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Evaluation of Antibacterial Effect of Aqueous, Hydro-Alcoholic and Alcoholic Extracts of *Morus nigra* on Gram-Positive and Gram-Negative Bacteria

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ABSTRACT

Background: Microbial resistance to antibiotics has led to serious efforts to discover novel drugs, which is why there is so much interest in the use of herbs. Therefore, the aim of this study was to investigate the antibacterial effect of aqueous, hydro-alcoholic, and alcoholic extracts of *Morus nigra* in comparison with some common antibiotics.

Method: In this experimental study, the zone of inhibition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the Morus nigra extracts against Staphylococcus epidermidis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans and Streptococcus pyogenes were determined using the disk diffusion and broth macro-dilution methods. Statistical analysis was performed using the ANOVA test by SPSS software.

Results: The results of this study showed that all studied strains were sensitive to different extracts of Morus nigra. The highest antibacterial effect was related to the aqueous extract of Morus nigra, which created an inhibition zone with a diameter of 22.25 against Streptococcus mutans and Streptococcus pyogenes. The lowest inhibition zone (9 mm) was related to the alcoholic extract of Morus nigra against Pseudomonas aeruginosa. The MIC values of different extracts of Morus nigra against test bacteria varied from 0.78 to 3.12 mg/ml and MBC values were between 3.12 and 50 mg/mL.

Conclusion: Based on the findings of this study, the aqueous extract of Morus nigra has shown strong bactericidal properties against all studied bacterial strains, which indicates its potential for therapeutic application.

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1. Introduction

Antibiotics refer to a group of drugs that were first discovered in 1928 by the Scottish physician Alexander Fleming. This class of drugs is particularly effective in killing a variety of pathogenic bacteria, but has no effect on viruses [1]. Improper administration of antibiotics causes bacterial resistance. This not only increases mortality, but also leads to the increasing production of new antibiotics [2]. Antibiotic resistance is a specific feature in a microorganism in which a particular antibiotic is no longer affected by the bacterium when it was previously sensitive to the drug [3]. In medicine, the use of plant extracts and compounds with biological properties has become common, and their compounds are also one of the valuable resources in medicine. As a result, with the spread of infectious diseases, the identification of most of these extracts and compounds is needed. Plant-based antimicrobial compounds have numerous therapeutic properties. Herbal compounds are effective in treating infectious diseases and do not have the side effects of chemical antimicrobial compounds [4, 5]. Nowadays, the study of antimicrobial effects of plant extracts, especially plants that have traditionally been used as medicine, is one of the topics of interest to researchers [6-8]. Due to the adverse effects of chemical drugs, most countries in the world, including developed countries, have thought about changing the pattern of drug use from chemical to herbal, so that now about 50% of the drugs used in Europe are herbal, while this figure is very low in the country of Iran should look for a way to expand the herbal pharmaceutical industry as much as possible is of the great importance [9]. *Morus nigra* contains biologically active compounds such as alkaloids, carotenoids and flavonoids, vitamins, fats, sugars, and minerals. Antioxidant compounds (such as anthocyanins) in *Morus nigra* have potential effects on reducing the risk of cardiovascular diseases and cancer. It also has anti-inflammatory and anti-metastatic properties. *Morus nigra* is used as a medicine for the treatment of dysentery and it is used as a laxative, antiemetic and hypoglycemic. This fruit contains essential fatty acids that the human body is unable to synthesize and must be obtained through diet [10].

Staphylococcus aureus has undergone many genetic changes over the past 50 years. In recent years, the important role of *Staphylococcus aureus* in the development of nosocomial infections and the community has led to increased research on this bacterium [11]. *Staphylococcus epidermidis* is a gram-positive cocci and is the most important member of the coagulase-negative staphylococcus group and is responsible for 75% of infections in this group [12]. *Staphylococcus epidermidis* has long been known as a saprophyte due to its relatively poor pathogenicity. However, in recent decades, with the expansion of the use of medical devices embedded in the body, such as catheters and prostheses, it is recognized as an important hospital pathogen [13].

Streptococcus mutans is a gram-positive coccus and facultative-anaerobic that is the flora of the oral cavity in humans. This bacterium is the most important cause of caries and tooth decay [14]. *Streptococcus mutans* damages tooth enamel by fermenting sucrose and producing lactic acid. The bacterium also uses sucrose to make dental plaque. Dental plaque is made of dextran, a type of polysaccharide [15]. *Streptococcus pyogenes* (Group A β-hemolytic Streptococcus) is an important species of gram-positive and extracellular pathogenic bacteria. Group A streptococci that settle in the throat and skin are responsible for a variety of purulent infections and non-purulent consequences [16]. These bacteria are the most common causes of bacterial inflammation of the throat [17], scarlet fever and jaundice [18].

Pseudomonas aeruginosa is a gram-negative bacterium and opportunistic pathogen that has the ability to live in all environments and is the cause of many infections in humans such as endocarditis, meningitis, sepsis and chronic lung infections in patients with cystic fibrosis. *Pseudomonas aeruginosa* is an important and serious factor in nosocomial infections and mortality in patients with leukemia, severe burns and patients with cystic fibrosis. This bacterium is directly related to the increase in antibiotic resistance, so that about 30% of the nosocomial infections are related to the antibiotic resistance of this bacterium [19].

