica* dry extract was dissolved in one milliliter of distilled water and after dissolving and centrifuging the extract. A waters liquid chromatography apparatus consisting of a Separations module: waters 2695 (USA) and a PDA Detector waters 996 (USA) was used for the HPLC analysis. Data acquisition and integration were performed with Millennium32 software. The injection was Auto sampler injector equipped The chromatographic assay was performed on a 15 cm×4.6 mm with pre-column, Eurospher 100-5 C18 analytical column provided by waters (sunfire) reversed phase matrix (3.5 µm) (Waters) and elution was carried out in a gradient system with methanol+ 0.02%TFA as the organic phase (solvent A) and distilled water+ 0.02%TFA (solvent B) with the flow-rate of 0.5 mL min-1. Peaks were monitored at 200-600 nm wavelength. The injection volume was 20 µL and the temperature was maintained at 25°C.

Bacterial strains

K. pneumoniae (ATCC 700603 and ATCC 10031 strains) from the Persian Type Culture Collection, Tehran, Iran.

Preparation of bacterial inoculation

To evaluate the antimicrobial activity, the microbial stock culture was inoculated for 24 hours before adding the experiments in the Trypticase Soy Broth (TSB,) culture medium and incubated at 37°C. It was then cultured in the Agar nutrient medium at 37°C. After the incubation time and the formation of separate colonies for each microbe, 4 to 5 colonies were selected and transferred to a tube containing normal saline. Its turbidity was compared with 0.5 McFarland turbidity.

Assessment of the microbial minimum inhibitory concentration (MIC)

The antimicrobial effect of the plant extract was measured by determining the MIC of the extract. First, specific concentrations of *Cressa cretica* were prepared using 5% Dimethyl sulfoxide (DMSO,) solvent and sterilized under sterile conditions by a syringe filter. *Klebsiella pneumoniae* (ATCC10031 and ATCC700603 strains) were cultured 24 hours before adding the experiment.

The separated inoculation of microbial stock culture was performed 24 hours before adding the experiments to activate it in the TSB culture medium. Then, the microbial suspension was matched to the standard 0.5 McFarland tube in terms of turbidity. It was diluted with a 1:20 ratio according to the Clinical and Laboratory Standards Institute (CLSI) protocol (7).

A 100 µl of Mueller Hinton Broth culture medium was added to all wells. Then, a serial dilution

(adding 100 µl of the thickest extract to the first well, discarding 100 µl of the last well) was done in the 96-well plate. A 10 µl of the diluted bacterial suspension was added to each of the wells in a 1:20 ratio. One of the wells was used as a positive control, and another as a negative control. It was placed in an incubator at 37°C for 24 hours. After the incubation period, the last well that was clear and had no turbidity was considered MIC.

It is difficult to detect the MIC of bacteria in the extracts due to their turbidity. To solve this problem, we added 5 µl of tetrazolium salt (8) with a concentration of 5 mg/ml to each well and put it in an incubator at 37°C for 2 hours to see its color change. No color change is observed if the microbes do not grow.

Evaluation of the synergistic effects of the extracts

The checkerboard method in broth was used to investigate the interaction between antibiotics and extracts against bacterial strains(9).

The fresh culture was prepared from *K. pneumoniae*. (ATCC 10031 and ATCC 700603) strains. The MIC of ampicillin and extracts were determined on bacterial strains.

A range of concentrations was determined according to the MIC of the strains relative to ampicillin and the extracts so that 3 to 4 concentrations higher than the MIC and 4 to 5 concentrations lower than the MIC were considered for ampicillin and the extracts.

In completely sterile conditions, 25 µl of Mueller Hinton Broth was added to the 96-well plate. ampicillin concentrations were poured horizontally and the extracts were poured vertically into the well.

A 25 µl of each concentration of ampicillin and the extract was poured into the wells, so the volume of each well containing the ampicillin and extract reached 50 µl.

In the well, where the extract and ampicillin were present separately, 25 µl of Mueller Hinton Broth medium was added to increase the volume to 50 µl, like the rest of the wells. A 0.5 McFarland turbidity was prepared from the pure culture of each strain and diluted 100 times in bacterial suspension in tubes containing Mueller Hinton Broth.

