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Antibacterial Effect of Combined Use of Amoxicillin Trihydrate And *Cupressus sempervirens* Leaf Extract on Some Gram-Negative Bacteria

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Abstract: Difficulties in the treatment of infections caused by Gram-negative bacteria have led to studies in recent years directed towards improving the effectiveness of existing antibacterial agents. In our study, the antibacterial activities of the combined use of *Cupressus sempervirens* leaf extract and amoxicillin trihydrate on *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922 *Salmonella typhimurium* ATCC 14028, *Salmonella abony* NCTC 6017 bacteria and 4 coliform bacteria isolated from Karaman wastewater facility were investigated using the disc diffusion method. It was determined that the mixture of amoxicillin trihydrate and *C. sempervirens* leaf extract caused a synergistic effect on *S. typhmurium* and *E. coli* ATCC 8739. In general, it was observed that *C. sempervirens* leaf extracts contributed to the existing antibacterial activity of the antibiotic amoxicillin trihydrate. In addition, three of the bacteria isolated from wastewater were found to be sensitive to the antibiotic amoxicillin trihydrate, while one was found to be resistant

Amoksisilin Trihidrat ve *Cupressus sempervirens* Yaprak Ekstraktının Birlikte Kullanımının Bazı Gram-Negatif Bakteriler Üzerindeki Antibakteriyel Etkisi

Anahtar Kelimeler
Antibakteriyel aktivite, *Cupressus sempervirens*, Koliform bakteri, Sinerjik etki, Atıksu

Öz: Gram-negatif bakterilerin neden olduğu enfeksiyonların tedavisindeki zorluklar son yıllardaki çalışmaları mevcut antibakteriyel ajanların etkisini geliştirilmeye yönelmiştir. Çalışmamızda *Cupressus sempervirens* yaprak ekstraktı ve amoksisilin trihidrat birlikte kullanımlarının *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922 *Salmonella typhimurium* ATCC 14028, *Salmonella abony* NCTC 6017 bakterileri ile Karaman atık su tesisinden izole edilen 4 koliform bakteri üzerindeki antibakteriyel aktiviteleri disk difizyon metodu kullanılarak araştırılmıştır. Amoksisilin trihidrat ve *C. sempervirens* yaprak ekstraktı karışımının *S. typhmurium* ve *E. coli* ATCC 8739 üzerinde sinerjik etkiye neden olduğu tespit edilmiştir. Genel olarak *C. sempervirens* yaprak ekstraktlarının amoksisilin trihidrat antibiyotiğinin mevcut antibakteriyel aktivitesine katkı sağladığı görülmüştür. Ayrıca amoksisilin trihidrat antibiyotiğine karşı atık sudan izole edilen bakterilerden üçünün duyarlı olduğu görülürken birinin dirençli olduğu belirlenmiştir.

1. INTRODUCTION

Water used by people during their daily activities and transferred to sewers is called domestic wastewater. Wastewater contains various pathogenic microorganisms such as viruses, bacteria, fungi, protozoa and helminths. Different types of polluting agents, such as detergents, personal care products, endocrine disruptors and pharmaceuticals are mixed into wastewater. Recent studies have focused on pharmaceuticals, particularly antibiotics, with their use increasing worldwide [1,2].

Once antibiotics get in ecosystems, they can affect the structure, development and genetic structure of microorganisms and, accordingly, change the ecological functioning of the aquatic ecosystem [3]. Generally, 30-90% of the dose taken as a result of the use of water-soluble antibiotics is excreted in human urine and feces. On the other hand, large amounts of antibiotics are mixed into wastewater due to the disposal of unused antibiotics [1,4]. Such antibiotic residues and other pharmaceutical active molecules are heavily discharged into the municipal sewage system and then transferred to the wastewater treatment plant. Antibiotics mixed into wastewater in this way may develop resistance in microorganisms in the environment and spread this resistance to sensitive microorganisms. Ultimately, this situation creates more threats to the environment and society [5-6]. In addition, unconscious and excessive use of synthetic antibiotics used in the treatment of infections causes increased resistance in bacteria. Difficult and slow healing of resistance-related infections has negative effects on human health. Therefore, the search for new antimicrobials has attracted worldwide attention. However, since such research is slow and costly, studies aimed at increasing the effects of existing antibiotics stans out [6-7].

In recent years, treatments with a single substance have been replaced by treatments with drug combinations in the treatment of some diseases. Investigating the synergistic effects of antimicrobial agents, especially with herbal extracts, is very important in finding new treatment combinations that increase the effects of existing antimicrobials.