Various studies have been conducted on the medicinal properties of different types of Morus, including *Morus alba*, *Morus nigra* and berries, which determine their effectiveness in the treatment of diabetes, atherosclerosis, hyperlipidemia and hypertension [20]. *Morus nigra* is traditionally used to treat microbial infections [21]. The antibacterial activity obtained from the extract of the fruit and leaves of the *Morus nigra* was tested against 77 clinical strains and five bacterial species including *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, and *Staphylococcus aureus* [22].

Consumption of synthetic antibiotics on a regular basis often causes the microbes to become resistant to a particular antibiotic. Due to the fact that natural compounds are more compatible with the physiological conditions

of the body, the use of plants and plant extracts is a very good choice. Doing this kind of research can reduce the use of chemical antibiotics. The present study was performed to evaluate the antibacterial properties of *Staphylococcus aureus* on *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*.

2. Materials and Methods

Extraction

For this study, unripe fruits of *Morus nigra* in Bahram village located in the city of Saqez were used. These fruits were dried in the shade and the vicinity of air, then pulverized by an electric grinder. For extraction, the soaking method was used, which was done with water and different solvents. In this study, distilled water and Ethanol 96% (Taghtirkhorasan, Iran) in ratios of 1 to 20 were used to prepare the aqueous extract as well as prepare alcoholic and hydro-alcoholic extracts (Distilled water 30% and Ethanol 70%). For this purpose, 50 g of dry powder was weighed in a ratio of 1 to 20, and 1000 cc of the solvent was then added to it. The lid of the glass bottle was closed and wrapped with foil so that the solution inside the bottle was away from light. The aqueous extract was soaked for 72 hours and alcoholic and aqueous-alcoholic extracts were soaked for 48 hours. The whole extracts were then filtered through Whatman 2 filter paper. In the next step, a rotary device (Buchi-R100, Germany) was used to concentrate the extract (rotation was performed for 40°C at 60 rpm. At this temperature, the solvent evaporated and was removed from the solution). Finally, the extract was obtained with the desired purity. Then, the prepared extracts were packed in special containers, and the extracts of dry powder were prepared by a freeze dryer device. In this method, the solvent of the sample is frozen and then sublimated, and finally, the physical and chemical properties of the material will remain unchanged.

Bacteria used

Bacteria used in this study were *Staphylococcus epidermidis* PTCC1114, *Pseudomonas aeruginosa* PTCC1707, *Staphylococcus aureus* PTCC1189, *Streptococcus mutans* PTCC1683, and *Streptococcus pyogenes* PTCC1762. All bacterial species were obtained from the Scientific and Industrial Research Organization of Iran. To prepare the stock of bacteria, first, tryptone soy broth powder (Merk, Germany) was dissolved in distilled water and after boiling, 15% glycerol was added and sterilized in an autoclave. Then, in a completely sterile space under the laminar hood, 2 mL of the medium was poured into cryotubes and a bacterial loop was added to it, and incubated, and the prepared stocks were kept at freezer temperature.

Antimicrobial test methods

In order to perform the disk diffusion test, the Müller Hinton agar culture medium (Merck, Germany) was first prepared and transferred to 8-cm plates, and the microbial suspension was then prepared according to 0.5 McFarland turbidity. After preparing the homogeneous solution with a sterile swab, the solution was stirred, and after dewatering (pressing the swab to the wall of the tube to eliminate its water), it was cultured on Müller-Hinton agar medium. After culturing, the dried discs (Padtan Teb, Iran) impregnated with the extract were selected and transferred to the medium under the hood and sterile conditions using sterile forceps. On each plate, three discs were placed on the medium at a distance of 4 cm. After placing the discs in the plate, they were closed and kept in an incubator at 37°C for 24 hours. Next, the plate was examined and the diameter of the growth inhibition zone was measured using an accurate ruler. This experiment was performed in four replications to calculate the mean and standard deviation. A one-way ANOVA test was used to analyze the mean growth inhibition zone.

After performing the qualitative stage of disk diffusion and recording the results of this stage and ensuring that the plant extracts had antibacterial properties, determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts on the tested bacteria was performed by the following procedure: in this method, seven tubes were used. First, all tubes were sterilized immediately at 170°C, and to ensure sterility, all work steps were then performed under the hood and next to the flame. Initially, a microbial suspension was prepared according to 0.5 McFarland of each bacterium. In this method, tube number 7 was used as a control and test control and in the remaining six tubes, serial dilutions were prepared. Inside all the tubes, 1 cc of the microbial solution was added according to 0.5 McFarland. Then, serial dilutions of 1.2, 1.4, 1.8, 1.16, 1.32, and 1.64 of the extract were added to tubes 1 to 6, respectively. Next, all of them were incubated for 24 hours at 37°C. After 24 hours, they were surface cultured on a nutrient agar medium, and all the prepared plates were again incubated for 24 hours at 37°C. Finally, all the plates were checked. The first plate with half the number of colonies in the control plate was considered as MIC, and the first plate with 5 or fewer colonies was considered as MBC.

