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ORIGINAL: In-vitro Antibacterial Effects of Aqueous, Ethanolic, Methanolic, Acetone and Hydro Ethanolic Extracts of *Achillea Millefolium* on Standard *Klebsiella, S. pyogenes,* and Oral Bacterias Strains

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ABSTRACT

Introduction: So far, a lot of attempts have been carried out to find antimicrobial compounds. In this study, it was also tried to investigate the antibacterial effects of *Achillea millefolium* on standard *Klebsiella, S. pyogenes*, and oral bacterias strain.

Material and Methods: The aerial parts of *Achillea millefolium* were used and the aqueous, ethanolic, methanolic, acetone and hydroethanolic extracts were prepared. After the preparation of standard strains of *Klebsiella, S. pyogenes*, and oral bacterias and sterilization of extracts by the Millipore filter, the antibacterial effects of these extracts on the mentioned microorganisms were assessed by minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and well diffusion at the concentration 50 mg/ml. The test was repeated three times for each bacterium.

Results: It was exposed that aqueous extract of *Achillea millefolium* had the most distinguished antimicrobial effects against all studied strains and methanolic extract had antimicrobial effects only on S. pyogenes. MIC and MBC of effective extracts were the basic concentration (50mg/ml), and non-growth zone was not observed in other serial dilution in case of all bacteria.

Conclusion: The *Achillea millefolium* can be admitted as an antibacterial medicinal herb. Thus, it can be concluded that after evaluating their effects in vitro, *Achillea millefolium* can be utilized as an alternative to the routine chemical drugs.

Introduction

Medicinal plants and medicines made from them have always been considered in human history due to their extensive healing properties Although chemical and industrial materials are increasingly used in pharmacy, the use of medicinal plants still has its fans (1). Research has shown that some herbs used in traditional medicine have antibacterial effects (2). Medicinal plants reservoirs are rich in the active ingredients that can be used in many drugs, these substances are mainly secondary metabolites of the plant (3). Among all the identified substances in the effective compounds of plants, phenolic compounds (secondary compounds without nitrogen) are the most important substances that have biological effects, including

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effective antibacterial activity (4).

The continued use of chemical drugs has led to highly resistant microbes and sometimes antibiotics have little effect on them; as a result, patients need stronger antibiotics and chemical drugs that enter the market every day (5). However, many medicinal plants, while having many positive effects, have fewer harms and side effects than industrial ones. For this reason, many researchers have studied the antibacterial effects of plant extracts (6).

Moreover, tooth decay is a disease that is actually caused by oral bacteria that dissolve and destroy tooth calcareous tissue. Although tooth decay is probably the most common chronic infectious disease in the world, no plans have yet been performed to eradicate it. Tooth loss is one of its continued disease complications (7,8), therefore, this issue is so important. Eliminating bacteria in the mouth can prevent tooth decay; for this purpose, in recent years, many herbal mouthwashes have been introduced by various manufacturers, which according to their brochure have antifungal, antibacterial, and antiviral properties.

Studies have shown that many plants, such as *Achillea millefolium* extract from the family *Asteraceae*, have antibacterial effects and can be used as antimicrobial agents in the treatment of infections. *Achillea millefolium* is a perennial plant that grows in various parts of Europe and Asia (9). *Achillea millefolium* contains alkaloids, saponins, and phenolic compounds such as flavonoids and phenolic carbons. The anti-inflammatory, antimicrobial and antioxidant properties of this plant are mainly attributed to flavonoids (10). The aim of this study was to evaluate the antibacterial activity of *Achillea millefolium* in comparison with standard and common therapeutic antibiotics against the standard bacterial strains of *Klebsiella*, *S. pyogenes*, and oral bacterias.

Methods

Preparation of plant samples for extraction

In this study, Achillea *millefolium* plant was prepared from Isfahan Medicinal Plants Center. They were then dried in the shade at room temperature and powdered by an electric grinder. Extraction of the extracts was performed by maceration method using water, ethanol, methanol, acetone and hydro ethanol solvents.