A 10 µl of Mueller Hinton Broth medium containing bacteria and 40 µl of Mueller Hinton Broth were spread in all microplate wells. So that the final volume of each well reached 100 µl and each well contained 〖1.5*10〗^5 cfu/ml. The microplates were incubated for 24 hours at 37°C. Then, the microplates were examined for bacterial growth. The turbidity indicates bacterial growth (9). A 96-well microplate was used for each bacterial strain.

The Fractional inhibitory concentration (FIC) index, which is the study of the synergistic effect using the MIC test, was calculated for the first well in which no growth was observed. The interaction of antibiotics and extracts is interpreted based on the number obtained from the calculation of the FICI.

FIC for the extract = MIC of plant extract in combination/MIC of plant extract alone

FIC for antibiotics = MIC of antibiotic in combination/MIC of antibiotics alone

 DOI: https://doi.org/10.18502/aptj.v3i2.13458

FIC extract + FIC antibiotic = FIC (fractional inhibitory concentration) index

The FICI is interpreted as follows: FICI≤0.5 is the average between two synergistic compounds

0.5 <FIC<1 indicates an additive effect

1<FICI <4 is indifferent and FICI>4 is known as antagonism (9).

Results

The extract compositions

The results of the analysis of the extract and determination of its compounds using the high-performance liquid chromatography (HPLC) method are given in **Table 1**.

As shown the compounds ethanolic extract of *C. cretica* are %9.5chlorogenic,0.62 % rutin, and %0.24 Catchin.

Table 1. The Compounds in *Cressa cretica* extract

	% of total	PPM	UV	Area	R.Time	Peak
Catchin	%0.24	24.40376	279.2	543361	11.3	1
Chlorogenic acid	%9.5	952.3288	326.7	6616346	14.8	2
Rutin	%0.62	64.19105	256.8	5369685	27	3

The MIC of ampicillin and ethanolic extract of *C. cretica*

The results showed that ethanolic extract of *C. cretica* with 100 and 400 mg/ml MIC and MBC were able to inhibit the growth of the standard strain of *K. pneumoniae* and did not affect the MDR strains of *K. pneumoniae*.

As shown in **Table 2**, the MIC of ampicillin was 8 and 2500µg/mL, respectively against the growth of the standard strain of *K. pneumoniae* and MDR strains of *K. pneumoniae*.

Table 2. The MIC of ampicillin and ethanolic extract of C. cretica

	Bacterial Strains	
	K. pneumoniae	*MDR strains of K. pneumoniae*
C. cretica (mg/ml)	100	nd
Ampicillin (µg/mL)	8	2500

nd: Not determined

The checkerboard method was used in broth to investigate the interaction between ampicillin and the extract. The FIC index was calculated and interpreted after combining ampicillin and the extracts in selected concentrations (**Table 3**).

Table 3. Interaction of ampicillin with *C. cretica* against the standard strain of *K. pneumoniae* the strain

Result of the synergistic effects of the extract and ampicillin	Final FIC	FIC of ampicillin	FIC of the extract	The effective concentration of extract in the compound (mg/ml)	The effective concentration of ampicillin in the compound (µg/ml)
Ineffective	1	0.5	0.5	50	4
Ineffective	1.2	0.5	0.7	70	4
Ineffective	1.07	0.37	0.7	70	3
Ineffective	1.4	0.5	0.9	90	4
Ineffective	1.27	0.37	0.9	90	3
Ineffective	1.15	0.25	0.9	90	2
Ineffective	1.025	0.125	0.9	90	1
Additive	0.906	0.0625	0.9	90	0.5

Discussion

Antibiotic resistance means that pathogenic microbes for which antibiotics are used to fight become resistant to these drugs through gene mutation, creating new generations that cannot be fought. One of the most important causes of this type of drug resistance is the arbitrary or excessive use of antibiotics. Among the serious problems in the treatment of bacterial infectious diseases is the resistance of bacteria to antibiotics, which increases treatment costs and wastes human, health, and medical time by increasing the treatment process or non-response to treatment. Bacteria can generally get resistant to antibiotics by three different mechanisms: Changes in bacterial cell surface protein pumps, Changes in the target structure of the drug, and Changes in drug targets due to plasmid or chromosomal transmission of bacteria. Today, antibiotic-resistant microbial infections are one of the most important and major challenges that threaten the health of communities (10).