Table 1. Mean diameter of growth inhibition zone (mL) of *Morus nigra* extracts and standard antibiotics for different bacteria

Bacteria	Penicillin (10 µg)	Cefixime (5 µg)	Gentamicin (10 µg)	Tetracycline (30 µg)	Mean±SD		
					Aqueous extract (40 µl)	Alcoholic extract (40 µl)	Hydroalcoholic Extract (40 µl)
Staphylococcus epidermidis	17	12	22	-	22.0±0.8	16.5±0.6	18.75±0.9
Staphylococcus aureus	40	18	17	31	18.75±0.5	16.5±1.6	17.5±1.3
Streptococcus mutans	17	23±0.9	16	-	22.25±0.6	17.5±0.6	18.75±0.5
Streptococcus pyogenes	16	23	17	-	22.25±0.5	17.0±0.8	17.75±0.5
Pseudomonas aeruginosa	-	-	17	17	13.5±1.3	9.0±0.8	10.5±0.6

Analysis of diameter of growth inhibition zone against Staphylococcus epidermidis

3. Results and Discussion

The mean diameter of the growth inhibition zone of aqueous, hydro-alcoholic (ratio 30 to 70), and alcoholic (96% ethanol) extracts of *Morus nigra* was evaluated. Also, the mean diameter of the growth inhibition zone of penicillin, cefixime, gentamicin, and tetracycline antibiotics has been given in Table 1. According to this table, the highest antibacterial effect was related to the aqueous extract of *Morus nigra*, which created an inhibition zone with a diameter of 22.25 against the bacterial species of *Streptococcus mutans* and *Streptococcus pyogenes*, and the lowest inhibition zone (9 mL) was related to the alcoholic extract of *Morus nigra* against *Pseudomonas aeruginosa*. It is noteworthy that the solvent-containing disks (ethanol and DMSO) (negative control) did not show any growth inhibition zone.

As shown in Table 1, the largest diameter of the growth inhibition zone was related to the aqueous extract for *Staphylococcus epidermidis* with an average diameter of 22 mm, which is equal to the antibiotic effect of gentamicin against this bacterium, and the hydroalcoholic extract with the average diameter of growth inhibition zone of 18.75 mm is placed in next rank. Statistical analysis showed that the mean diameter of the growth inhibition zone of the aqueous extract was significantly higher than that of the alcoholic, hydroalcoholic extracts, and the penicillin, cefixime, and tetracycline antibiotics ($P<0.01$). Also, according to the table of standards for interpreting the diameter of growth inhibition zone (CLSI) [23], it was determined that *Staphylococcus epidermidis* is resistant to the penicillin, cefixime, and tetracycline standards so that the diameter of growth inhibition zone for them were 17, 12 and zero millimeters, respectively; but it is sensitive

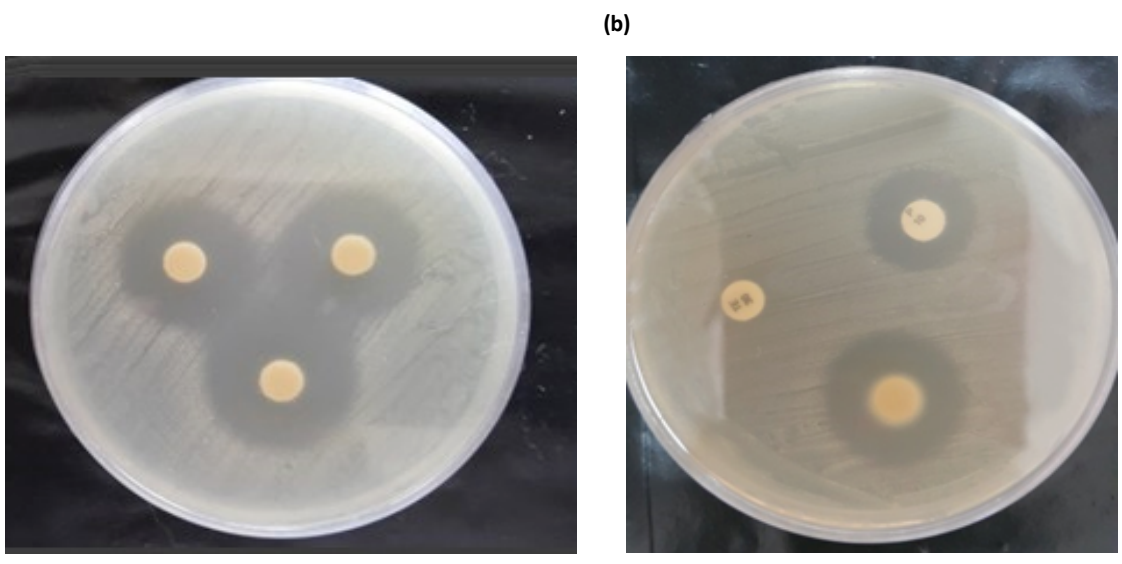

Figure 1. (a) The growth inhibition zone of aqueous-alcoholic extract of blackberry on Staphylococcus epidermidis and (b) the growth inhibition zone of aqueous extracts of *Morus nigra,* penicillin and tetracycline on *Staphylococcus epidermis*

to the gentamicin with a diameter of growth inhibition zone of 22 mm. The aura of lack of growth of aqueous-alcoholic extract of blackberry on *Staphylococcus aureus* and the aura of lack of growth of aqueous extracts of *Morus nigra*, penicillin and tetracycline on Staphylococcus epidermis have been presented in Figure 1.