Aqueous, ethanolic, methanolic, acetone and hydro ethanolic extracts of Achillea millefolium

For preparing the cold extract, 50 g of the plant was first poured into a sterilized glass jar and 300 ml of solvent was added. It was maintained for four days at room temperature without light, and the glass containing the plant and solvent was gently stirred every day without opening the container. After extracting, all extracts were filtered using sterile gauze. The extra ingredients in the extracts were then smoothed out with Whatman filter paper and a glass funnel. In the next step, the extracted extracts were poured into a sterile plate and dried at room temperature, then 50 mg of the dried extract was transferred to 1 ml of solvent. Two types of solvents including water and Dimethyl sulfoxide (DMSO) were used to dissolve the various extracts. The aqueous extract of *Achillea millefolium* in water and other extracts in DMSO were completely dissolved.

Preparing the bacteria

In this study, two bacteria, *Klebsiella* and *S. pyogenes* were provided from the Isfahan University of Medical Sciences and were examined. The bacteria were first cultured on QUELAB's Mueller Hinton Agar medium. To prepare a microbial suspension, one to several clones of the microorganism were added to a sterile tube containing 5 ml of sterile water so that it had a standard equivalent of 0.5 McFarland, then it was checked by spectrophotometer.

Also, in order to investigate the effect of *achillea millefoliumon* extracts on bacteria that cause tooth decay, oral sampling was performed by a sterile swab and cultured on the environment of Mueller Hinton Agar

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medium while maintaining sterile conditions.

Investigation of the antibacterial effect of extracts

The Agar Well Diffusion method was used according to the Bakri & Douglas protocol to investigate the effect of *Achillea millefolium* extracts on *Klebsiella*, *S. pyogenes*, and oral bacteria. To do this, first, the Mueller Hinton Agar culture medium was prepared on an 8 cm plate, Bacteria were then cultured on each plate, and five well were created in the medium using a pasteurized Pasteur pipette. To ensure the accuracy of the test and comparison conditions, Gentamicin and Nalidixic acid Antibiotic discs from Padtan Teb Company were located by distance in two wells (positive control). DMSO was also used as a negative control sample in one of the wells. To ensure the result obtained, the plant extract suspension was poured into two wells. Each extract was studied on *Klebsiella*, *S. pyogenes*, and oral bacteria, and each test was repeated twice. Finally, all the plates were incubated for 24 hours at 37˚C.

Investigation of MIC and MBC

Due to the color of the plant extracts and the possibility of misunderstanding of turbidity and growth, in this study, instead of using culture tubes, the well diffusion method were used to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). For this purpose, wells were made on the Mueller Hinton Agar media and 0.5 McFarland microbial suspension was prepared. Then, complete cultivation was performed by Swab Sterile from this suspension. Then 5 dilutions of each extract were prepared and different dilutions of each extract were added to the wells by a sampler. Next, the plates were incubated for 24 hours at 37˚C. Finally, the ability of the extracts to inhibit bacterial growth was assessed on the basis of a non-growth halo around the discs. The lowest dilution of any extract that caused a halo of non-growth was considered as the MIC of that extract. To determine the lowest concentration of extracts, samples from the non-growth area of each dilution by Loop Sterile were obtained and linearly cultured on Mueller Hinton Agar medium and incubated for 24 hours at 37 ˚C. The lowest concentration of the extracts, which inhibit bacteria growth and had bactericidal effect was considered as the MBC of the extract.

Results

According to ***Table 1***, the antibacterial activity of Aqueous, ethanolic, methanolic, acetone and hydro ethanolic extracts of *Achillea millefolium* and qualitative methods showed that some of this extract has a significant inhibitory effect on *Klebsiella*, *S. pyogenes*, and oral bacteria showed in ***table1***.

Table 1. Mean diameter of non-growth zone of Aqueous, ethanolic, methanolic, acetone and hydro ethanolic extracts of *Achillea millefolium* against selected bacteria in millimeters.