β -lactam antibiotics are the most widely used antibacterial compounds (approximately 70% of prescriptions) due to their ability to act on many different types of bacteria and their inhibition mechanism that disrupts bacterial cell wall formation. On the other hand, β-lactamase enzymes, which are frequently detected in Gram-negative bacilli, hydrolyze the β-lactam ring in the chemical structure of these antibiotics and render them ineffective. These enzyme groups, which hydrolyze broad-spectrum β-lactam antibiotics, negatively affect the clinical use of antibiotics [8,9].

Cupressus sempervirens L. is a decorative tree belonging to the Cupressaceae family and commonly used in parks and gardens, growing mostly in the Mediterranean region. It is also used as a pharmaceutical agent due to its antiseptic, antispasmodic, antipyretic and anti-inflammatory properties. In addition, it has been proven that extracts obtained from different parts of this plant have antibacterial properties [10,11]. Therefore, it can be used as a natural antimicrobial agent in food preservation and to treat humans against infectious diseases [12]. The use of natural antimicrobial agents together with antibiotics can also help reduce negative effects such as environmental pollution, toxicity to humans, and resistance to synthetic chemicals and drugs.

In our study, it was aimed to determine the antibacterial activity of *Cupressus sempervirens* extract, whose antibacterial activity has been proven in previous studies, and amoxicillin trihydrate antibiotic, which is widely used among the broad-spectrum penicillin group antibiotics, on gram-negative bacteria, both separately and together for the first time..

2. MATERIAL AND METHOD

2.1. Preparation of The Extract

Cupressus sempervirens leaves collected from Sakarya University Esentepe Campus were washed, cleaned and dried in a drying oven at 40ºC for 10 days. Then, 10 grams of the sample from the leaves pulverized with the help of an electric grinder was placed in soxhlet cartridges and extracted with 200 mL 70% ethyl alcohol in the soxhlet apparatus for 8 hours. The solvents of the extracts filtered through the filter paper were removed in a rotary evaporator at 55º C for 15 minutes [13]. Stocks were prepared from the dried extracts at a concentration of 10 mg/mL(1:1 ratio of distilled water and DMSO) and used in activity studies.

2.2. Isolation of Gram Negative Coliforms from Wastewater

Water samples taken from the Karaman wastewater treatment plant effluent were brought to the laboratory in the cold chain and analyzed on the same day. 100 mL of the collected wastewater was filtered through cellulose nitrate filters with a pore size of 0.45 µm using a membrane filtration system. To detect total coliform bacteria, the filter placed on Chromocult Coliform Agar (CCA)(Bioneks) medium was incubated at 37±0.5°C for 24±2 hours. After incubation, the bacteria growing on the petri dish were subjected to oxidation test [14]. For the oxidation (Merck 1.13300) test, a random colony thought to be coliform bacteria was selected from the colonies growing on CCA medium, and it was planted in Tryptic Soy Agar (Merck) and incubated at 37±0.5°C for 24±1 hours. 10 possible coliform bacteria were selected and isolated from the bacteria with negative oxidation results. Additionally, gram negativity was confirmed by gram staining.

2.3. Antibacterial Activity

In determining the antibacterial activity, 4 standard strains (*Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922 *Salmonella typhimurium* ATCC 14028, *Salmonella abony* NCTC 6017) used from Sakarya University Microbiology laboratory and 4 coliform bacteria isolated from Karaman wastewater treatment facility (wastewater treatment plant) were used. In this study, the antibacterial activity of *C. sempervirens* leaf extract, amoxicillin trihydrate and leaf extract + amoxicillin trihydrate combinations was evaluated using the disc diffusion method.

2.3.1. Disc Diffusion Methods

First of all, the leaf extract prepared at a concentration of 5 mg/mL (1:1 ratio of distilled water and DMSO), 0.5 mg/mL amoxicillin trihydrate and the binary mixture were absorbed (20µL) into empty sterile discs (Himedia) with a diameter of 6 mm. Suspensions were prepared from 24-hour fresh bacterial cultures at a density of 0.5 McFarland. Then, bacteria were inoculated from these bacterial suspensions into Mueller Hinton Agar (Merck) with sterile swabs and previously prepared sample-impregnated disks were placed. After 24 hours of incubation at 37±0.5°C, the zone diameters formed in the petri dishes were measured in mm using a digital caliper [10]. Discs loaded with 50% DMSO(Merck) were used as negative control, and discs loaded with gentamicin (Oxoid) were used as positive control. The antimicrobial activity test was performed three times under aseptic conditions and the diameter of inhibition zone measured was the average of the three replicates.

3. RESULTS AND DISCUSSION

Gram-negative bacteria are more resistant than Gram-positive bacteria due to their unique morphological structure and cause morbidity and mortality worldwide. Various strategies have been reported to combat and control resistant Gram-negative bacteria, such as the development of adjuvants to antimicrobial agents, structural modification of existing antibiotics, investigation of new mechanisms of action, etc. [15].