Analysis of halos against *Staphylococcus aureus*

For *Staphylococcus aureus*, the strongest antibacterial effect with a growth inhibition zone of 40 and 31 mm was assigned to penicillin and tetracycline antibiotics, respectively, followed by aqueous extract of *Morus nigra*, which exhibited a relatively weak growth effect against this special bacterium by creating a growth inhibition zone of 18.75 mm. Also, statistical analysis showed that there was no significant difference between the mean diameter of the growth inhibition zone of the aqueous extract with hydroalcoholic and the antibiotic cefixime (P>0.05). According to the CLSI table, *Staphylococcus aureus* is sensitive to the antibiotic's penicillin, cefixime, and tetracycline, with a diameter of growth inhibition zones of 40, 18, and 31 mm, respectively; but it is resistant to the gentamicin antibiotic with a diameter of growth inhibition zone of 17. The aura of no growth of *Morus nigra* aqueous extract on *Staphylococcus aureus* and the aura of no growth of aqueous extract of *Morus nigra*, Cefixime and gentamicin on *Streptococcus pyogenes* have been presented in Figure 2.

Analysis of diameter of growth inhibition zone against *Streptococcus mutans*

For *Streptococcus mutans* bacteria, the largest diameter of the growth inhibition zone was related to the aqueous extract of *Morus nigra* with an average diameter of growth inhibition zone of 22.25 mm; it was slightly weaker than the cefixime, which produced a growth inhibition zone with a diameter of 23 mm against this bacterium. Statistical analysis showed that the mean diameter of the growth inhibition zone of aqueous extract was significantly higher than alcoholic, hydroalcoholic extracts, and antibiotics of penicillin, gentamicin, and tetracycline (P<0.01); but there was no significant difference between the mean diameter of growth inhibition zone of the aqueous extract with cefixime antibiotic (P>0.05). The inhibitory effect of hydroalcoholic extract of *Morus nigra* against *Streptococcus mutans* with an average diameter of growth inhibition zone of 18.75 mm was significantly different from all antibiotics (P<0.01) but differed only from the alcoholic extract by 5%. The effect of alcoholic extract for *Streptococcus mutans* was determined with an average diameter of 17.5 mm, which showed a weaker effect than aqueous and hydroalcoholic extracts. According to the CLSI table, this bacterium is resistant to penicillin and tetracycline antibiotics with a diameter of growth inhibition zone of 17 and zero, respectively, and was sensitive to cefixime and gentamicin with a diameter of growth inhibition zone of 23 and 16 mm, respectively. The aura of no growth of aqueous extract of *Morus nigra*, tetracycline and penicillin on *Streptococcus mutans* have been presented in Figure 3.

Analysis of diameter of growth inhibition zone against *Streptococcus pyogenes*

The largest diameter of the growth inhibition zone was related to the aqueous extract of *Morus nigra* with an average halo diameter of 22.25 mm, which is significantly higher than the effect of alcoholic, hydroalcoholic extracts and penicillin, gentamicin, and tetracycline antibiotics against *Streptococcus pyogenes* (P<0.01); but there

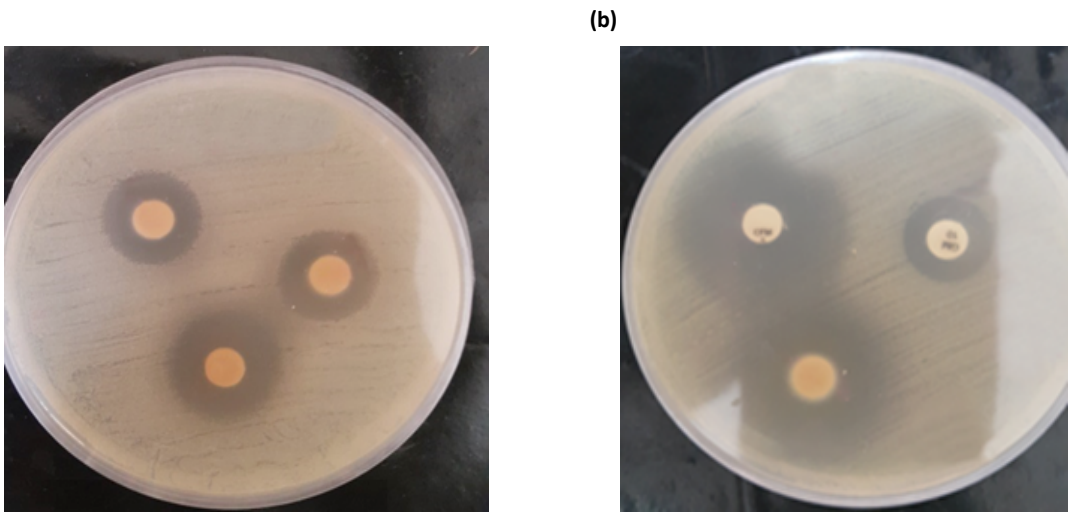

Figure 2. (a) The growth inhibition zone of Moros nigra aqueous extract on Staphylococcus aureus, and (b) the growth inhibition zone of aqueous extract of *Morus nigra*, cefixime and gentamicin against *Streptococcus pyogenes*.