Bacteria Strain	Concentration (mg/ml)						
	Aqueous extract 50 mg/ml	Ethanolic extract 50 mg/ml	Methanolic extract 50 mg/ml	Acetone extract 50 mg/ml	Hydro ethanolic extract 50 mg/ml	Nalidixic acid	Gentamicin
Klebsiella	12	-	-	-	-	20	20
S. pyogenes	12	-	12	-	-	15	30
oral bacteria	11	-	-	-	-	N/A	N/A

Table 2. MIC values of Aqueous, ethanolic, methanolic, acetone and hydro ethanolic extracts of *Achillea millefolium* (mg/ml)

Bacteria Strain	Substance				
	Aqueous extract	Ethanolic extract	Methanolic extract	Acetone extract	Hydro ethanolic extract
Klebsiella	50	-	-	-	-
S. pyogenes	50	-	50	-	-
oral bacteria	50	-	-	-	-

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Table 3. MBC values of Aqueous, ethanolic, methanolic, acetone and hydro ethanolic extracts of *Achillea millefolium* (mg/ml)

Bacteria Strain	Substance				
	Aqueous extract	Ethanolic extract	Methanolic extract	Acetone extract	Hydro ethanolic extract
Klebsiella	50	-	-	-	-
S. pyogenes	50	-	50	-	-
oral bacteria	50	-	-	-	-

Discussion

In recent years, extensive research has been conducted on the antimicrobial effects of various plants and it has approved that some plants have effects similar to or far more than chemical drugs (11). *Achillea millefolium* is also a traditional medicinal plant that has various uses in traditional medicine and is used for fever, nasal congestion, stomach pain, bleeding, and so on. According to a study, methanolic extracts of the leaves and flowers of this plant had significant effects against gram-positive bacteria such as Staphylococcus aureus and Bacillus cereus, but this extracts had a weaker effect on gram-negative bacteria (12). Cellular wall polysaccharides were likely to prevent the active ingredients of essential oils and extracts from reaching the cytoplasmic membrane of gram-negative bacteria (13). In general, plant products lead to cell membrane rupture (14), inactivation, or preventing the activity of the intracellular and extracellular enzyme (15), and cell membrane elimination (16) that these results have shown many times in different researches. Most herbal extracts have a more deterrent effect on gram-positive bacteria and less on gram-negative bacteria, of which Pseudomonas aeruginosa is the most resistant bacteria to plant extracts (12).

According to reports, the *Achillea millefoliumon* plant is rich in flavonoids and sesquiterpene lactone. The best solution for extraction of flavonoids is methanol. On the other hand, methanolic extracts, in addition to flavonoids, alkaloids, saponins, tannins, and anthraquinones, could extract tarpaulins (17). It appears that the antimicrobial properties of methanolic extracts are related to the presence of secondary metabolites, especially flavonoids, terpenes, Saponins.

In a study by Khalili Dehkordi et al. to investigate the effect of plant extracts of *Achillea millefolium*, on the Trichomonas vaginalis parasite under laboratory conditions, the results showed the extract of this plant reduced significantly the number of parasites (18). Also, Aljancic et al. reported that *Achillea millefolium* has a significant inhibitory effect on Candida albicans and Bacillus soblis. According to this researcher, the flavonoids in *Achillea millefolium* essential oil, in addition to the inhibitory effect on the two microorganisms mentioned above, also have an inhibitory effect on Aspergillus niger (19). Another study looked at the antifungal effect of the aqueous and alcoholic extract of *Achillea millefolium* on Candida albicans. In this study, the antifungal effect of alcoholic extract was significantly higher than the aqueous extract of *Achillea millefolium*. Also, the results showed that the effect of miconazole 2% was significantly higher than the concentrations of aqueous and alcoholic extracts of *Achillea millefolium* (20). Fathi et al. examined the antimicrobial properties of chloroform, water, and ethyl acetate extracts and said that there was no significant antifungal activity in the water-soluble parts of the plan (21). Tuberoso obtained a similar result and reported little activity on its antifungal properties, including Candida albicans (22).

Conclusion

The results of this study showed that aqueous extract of *Achillea millefolium* flower has antibacterial effects on *Klebsiella*, *S. pyogenes*, and oral bacteria. It seems that the antimicrobial effects of aqueous extract was

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greater than those of other extracts. It is hoped that in the future the effective ingredients of yellow will be used as antimicrobial substances, but more in vitro and in vitro research needs to be done before applying them.

Conflicts of interest

Authors declare that there is no conflict of Interests.

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