In recent decades, due to the high prevalence of bacterial resistance in most parts of the world, traditional medicine, as one of the oldest treatment methods, has become popular. Therefore, the interest in plant products to fight infectious diseases has increased. Researchers have tested a variety of plants to combat bacterial resistance to antibiotics, which are mainly used to treat most infections (15).

The antimicrobial properties of plants have attracted the attention of some scientists. In addition to being available, this antimicrobial ability has made medicinal plants an alternative to chemical antibiotics. In some pharmaceutical factories, products in the range of synthetic antibiotics are prepared from these herbs (16). In the treatment of resistant infections, an alternative method has been proposed, which is the use of antibiotics along with resistance-destroying agents that remove the resistance of pathogens to known antibiotics (10-17).

Klebsiella is an immobile, gram-negative, oxidase-negative, rod-shaped bacteria with a differentiated polysaccharide capsule. Members of the Klebsiella species are part of the natural flora in the nose, mouth, and intestines of humans and animals. This species is shorter and thicker than other members of the Enterobacteriaceae family A major share of Klebsiella infection is related to *Klebsiella pneumoniae*, which often occurs in children, older people, and patients with underlying conditions such as cancer (19). Physicians will usually prescribe antibiotics to treat the infection. However, Klebsiella is resistant to the drug by having drug-degrading enzymes such as carbapenemase. According to the Center for Disease Control and Prevention, this species of Klebsiella is responsible for 7,900 cases of refractory infections annually, of which 520 lead to death. Therefore, the physician has to use a variety of different antibiotics, which will have a significant effect on the microbe according to the Journal of Diagnostic Microbiology and Infectious Diseases (18).

The present study was conducted to evaluate the synergistic effect of ethanolic extract of *C. cretica* and ampicillin against the standard and multidrug-resistant (MDR) strains of *Klebsiella pneumoniae*. The ethanolic extract of *C. cretica* with MIC, 100 could inhibit the growth of the standard strain of

 DOI: https://doi.org/10.18502/aptj.v3i2.13458

K. pneumoniae and did not affect the MDR strains of *K. pneumoniae*.

The fractional inhibitory concentration (FIC) had an additive effect with ampicillin plus *C. cretica* on the standard strain of *K. pneumoniae*. The MIC of ampicillin in combination with *C. cretica* was reduced Eightfold. *C. cretica* ethanolic extract can reduce the MIC of this antibiotic and can be used as a supplement with a low dose of this antibiotic in the treatment of Klebsiella-induced infections.

Previous studies have shown that any measurement with ethanol solvent can lead to the extraction of polar and non-polar compounds from this plant (18-20). The phenolic and flavonoid compounds of ethyl acetate and ethanolic extracts of *C. cretica* extract were identified by HPLC. Analysis of *C. cretica* ethanolic extract showed 12 phenolic compounds and 4 flavonoid compounds with apigenin-7-glucoside as the main component (21). In this study, chlorogenic acid, rutin, and catechin compounds were obtained by analyzing the ethanolic extract *C. cretica*. chlorogenic acid is a phenolic compound potent antimicrobial (22).

Rutin is a bioflavonoid compound with antioxidant and antibacterial effects (23). Catechin is a flavan-3-ol, a type of phenol, a natural antioxidant, and a plant secondary metabolite with antibacterial properties.

Numerous studies have shown that some plant extracts are more active against gram-positive than gram-negative bacteria. The main reason for this difference is due to the outer membrane surrounding the cell wall in gram-negative bacteria, which prevents the spread of compounds through the lipopolysaccharide coating. Also, the periplasmic space contains enzymes that can break down molecules that enter from the outside (24, 25). In our study, *C. cretica* had a low antibacterial effect on the ATCC 10031 strain of *K.pneumoniae*, which is a gram-negative bacterium.