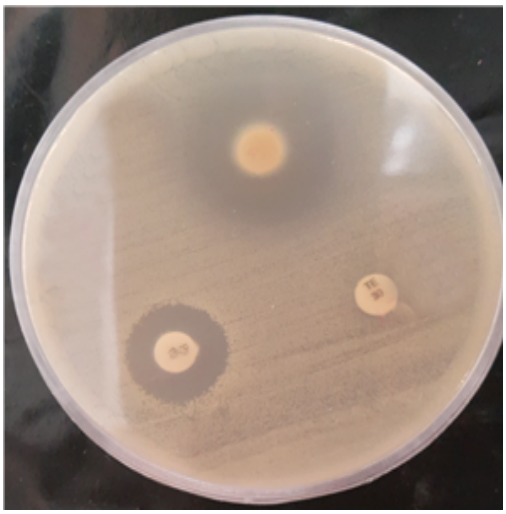

Figure 3. Growth inhibition zone of aqueous of *Morus nigra,* tetracycline and penicillin on *Streptococcus mutans*

was no significant difference between the mean diameter of the growth inhibition zone of the aqueous extract with cefixime antibiotic (P>0.05). The inhibitory effect of the hydroalcoholic extract on *Streptococcus pyogenes* with a mean diameter of growth inhibition zone of 17.75 mm was significantly different at 1% level with penicillin, cefixime, and tetracycline antibiotics but there was no significant difference with *Morus nigra* alcoholic extract and gentamicin antibiotic. The effect of the alcoholic extract on *Streptococcus pyogenes* with an average diameter of the inhibition growth zone of 17 mm was found to be weaker than that of aqueous and hydro-alcoholic extracts (P<0.01). According to the CLSI table, this bacterium is resistant to penicillin and tetracycline antibiotics with a diameter of the inhibition growth zone of 16 and zero, respectively, and was sensitive to cefixime and gentamicin antibiotics with a diameter of the inhibition growth zone of 23 and 17 mm, respectively. The aura of no growth of the aqueous extract of *Morus nigra*, tetracycline and penicillin on *Streptococcus pyogenes* are presented in Figure 4.

Analysis of diameter of growth inhibition zone against *Pseudomonas aeruginosa*

The antibiotics gentamicin and tetracycline had a similar resistance effect against *Pseudomonas aeruginosa*, with an average diameter of 17 mm, which is much higher than the resistance of the aqueous extract of *Morus nigra* with a diameter of growth inhibition zone of 13.5 against this bacterium and significance level of 1% was observed. According to the CLSI table, *Pseudomonas aeruginosa* is resistant to penicillin and cefixime with a diameter of zero; but it is semi-sensitive to tetracycline antibiotics with a diameter of growth inhibition zone of

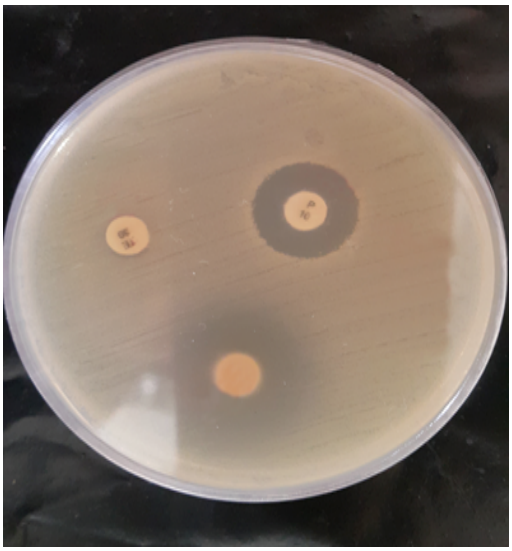

Figure 4. Growth inhibition zone of aqueous extract of *Morus nigra,* tetracycline and penicillin on *Streptococcus pyogenes*

Table 2. MIC (mg/mL) of different extracts of *Morus nigra* fruit against the tested bacteria

Bacteria \ Extract s	Aqueous	Alcoholic	Hydroalcoholic
Staphylococcus epidermidis	1.56	3.12	1.56
Staphylococcus aureus	1.56	3.12	1.56
Streptococcus mutans	1.56	3.12	1.56
Streptococcus pyogenes	1.56	3.12	1.56
Pseudomonas aeruginosa	0.78	3.12	1.56

17 and is sensitive to gentamicin antibiotics with a diameter of growth inhibition zone of 17 mm.

According to Table 2, the aqueous and hydroalcoholic extracts of *Morus nigra* showed a similar antibacterial effect and the corresponding MIC value against Staphylococcus and *Streptococcus genus* bacteria was 1.56 mg/mL. The MIC of the aqueous extract was lower against *Pseudomonas aeruginosa* (MIC=0.78); this indicates that this species is more sensitive to the aqueous extract of *Morus nigra* compared to other species.

For two bacteria belonging to the genus Staphylococcus, the MBC had a similar pattern, and the lowest bactericidal concentration was related to the aqueous and hydro-alcoholic extract of *Morus nigra* with a dose of 12.5 mg/mL. Aqueous extract of *Morus nigra* had a MBC of 12.5 mg/mL against mutans and pyogenes strains of the *Streptococcus genus*. The highest antibacterial activity was related to aqueous extract on *Pseudomonas aeruginosa* with an MBC of 3.12, and the hydroalcoholic extract with MBC equal to 6.25 mg/mL was in the next rank (Table 3).

