Journal of Nuts
Journal homepage: ijnrs.damghan.iau.ir

ORIGINAL ARTICLE

Examining the Antibacterial Efficacy of Aqueous Extracts of Chestnut Leaves and Catkins in a Variety of Wild and Cultivated Environments

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KEYWORDS

Aqueous extract;
Bacteriology;
Chestnut;
Castanea sativa;
Irrigation;
Natural forests

ABSTRACT

Chestnuts are highly valued for their nutritional and practical qualities in addition to their organoleptic qualities. Chestnut tree is extremely susceptible to pollutants, summer droughts, high soil moisture, and harsh frosts. Natural forests that are home to chestnuts can be found in Kurdistan region of Iraq. Hand-planted chestnuts are a common sight in several provinces around the country. It is possible to get medical benefit from chestnut leaves (*Castanea sativa*) and catkins. The purpose of this study was to investigate the impact that aqueous extracts of chestnut leaves and catkins have on seven different strains of bacteria: *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa,* and *Yersinia enterocolitica*. This study selected three harvesting locations: Babulah, Argen, and Charbut. The samples collected from chestnut trees were dried and processed. Antimicrobial effects of the extracts were tested on various gram-positive and gram-negative bacteria using the Kirby-Bauer disk diffusion method. Ciprofloxacin (5 µg) and ceftizoxime (30 µg) discs were used to compare the antibacterial properties of the extracts. Statistical analyses were performed using SPSS software. According to the findings, the antibacterial qualities of chestnuts shifted depending on the environment in which they were grown. The catkins, but not the leaves, exhibited antibacterial activity. The leaves did not show any antibacterial activity. Gram-positive bacteria were more sensitive, and antibiotics had greater antibacterial effects than extracts.

Introduction

Chestnuts (Castanea spp.) are a widely recognized and esteemed nut species, boasting a rich heritage of cultivation and consumption across diverse regions globally (Freitas *et al*., 2021). Despite their historical

***Corresponding author**: Email address: zead3133@gmail.com
Received: 8 February 2023; Received in revised form: 21 June 2023; Accepted: 16 July 2023
DOI: 10.22034/jon.2023.1979726.1213

significance, chestnuts do not rank among the top nut varieties in terms of consumption within Iraq (Massantini *et al*., 2021). As well as being a fruit, chestnuts are also an essential and versatile ingredient used in a variety of food products (Poljak *et al*., 2021). According to Food and Agriculture Organization (FAO), countries such as China, Korea, and Japan are the leading producers of chestnut. These countries produce more than two million tons, or nearly 70% of global production, followed by Italy (16%), the United States (4%), and the Republic of Azerbaijan (3.4%). Other countries, such as Spain, Georgia, and Iraq, produce 6.6% of the total global production (Baser and Bozoglu, 2020; Bozoglu *et al*., 2020). The chestnut is indigenous to Europe, Anatolia, and the Caucasus, although it has long been found growing wild throughout the majority of the Northern Hemisphere, from Iraq and Turkey to Japan and China (Fig. 1).

Fig. 1. Global chestnuts market by region, 2022.

At a height of 3600 meters, near the mount Cheekha Dar in Iraqi Kurdistan, Yousif (2010) has studied the occurrence of several seeds from the early Pleistocene era in the clay layers. Many chestnut seeds, which are now one of the region's emblematic trees, were found in the upper reaches of the Pendro and Mount Butin (Al-Ameri *et al*., 2011). Iraq has long practiced the cultivation of chestnut trees; however, the actual beginning of this practice is unknown. Since ancient times, chestnut leaves (*Castanea sativa*) and stems have been utilized as medicines (Idris *et al*., 2020). Also, chestnuts are used as a rootstock for grafting of fruit trees that can have successfully vascular connections with oak scion (Farsi *et al*., 2016). Indians use chestnut, which have a toasty, dry nature, to strengthen their stomachs. Chestnut is known for its astringent and anti-diarrheal properties (Antani and Mahapatra, 2022). When ingested with honey, chestnut has a longer-lasting effect and is highly beneficial in treating situations of shortness of breath and repeated coughing. Chestnut also boosts sexual potency (VM *et al*., 2009). Additionally, it detoxifies the body and prevents inflammation of the urinary system (Khar'kov *et al*., 2022). Chestnut oil has been found to strengthen the liver and can also heal coughs and reduce inflammation. It also has the ability to reduce blood pressure (Zhou *et al*., 2021). The external application of chestnut leaves as a compress on open and closed wounds stimulates faster wound healing. Chestnut leaves are also a blood purifier when infused (Yadav *et al*., 2021).