In a 2021 study by Kavitha Sagar, the antibacterial properties of ethanolic extracts of Mill *Senna italica*, *Prosopis juliflora*, *Schouwia purpura*, and *C. cretica* were evaluated on *Escherichia coli*, *K. pneumoniae*, *Candida albicans*, *Pseudomonas aeruginosa* and Citrobacter species. The results showed that all four plants had good activity against the relevant microorganisms. The ethanolic extract of *C. cretica* at a concentration of 1 mg/ml inhibited Klebsiella with a 1.8 cm growth inhibition zone diameter followed by *Pseudomonas aeruginosa* with a growth diameter of 1.75 cm, Citrobacter (1.6 cm), E-coli (1.45 cm), and Candida (1.4 cm). At a concentration of 0.5 mg/ml, it inhibited Pseudomonas (1.55 cm) followed by E-coli (1.35 cm), *K.pneumoniae* (1.2 cm), Citrobacter (1.15 cm) and Candida (1 cm in diameter) (25).

In a study by Saeedeh Saeedi and Bahman Fazeli Nasab (2019), the antibacterial properties of *C. cretica* Capparis spinosa L. and *Rhazya stricta* were evaluated. According to the results of this study, these plants can be used to treat bacterial and infectious diseases. In their study, *C. cretica* had an inhibitory effect on *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Shigella dysentery*, and *Candida albicans* (26).

Fatema Shah et al. studied the synergistic property of olive plant and ampicillin on *Pseudomonas*

aeruginosa and Escherichia coli with acceptable results, including the synergistic property of methanolic extract of olive leaf and ampicillin with FIC≤ 0.5 which inhibited the growth of *P. aeruginosa* and *Escherichia coli* (27).

Mahmoud Bahmani et al. (2019) conducted a study to evaluate the synergistic activities of hydroalcoholic extracts of *Origanum vulgare*, *Hypericum perforatum*, and their active ingredients, carvacrol, and hyperspin, against *Staphylococcus aureus*. The fractional inhibitory concentration (FIC) was 0.5 for *O. vulgare* and *H. perforatum* and 0.49 for the carvacrol and hyperspin ingredients, both of which indicated a synergistic effect. This study shows that a combination of plants, as well as carvacrol and hyperspin may be used as a new antibacterial strategy against *S. aureus* (28).

Atefeh Salari *et al.* examined the effect of 19 plant extracts on standard and clinical strains of *P. aeruginosa*. Methanolic and ethanolic extracts of *Quercus infectoria* with a concentration of 1000 µg/ml were able to inhibit the growth of both strains. The aqueous extract at this concentration also reduced the growth of both strains. Moreover, methanolic and ethanolic extracts of *Myrtus communis* L. and eucalyptus reduced the growth of both strains at this concentration but did not completely inhibit growth. As a result, the hydroalcoholic extract of *Quercus infectoria* was the most effective extract on both bacterial strains and had significant synergistic effects with ceftazidime (29).

Conclusion

C. cretica ethanolic extract is containing chlorogenic, rutin, and catechin. The extract in combination with ampicillin can reduce the MIC of ampicillin. It can be used as a supplement to reduce the dose of ampicillin in the treatment of Klebsiella infections. The exact mechanism of the additive effect of the combination of the extract and ampicillin is not fully understood and needs further research.

Conflict of interest: The authors declare no conflict of interest in conducting this study.

Funding: This study was funded by Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Acknowledgments: This study was financially supported by Shahid Sadoughi University of Medical Sciences, Yazd, Iran (Grant No. 6654).

Ethical considerations: This study was reviewed and approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences, Yazd, Iran. (Registration Code: IR.SSU.MEDICINE.REC.1398.178).

Authors' contribution: MZ and MFB designed the research and prepared the manuscript, ZB, MFB, RZ, and AE performed the experimental studies and helped in manuscript preparation; ZB and RZ collected the plants and performed extraction.

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