Among all the extracts, according to the results, the aqueous extract of *Morus nigra* had the highest yield and efficiency. The extraction efficiency seems to have increased with increasing solvent polarity. Among the solvents used, water has the highest polarity index, which may be effective in obtaining high efficiency of the aqueous extraction, and probably the major part of the composition of *Morus nigra* is polar material.

There is evidence in the literature that gram-positive bacteria are more sensitive to essential oils and plant extracts than gram-negative bacteria [24, 25]. Due to the hydrophobic lipopolysaccharide in the outer membrane, which provides protection against various factors [26]; in our results, it was found that only the gram-negative bacteria (*Pseudomonas aeruginosa*) showed less sensitivity to the extract of *Morus nigra* compared to the gram-positive bacteria (Staphylococcus and *Streptococcus* studied in this study).

However, the aqueous extract of the *Morus nigra* fruit in this study with an average diameter of growth inhibition zone of 13.5 mm showed a greater inhibitory effect compared to the results of Sj and Mahmood [27] that obtained the diameter of growth inhibition zone of 11 mm for the methanolic extract of *Morus nigra* leaves against *Pseudomonas aeruginosa*.

Yiğit and Yiğit tested the antibacterial activity of aqueous and methanolic extracts of *Morus alba* and *Morus nigra* fruit and leaves against 5 bacterial species and 77 clinical strains by the disk diffusion method. Using aqueous and methanolic extracts of *Morus nigra*, Enterobacter aeruginosa, Proteus mirabilis, and *Pseudomonas ae-*

Table 3. MBC (mg/mL) of different extracts of *Morus nigra* fruit against the tested bacteria

Bacteria \ Extracts	Aqueous	Alcoholic	Hydroalcoholic
Staphylococcus epidermidis	12.5	25	12.5
Staphylococcus aureus	12.5	25	12.5
Streptococcus mutans	12.5	50	25
Streptococcus pyogenes	12.5	50	12.5
Pseudomonas aeruginosa	3.12	12.5	6.25

ruginosa showed no growth inhibition zone. However, in our study, the aqueous, hydro-alcoholic, and alcoholic extracts of *Morus nigra* with a mean diameter of growth inhibition zone of 13.5, 10.5, and 9 mm, respectively, showed a stronger effect on *Pseudomonas aeruginosa*. The highest diameter of growth inhibition observed for the aqueous extracts of *Morus nigra* fruit was related to aqueous *Morus nigra* against *Staphylococcus aureus* with a diameter of 10 mm and aqueous extract of *Morus nigra* leaf with a diameter of 15 mm [22]. In the results of the present study, aqueous, hydro-alcoholic, and alcoholic extracts of *Morus nigra* showed a stronger effect against *Staphylococcus aureus* with a growth diameter of growth inhibition zone of 18.75, 17.5, and 16.5 mm, respectively. This difference may be due to differences in the extraction method and the fruit used.

In the study by Khalid et al., the antimicrobial activity of fresh *Morus nigra* juice in vitro against gram-positive and gram-negative bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Bacillus spizizenii*, *Bacillus subtilis*, and *Corynebacterium diphtheria*) was studied. In their results, the diameter of growth inhibition zone was observed against all the bacterial species, illustrating the confirmation of the antibacterial activity of *Morus nigra* water. The maximum inhibition zone against *Pseudomonas aeruginosa* was 19.87 mm. This is in contrast to the results of the present study in which the least growth inhibition zone was observed for *Pseudomonas aeruginosa* [28]. The main reason for this difference could be that they used fresh *Morus nigra* juice and did not extract it. In the present study, the mean diameter of growth inhibition zone in *Staphylococcus aureus* for the methanolic extracts of *Morus nigra* fruit and aqueous-alkyl extract (30% distilled water - 70% ethanol) of immature *Morus nigra* fruit were 10 and 17.5 mL, respectively, for *Pseudomonas aeruginosa* was also obtained 0 (None) and 10.5 mL, respectively.

In 2015, Shukla et al. performed the approximate and phytochemical analysis of *Morus nigra* using the standard protocols as well as antimicrobial properties of the seeds on gram-negative and gram-positive bacteria. The antibacterial activity of the *Morus nigra* seed was measured against two gram-positive bacteria, i.e., Bacillus cereus and *Staphylococcus aureus*, and two gram-negative bacteria, i.e., Salmonella and *Pseudomonas aeruginosa*. The largest diameter of the growth inhibition zone of ethyl acetate extract among gram-negative bacteria was related to *Pseudomonas aeruginosa* with a diameter of the growth inhibition zone of 13.82 mm, which was more than the reported 13.5 mm in the extract of *Morus nigra* fruit in our work. Among gram-positive bacteria, the largest diameter of growth inhibition zone was related to *Staphylococcus aureus* with a diameter of growth inhibition zone of 12.89 mm [29], which was less than the bacterial resistance of *Morus nigra* extracts against gram-positive bacteria tested in present study.