The antioxidant and antibacterial properties of chestnut have been confirmed based on its chemical composition (Ronsisvalle *et al*., 2019; Kobya *et al*., 2021). Its antimicrobial capacity against Gram-positive bacteria (*Bacillus cereus, Bacillus subtilis, Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae*) and fungi (*Candida albicans, Cryptococcus neoformans*) was studied (Razvy *et al*., 2011; Fernández-Agulló *et al*., 2014). It was discovered that chestnut has a high antibacterial property against gram-positive bacteria (Zhan *et al*., 2014). Gram-positive bacteria in this study included *Bacillus subtilis, Bacillus cereus, Staphylococcus* *aureus, and Micrococcus luteus*. In individuals who have a weakened immune system, such as patients with HIV virus, these bacteria are the cause of diseases like septic shock, endocarditis, meningitis, and pneumonia. They are also the cause of dizziness, diarrhea, and food poisoning (Heinrichs *et al*., 2004). Gram negative bacteria include *Yersinia enterocolitica, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Escherichia coli*. These bacteria are the cause of many natural infections in humans, as well as various animals (Clancy and Nguyen, 2022).

Using aqueous extracts of plants for inhibiting enzymatic or bacteria activity and medicinal usage has been reported in the other nut trees (Khodadadi *et al*., 2016; Chatrabnous *et al.*, 2018; Jahanbani *et al.*, 2018; Habibie *et al.*, 2019; Habibi *et al.*, 2022). This research examined at how seven distinct bacterial strains—*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa*, and *Yersinia enterocolitica*—reacted to aqueous extracts of chestnut leaves and catkins. The efficacy of the extracts was assessed and compared to that of two medicines, namely ceftizoxime and ciprofloxacin.

Material and Methods

Three places of harvesting were selected in this study, including (1) Babulah area situated at an elevation of 1610 meters above sea level, 100 kilometers from Akre city (36°58'07.1"N 43°44'05.9"E). The region has a very humid, extremely cold climate. The silt loam soil on the surface has an acidity of about 6.7; (2) Argen area situated at an altitude of roughly 1400 to 1500 meters, it is situated 40 kilometers south of Babulah (36°56'12.7"N 43°35'40.8"E). There is no dry season in the area, and it has cold climate. The majority of the region's topsoil is loamy, and its pH is 6.8 on average; (3) Charbut area situated at an altitude of 1400 meters (36°53'59.6"N 43°54'00.0"E), it has an average annual rainfall of 270 mm, a semi-arid climate, and annual minimum and maximum temperatures of -19.4^{o}C and 39^{o}C, respectively. The station is located in an area of 65 hectares of loam to clay loam with an acidity of 8.1. Due to the extremely dry summer climate, the chestnut trees that were planted in this area require irrigation once every 6 to 9 days.

Sampling from the three different habitats of Argen, Babulah, and Charbut was performed in September. The collected samples consisted of leaves and catkins from these habitats. The samples that were gathered should first be placed in the shade to dry out and lose their water. The catkins took longer to dry—about a week—than the leaves, which required 4 days. Using a grinder, the samples of leaves and catkins were put into independent batches. The extraction process employed 20 g of dry, grinded samples. Each region's grind leaves and catkins were submerged separately in water, methanol, ethanol, and chloroform solutions for 48 hours. Then, we filter the leaf and catkins solution through Whatman grade 20 paper before adding it to a volumetric flask. The extract is then obtained using a rotary machine. The resultant extracts are put in sealed vials and kept in a refrigerator at 3^{o}C to be tested for microbes. The rotating device's water temperature and pressure were both set to 67^{o}C degrees and 35^{o}C, respectively. Distillation of the water took around 20 min. The extracts were further diluted (1:2, 1:5, and 1:10), and utilized for comparison (Fernández-Agulló *et al*., 2014).