In our study, the antibacterial activity results of C. sempervirens leaf extract and amoxcillin trihydrate combinations on gram-negative coliform bacteria isolated from wastewater environment and *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922 *Salmonella typhimurium* ATCC 14028, *Salmonella abony* NCTC 6017 bacteria used as standard strain are given in Table 1.

Table 1. Inhibition zone diameters of extracts and amoxcillin trihydrate

Test bacteria	Inhibition Zones(mm)(±SD)			
	Extract	Amoxcillin trihydrate	Extract+ amoxcillin trihydrate	Negative control
E. coli ATCC 25922	9±0.1	28.3±0.7	28.3±0.6	-
E. coli ATCC 8739	-	18±1.0	19±0.1	-
S. abony NCTC 6017	-	23.7±0.6	23.5±0.3	-
S. typhimurium ATCC 14028	-	25.6±0.2	27±0.1	-
Coliform-1	-	8±0.2	8±0.1	-
Coliform-2	9±0.1	36±0.3	37.5±0.3	-
Coliform-3	10±0.3	31.5±0.1	32.7±0.6	-
Coliform-4	8±0.3	22±0.1	22.3±0.1	-

Amoxicillin, which is among the beta-lactams that constitute the largest antibiotic group, is used in veterinary medicine as well as being frequently used in human treatment. Amoxicillin trihydrate created an inhibition zone diameter of 23.7 mm in *S. abony*, 25.6 mm in *S. typhimurium*, 8 mm in Coliform-1, 36 mm in Coliform-2, 31.5 mm in Coliform-3, and 22 mm in Coliform-4. Standard strains, except *E. coli* ATCC 8739, were found to be highly sensitive to the antibiotic amoxicillin trihydrate. While amoxicillin trihydrate created an 18 mm inhibition zone in *E. coli* ATCC 8739, it created a 28 mm inhibition zone in *E. coli* ATCC 25922. There are many studies showing differences in the antibacterial activity of different strains of the same bacteria. In a study evaluating the antibacterial effect of cystatin on three *E. coli* strains, it was reported that cystatin showed different effects in the three strains [16]. In a study examining the activity of *C. sempervirens* leaf extracts, it was reported that the ethanolic extract created an 8 mm inhibition zone on *E. coli* ATCC 35218 [17]. Similarly, Mogna et al. [18] reported in their study that probiotic bacteria exhibited different antibacterial activities on *E. coli* strains (ATCC 8739, ATCC 10536, ATCC 35218 and ATCC 25922).

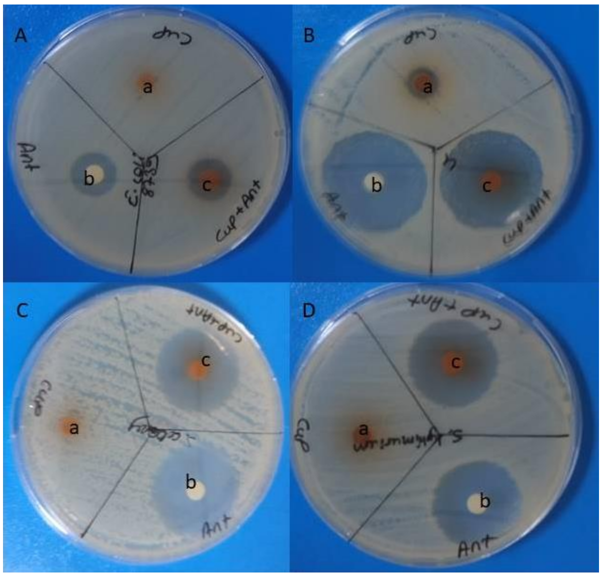

Figure 1. Antibacterial activity of samples (a=leaf extract, b= amoxicillin trihydrate, c) leaf extract + amoxicillin trihydrate) against (A= *E. coli* ATCC 8739,B=Coliform-3, C= *S. abony*, D= *S. typhimurium*).

In our study, we isolated fecal indicator bacteria (coliform bacteria) from the Sakarya Karaman Wastewater Facility and evaluated the effect of amoxicillin trihydrate, and *C. sempervirens* extracts on these bacteria. Because most of this group of bacteria threaten public health. In addition, *E. coli* and other coliform bacteria can relatively easily incorporate resistance genes contained in plasmids. This may cause the spread of resistance in sensitive bacteria in Wastewater Treatment Plants [1,8].The results obtained showed that Coliform-1 bacteria isolated from wastewater were resistant to the antibiotic amoxicillin trihydrate, while other coliform bacteria were found to be sensitive. In particular, the fact that *C. sempervirens* leaf extract creates an inhibition zone of 9 mm in Coliform-2, 10 mm in Coliform-3 and 8 mm in Coliform-4 is important in terms of demonstrating that phytochemical resources can be used as antibacterial agents. Many studies in the literature show that antibiotic-resistant bacteria are present in drinking water, surface water, groundwater and wastewater along with antibiotics [19-21].