Mazimba et al. studied the antioxidant and antibacterial compounds of the root and bark of the *Morus nigra* tree. The aim of their study was to isolate specific compounds from *Morus nigra* to compare antioxidant compounds and antibacterial activities with other types of Morus. The MIC of the bark of the stem was reported against *Staphylococcus aureus* was reported to be 125 µg/mL and the MIC of the bark of the stem of *Morus nigra* for *Pseudomonas aeruginosa* was reported to be 500 µg/mL. The MIC of *Morus nigra* wood against *Staphylococcus aureus* was 62.5 and was 500 µg/mL against *Pseudomonas aeruginosa* [30]. In the results of the present study, the MIC value of aqueous extract of *Morus nigra* fruit (0.78 mg/mL) for Pseudomonas gram-negative bacteria was relatively higher than other studies, which was 0.625 mg/mL for aqueous extract of *Morus nigra* against *Pseudomonas aeruginosa* and was also 0.625 mg/mL for its methanolic extract.

In a 2008 study by Yiğit and Yiğit, the MIC of the aqueous extract of *Morus nigra* for *Staphylococcus aureus* was 0.625 mg/mL and the MIC of its methanolic extract against this bacterium was 1.25 mg/mL, which had a significant difference in comparison with MIC results of different extracts against gram-positive bacteria obtained in our study. The extract of *Morus nigra* fruit in the present study had a greater effect compared to the aqueous extract of *Morus nigra* leaves in the study by Yigit, which had a MIC of 0.156 mg/mL to stop the growth of Staphylococcus, and the MIC of its methanolic extract was 0.312 mg/mL [22]. The disk diffusion results showed that the extracts had less effect on gram-negative bacteria; on the other hand, the broth dilution results showed that the extracts had a greater effect on gram-negative bacteria. This inconsistency can only be explained by the fact that polar compounds are better released in the broth by the broth dilution method and had an antimicrobial effect.

4. Conclusion

However, the results obtained from this study confirmed the antimicrobial activity of the *Morus nigra* extract. Aqueous, alcoholic, and hydroalcoholic extracts of *Morus nigra* had a growth inhibitory effect on all five species of bacteria studied, and in most cases, the *Morus nigra* aqueous ex-

tract showed stronger antibacterial activity than chemical antibiotics. This antimicrobial property is probably related to polar and phenolic compounds (including flavonoids, flavones, isoflavones, isoprenylates, acetylbones, quarines, chromiums and xanthans) which are associated with the effect on the bacterial wall causes antimicrobial properties on these bacteria. Hence, further laboratory research for clinical use of this herbal medicine is required.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Ardabil University of Medical Sciences (Code: 117482739377495139912719 8).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] Dillard LK, Wu CZ, Saunders JE, McMahon CM. A scoping review of global aminoglycoside antibiotic overuse: A potential opportunity for primary ototoxicity prevention. Res Social Adm Pharm. 2022; 18(8):3220-9. [PMID]

[2] Shama G. La Moisissure et la Bactérie: Deconstructing the fable of the discovery of penicillin by Ernest Duchesne. Endeavour. 2016; 40(3):188-200. [PMID]

[3] Gopal P, Dick T. Targeted protein degradation in antibacterial drug discovery? Prog Biophys Mol Biol. 2020; 152:10-4. [PMID]

[4] Prasathkumar M, Anisha S, Dhrisya C, Becky R, Sadhasivam S. Therapeutic and pharmacological efficacy of selective Indian medicinal plants-A review. Phytomed Plus. 2021; 1(2):100029. [DOI:10.1016/j.phyplu.2021.100029]

[5] Rajput M, Kumar N. Medicinal plants: A potential source of novel bioactive compounds showing antimicrobial efficacy against pathogens infecting hair and scalp. Gene Rep. 2020; 21:100879. [DOI:10.1016/j.genrep.2020.100879]

[6] Xu WH, Liang ZS, Su XM, He RX, Liang Q. Genus Picrasma: A comprehensive review on its ethnopharmacology, phytochemistry and bioactivities. J Ethnopharmacol. 2021; 280:114441. [DOI:10.1016/j.jep.2021.114441] [PMID]

[7] Dai J, Han R, Xu Y, Li N, Wang J, Dan W. Recent progress of antibacterial natural products: Future antibiotics candidates. Bioorg Chem. 2020; 101:103922. [PMID]

[8] Moshafi M, Forutan H, Mehrabani M. Investigation of antibacterial effects of *Hibiscus Sabdariffa* L. dried calyx by agar diffusion and bioautographic methods. J Kerman Univ Med Sci. 2006(2):103-10. [Link]

[9] Azaizeh H, Saad B, Khalil K, Said O. The state of the art of traditional Arab herbal medicine in the Eastern region of the Mediterranean: A review. Evid Based Complement Alternat Med. 2006; 3(2):229-35. [PMID]

[10] Farahani M, Salehi-Arjmand H, Khadivi A, Akramian M. Chemical characterization and antioxidant activities of *Morus alba* var. nigra fruits. Sci Hortic. 2019; 253:120-7. [DOI:10.1016/j.scienta.2019.04.040]

[11] Kathirvel K, Rudhra O, Rajapandian SGK, Venkatesh Prajna N, Lalitha P, Devarajan B. Characterization of antibiotic resistance and virulence genes of ocular methicillin-resistant Staphylococcus aureus strains through complete genome analysis. Exp Eye Res. 2021; 212:108764. [PMID]