This study examined the antimicrobial effects of the aqueous extract of the leaves and catkins of chestnut trees grown in Argen, Babulah and Charbut habitats on four types of gram-positive bacteria, including *Bacillus subtilis, Bacillus cereus, Staphylococcus, Micrococcus luteus*, as well as three types of gram-negative bacteria, including *Escherichia coli, Pseudomonas aeruginosa,* and *Yersinia enterocolitica*. The Kirby-Bauer disk diffusion method of the agar diffusion method was employed to examine the antibacterial impact of the extracts. For this purpose, using dimethyl sulfoxide

(DMSO) as a solvent, the extracts were diluted in the ratio of 1:2, 1:5, and 1:10. The Tryptic Soy Agar culture medium was infected with 500 microliters of an 18-hour microbial suspension that was dispersed uniformly using a sterile swab and had a concentration equal to McFarland 0.5 standard. After that, 8 mm diameter blank discs containing 30 microliters of the aforementioned extract concentrations were put on the plate. After 24 hours, the diameter of the growth halo was measured in milliliters. For each of the bacteria, these concentrations were repeated three times. The negative control was a blank disc that was filled with 30 microliters of DMSO. Ciprofloxacin (5 µg) and ceftizoxime (30 µg) discs were used to compare the antibacterial properties of the extracts (Khar'kov *et al*., 2022). All statistical analyses including ANOVA analysis with Duncan grouping were conducted using SPSS software version 23.0.

Results

Investigations were conducted into the antimicrobial properties of aqueous extracts against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, and *Yersinia enterocolitica* (Fig. 2).

Fig. 2. An example of antimicrobial activity of aqueous extract of chestnut leaves from Babulah habitat against *Escherichia coli*.

Along with the aforementioned cases, the antibacterial qualities of the extracts were examined in comparison to the two antibiotics ceftizoxime and ciprofloxacin. According to the findings, there is no difference between Argen, Babulah and Charbut habitats and no effect is seen in the aqueous extracts of chestnut leaves. However, the two control antibiotics had a significant difference (positive), and ceftizoxime was assigned to group A (Table 1).

Table 1. Grouping of aqueous extracts of leaves in accordance with their ability to prevent the development of bacteria in various habitats

Extract	Groups
Concentration 1:10	C
Concentration 1:5	C
Concentration 1:2	C
Ceftizoxime	A
Ciprofloxacin	B

In contrast to the aqueous extract of the leaves, the chestnut catkins extract had an antibacterial activity. The investigation of the variance of the aqueous extract of chestnut catkins in the Argen, Babulah and Charbut (Argen and Babulah origins) habitats are presented in Tables 2-5. These findings demonstrate a significant difference in bacterial sensitivity to aqueous extracts of chestnut catkins, as well as different habitats, different concentrations of extracts, and two antibiotics (positive control).

Table 2. Variance analysis of the aqueous extract of chestnut catkins in Argen habitat.

Sources of variation	Sum of squares	Degrees of freedom	Mean of squares	p-value
Bacteria	1278.23	5	255.65	0
Extract	4680.51	5	936.10	0
Extract × Bacteria	1139.96	18	63.33	0
Error	87.12	45	1.94	
Total	7185.83	75		

Table 3. Variance analysis of the aqueous extract of chestnut catkins in Babulah habitat.

Sources of variation	Sum of squares	Degrees of freedom	Mean of squares	p-value
Bacteria	192.33	5	38.47	0
Extract	4297.33	5	859.47	0
Extract × Bacteria	1661.82	18	92.32	0
Error	147.60	45	3.28	
Total	6299.08	75		

Table 4. Variance analysis of the aqueous extract of chestnut catkins in Charbut (Argen origin) habitat.

Sources of variation	Sum of squares	Degrees of freedom	Mean of squares	p-value
Bacteria	212.83236	5	42.57	0
Extract	4620.24	5	924.05	0
Extract × Bacteria	1370.52	18	76.14	0
Error	216	45	4.80	
Total	6419.59	75		

Table 5. Variance analysis of the aqueous extract of chestnut catkins in Charbut (Babulah origin) habitat.

Sources of variation	Sum of squares	Degrees of freedom	Mean of squares	p-value
Bacteria	836.99244	5	167.40	0
Extract	6184.21716	5	1236.84	0
Extract × Bacteria	864.25164	18	48.01	0
Error	490.32	45	10.90	
Total	8375.78	75		

The grouping of bacteria using Duncan's approach revealed that various bacteria responded differently to the effects of aqueous extract of chestnut catkins from diverse habitats. Bacteria in various habitats are grouped in Table 6.

Table 6. Grouping of bacteria based on sensitivity to the aqueous extract of chestnut catkins in the extraction habitats of Argen, Babulah, Charbut (Argen origin), and Charbut (Babulah origin).