The effect of antibiotics on bacteria can be enhanced by their combined use with phytopharmaceuticals and modern drugs. It was also observed that different combinations could help reduce the emergence of resistant bacteria and drug toxicity [22]. As a result of combining two drugs or phytochemical products, there may be a synergistic, additive or antagonistic effect on antibacterial activity, depending on the interaction between the drugs.

In our study, the contributions of the combined use of *C. sempervirens* leaf extract and the antibiotic amoxicillin trihydrate to the activity of the antibiotic are given in Figure 2. If the antibacterial activity of two agents used together is greater than the sum of the antibacterial activities of the individual agents, it shows a synergistic effect. While *C. sempervirens* leaf extract did not show activity on *S. typhimurium* and *E. coli* ATCC 8739 bacteria, the leaf extract used together with the antibiotic (amoxicillin trihydrate) showed a synergistic effect by increasing the existing activity of the antibiotic.

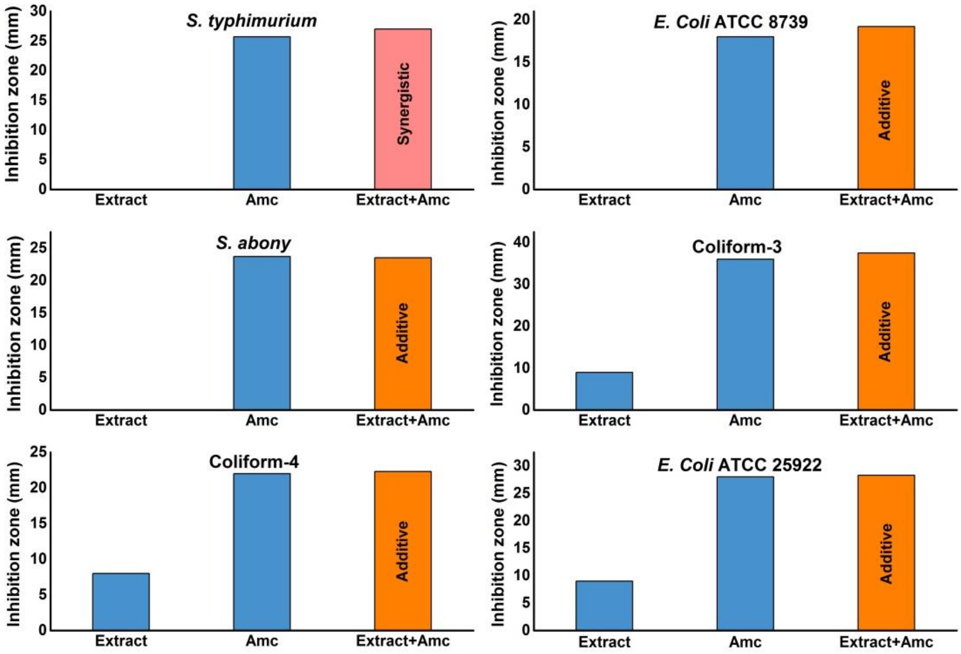

Figure 2. Antibacterial activity of combined use of amoxicillin trihydrate and *C. sempervirens* leaf extracts on bacteria

When *C. sempervirens* leaf extract was used together with the antibiotic amoxicillin trihydrate, it was observed that it contributed to the existing effect of the antibiotic on Coliform-3, Coliform-4, *E. coli* ATCC 25922 and *S. abony* bacteria. Studies in the literature have determined that *C. sempervirens* leaf extracts have antibacterial activity on many bacteria [23-25]. In our study, the effectiveness of *C. sempervirens* leaf extract on 3 of the 4 coliform bacteria isolated from wastewater emphasizes the importance of plant extracts against microbial resistance. In addition, our study provides important data to the literature by revealing that the use of *C. sempervirens* leaf extract together with amoxicillin trihydrate increases the antibacterial activity.

Our study confirmed that plants can indeed be a source of compounds that can potentiate the activity of antibiotics against bacterial pathogens. From studies in the literature, it was seen that synergistic interactions between plant extracts and antibiotics could support β-lactamase inhibition, blocking drug flow, increasing permeability and antibacterial activity due to alternative mechanisms of action [26,27].

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