[12] Doyle M, Boyle B, Brennan C, Holland J, Mifsud A, Hell M, van Tiel F, Leegaard TM. Specialist training in medical microbiology across Europe in 2021-an update on the actual training situation based on a survey. Clin Microbiol Infect. 2021; 27(11):1576-1580. [PMID]

[13] Noshak MA, Rezaee MA, Hasani A, Mirzaii M, Memar MY. Biofilm formation capacity in common SCCmec types of coagulase-negative staphylococci isolated from hospitalized patients and health-care workers in northwest of Iran. Gene Rep. 2019; 17:100531. [DOI:10.1016/j.genrep.2019.100531]

[14] Atafar Z, Almasi A, Sarkhosh M, Dargahi A. Microbiological quality trend of drinking water in rural areas of Kermanshah during 2004-2013. J Environ Health Engin. 2015; 3(1):10-9. [Link]

[15] Amissah F, Andey T, Ahlschwede KM. Nanotechnology-based therapies for the prevention and treatment of Streptococcus mutans-derived dental caries. J Oral Biosci. 2021; 63(4):327-36. [PMID]

[16] Lam PW, Chan AK, Chau NE, Clark ST, Kozak RA. Streptococcus pyogenes secondary impetigo due to loofah sponge use. IDCases. 2022 ; 27:e01395. [PMID]

[17] Fox JW, Marcon MJ, Bonsu BK. Diagnosis of streptococcal pharyngitis by detection of Streptococcus pyogenes in posterior pharyngeal versus oral cavity specimens. J Clin Microbiol. 2006; 44(7):2593-4. [PMID]

[18] McLellan D, Dowse GK, Feeney KT, Mackaay C, Keil AD. Epidemiological features and control of an outbreak of

scarlet fever in a Perth primary school. Commun Dis Intell Q Rep. 2005; 29(4):341-452. [**Link**]

[19] Dey N, Kamatchi C, Vickram AS, Anbarasu K, Thanigaivel S, Palanivelu J, et al. Role of nanomaterials in deactivating multiple drug resistance efflux pumps: A review. Environ Res. 2022; 204(Pt A):111968. [PMID]

[20] Nair N, Yadav S, Biharee A, Prathap VM, Majeed J. Updated Ethnobotanical Notes, Phytochemistry and Phytopharmacology of plants belonging to the *genus Morus* (Family: Moraceae). Phytomed Plus. 2022; 2(1):100120. [DOI:10.1016/j.phyplu.2021.100120]

[21] Atchudan R, Edison TN, Perumal S, Vinodh R, Sundramoorthy AK, Babu RS, et al. Morus nigra-derived hydrophilic carbon dots for the highly selective and sensitive detection of ferric ion in aqueous media and human colon cancer cell imaging. Colloids Surf. A Physicochem Eng Asp. 2022; 635:128073. [DOI:10.1016/j.colsurfa.2021.128073]

[22] Yiğit D, Yiğit N. Antibacterial activty of black mulberry (*Morus nigra*) fruits and leaves. Erzincan Univ J Sci Technol. 2008; 1(1):39-48. [Link]

[23] Hsueh PR, Ko WC, Wu JJ, Lu JJ, Wang FD, Wu HY, et al. Consensus statement on the adherence to Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Testing Guidelines (CLSI-2010 and CLSI-2010-update) for Enterobacteriaceae in clinical microbiology laboratories in Taiwan. J Microbiol Immunol Infect. 2010; 43(5):452-5. [DOI:10.1016/S1684-1182(10)60070-9]

[24] Bouyahya A, Et-Touys A, Bakri Y, Talbaui A, Fellah H, Abrini J, et al. Chemical composition of Mentha pulegium and Rosmarinus officinalis essential oils and their antileishmanial, antibacterial and antioxidant activities. Microb Pathog. 2017; 111:41-9. [PMID]

[25] Brahmi F, Abdenour A, Bruno M, Silvia P, Alessandra P, Danilo F, et al. Chemical composition and in vitro antimicrobial, insecticidal and antioxidant activities of the essential oils of *Mentha pulegium* L. and *Mentha rotundifolia* L. Huds growing in Algeria. Ind Crops Prod. 2016; 88:96-105. [DOI:10.1016/j.indcrop.2016.03.002]

[26] Fatima H, Goel N, Sinha R, Khare SK. Recent strategies for inhibiting multidrug-resistant and β-lactamase producing bacteria: A review. Colloids Surf B Biointerfaces. 2021; 205:111901. [PMID]

[27] Sj AR, Mahmood R. Anthelmintic and antimicrobial activities in some species of mulberry. Int J Pharm Pharm Sci. 2012; 4:335-8. [Link]

[28] Khalid N, Fawad SA, Ahmed I. Antimicrobial activity, phytochemical profile and trace minerals of black mulberry (*Morus nigra* L.) fresh juice. Pak J Bot. 2011; 43(6):91-6. [Link]

[29] Shukla RK, Painuly D, Shukla A, Kumar V, Singh J, Porval A, et al. Physical evaluation, proximate analysis and antimicrobial activity of *Morus nigra* seeds. Int J Pharm Pharm. 2015; 191-7. [Link]

[30] Mazimba O, Majinda RRT, Motlhanka D. Antioxidant and antibacterial constituents from *Morus nigra*. Pharm Pharmacol. 2011; 5(6):751-4. [DOI:10.5897/AJPP11.260]