Bacteria	Argen	Babulah	Charbut (Argen origin)	Charbut (Babulah origin)
Escherichia coli	-	-	-	A
Bacillus cereus	B	B	A	B
Staphylococcus aureus	A	A	A	-
Bacillus subtilis	-	-	B	-
Pseudomonas aeruginosa	C	A	-	-
Micrococcus luteus	D	C	C	C
Yersinia enterocolitica	D	B	C	C

Bacillus subtilis, and *Escherichia coli* bacteria did not exhibit a halo of non-growth and were entirely resistant to the aqueous extract of chestnut catkins from the Argen and Babulah habitats. In contrast to their mother trees, the chestnut of the Charbut (Argen and Babulah origins) habitat yielded distinct aqueous extracts of chestnut catkins. Table 6 demonstrates that the aqueous extract of Argen origin chestnut trees' catkins in the Charbut habitat reacted with *Bacillus subtilis*. However, *Pseudomonas aeruginosa* was completely resistant. The bacteria *Escherichia coli*, which is the origin of the fungus, shown exceptional sensitivity in the Charbut habitat; in fact, it was more sensitive than all other bacteria (group A). While *Staphylococcus aureus* bacteria exhibited the highest sensitivity in the Duncan grouping of the other three habitats (group A), aqueous extracts of chestnut catkins from Charbut (Babulah origin) habitats did not react against these bacteria. In general, the grouping of sensitive bacteria revealed that *Staphylococcus aureus* and *Bacillus cereus* were very sensitive in the habitat of Argen, Babulah, and Charbut, with Argen as the origin (Table 6). The results of Duncan's grouping of the extracts and the two antibiotics (positive controls) revealed a significant difference, with the ceftizoxime antibiotic being placed in group A having the highest antimicrobial effect and the chestnut aqueous extract with a concentration of 1:10 being placed in group E having the lowest antimicrobial effect (Table 7).

Table 7. Grouping of aqueous extracts of chestnut catkins in various habitats.

Extract	Groups
Concentration 1:10	E
Concentration 1:5	D
Concentration 1:2	C
Ceftizoxime	A
Ciprofloxacin	B

Discussion

The activity of plant extracts and essential oils depends on the existing compounds of the spatial form and active groups and the mutual relationships between them (Alizadeh-Salteh, 2015; Azimi Zadeh and Ahmadi, 2018). The findings of this study indicate that all of the extracts investigated, with the exception of the aqueous extract of chestnut leaves, demonstrated antibacterial action against certain bacteria. From two environmental and microbiological perspectives, the differences can be explored. From the perspective of the habitat, it can be claimed that a number of variables, including the environmental and nutrient conditions of the habitats, have an impact on the quantity and type of secondary metabolites, including compounds with antimicrobial potential. A result of this, many bacteria exhibit sensitivity or resistance in various environments. The variations in antimicrobial activity demonstrate that, in addition to the habitats' nutritional and environmental parameters, a species' genetics and place of origin have an impact on its secondary metabolites and biological activity. In most of the studies conducted in the field of antimicrobial activity of plants, including the antimicrobial effects of plant essential oils, Gram-positive bacteria have a higher sensitivity compared to Gram-negative bacteria and fungi, which is due to the cell wall structure of Gram-positive bacteria and their higher permeability (Kazemi, 2015; Semeniuc *et al*., 2017; Abers *et al*., 2021). In this study, gram-positive bacteria exhibited higher sensitivity in the majority of cases, although gram-negative bacteria also occasionally displayed higher sensitivity, which may be related to their genetic makeup.

According to the data gathered, there have only been a few investigations on the chestnut's antibacterial ability (Agarwal *et al*., 2021; Yuan *et al*., 2022). The findings of the research on the aqueous extract of chestnut leaves presented in this article are in agreement with those of Borchardt *et al*. (2008), who looked into the antibacterial activities of native and local plants in the states of Wisconsin and Minnesota. Regarding *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*, they did not report any antimicrobial activity for chestnuts.

Conclusions

This study aimed to investigate the antimicrobial properties of the aqueous extract obtained from the leaves and catkins of chestnut trees cultivated in three different habitats, namely Argen, Babulah, and

Charbut. The effects of the extract were evaluated against four strains of gram-positive bacteria, including *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus*, and *Micrococcus luteus*, as well as three strains of gram-negative bacteria, namely *Escherichia coli*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica*. According to the findings of this study, the observed variations in antimicrobial activity indicate that, alongside environmental and nutritional factors pertaining to habitats, genetic makeup and species origin exert significant influence on the production of secondary metabolites and their resultant biological activity.

Acknowledgements

We would like to express our sincere gratitude to all those who have contributed to the successful completion of this project.

Conflict of interests

The authors declare no conflict of interest.

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