



Biological and Antibacterial Features of *Acroptilon repens* (L.) DC

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Abstract

Objectives: *Acroptilon repens* (L.) DC is an Asian native plant belongs to the Asteraceae family. It has been cultivated the United States and Canada in recent years. This study investigated the effects of antibacterial, anti-biofilm, and cytotoxicity of *A. repens* ethanolic extract and its components.

Materials and Methods: We used disk diffusion and well diffusion strategies to screen antimicrobial activity of *A. repens* against *Staphylococcus aureus* and *Staphylococcus epidermidis*. Then, the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of extract and fractions were determined by the small dilution broth methodology for *S. aureus* and *S. epidermidis*. The impact of extract and fractions on biofilm formation was evaluated by microtiter plate assay. The MTT assay determined the toxicity effect of samples on Vero cells. The results are expressed because of the viability.

Results: Results showed that *A. repens* extract and fragments inhibit the growth of gram-positive bacteria. The ethanolic extract and its fractions showed stronger inhibition zones for *S. aureus* and *S. epidermidis*. According to the antibiofilm test, the ethanolic extract and water fraction of *A. repens* have the highest antibiofilm effect on *S. aureus* and *S. epidermidis*. The value of LC₅₀ (50% mortality) in MTT assay results was 30, 60, 30, and 2/5 mg/mL for ethanolic extract, chloroform, ethyl acetate and water fractions of *A. repens*, respectively.

Conclusions: Extract of *A. repens* and its fractions have an antibacterial and antibiofilm effect on *S. aureus* and *S. epidermidis* without significant cytotoxic effect.

Keywords: *Acroptilon repens*, Antibacterial, Antibiofilm, MTT assay

Introduction

The antimicrobial effects of herbal medicine have been seen in treating various human diseases (1). About 60%-90% of people in developing countries use herbs because they believe that herbs are a potential source of antimicrobial agents. Crude plant extract is used to treat microbial diseases. Plant chemicals include tannins, terpenoids, alkaloids, and flavonoids. The results of laboratory studies show that these substances have antimicrobial properties. However, the mechanism of action and effectiveness of these plant extracts in most cases have not yet been scientifically confirmed (2). However, in terms of their chemical, pharmacological, and toxicological effects, the majority of these plants have not been studied yet (3). Plants have bioactive secondary metabolites and have attracted the attention of scientists due to their complex structure (1, 4). Scientists are trying to make plant extracts to treat cancer and viral and microbial infections. Plant cytotoxic screening is one of the methods to identify the active compounds of plants (5, 6).

Acroptilon repens (Russian knapweed) belong to the Asteraceae family. This plant is perennial herbaceous. Only one species of this plant has been known in Iran, found in West Azarbaijan, Zanjan, and Tehran. *A. repens*

is a medicinal plant with antipyretic properties (7). *A. repens* is a perennial plant with straight stems up to 80 cm (Figure 1). The branches are broad. The flowers are in the form of flowers. The color of this plant is pink to purple. The common name of this plant is Talkhe, and it is considered a noxious weed. This plant is widely grown in northwestern Iran (8). The plant is reported to be allelopathic as well as toxic to horses. The genus *Acroptilon* has been the subject of several investigations because of different sesquiterpene lactones and flavones with substantial toxicity. The phytotoxin in the root exudates of *A. repens* has been identified as 7, 8-benzoflavone. *A. repens* has been described as *Centaurea repens* in some literature (7).

Our study aimed to evaluate the antimicrobial, anti-biofilm, and cytotoxicity activity of the extract and fractions of *A. repens* and to introduce a new antimicrobial candidate.

Materials and Methods

Plant Materials

The aerial parts of *A. repens* were collected from farms of Maragheh city (East Azarbaijan, Iran) during the flowering period. Botanical identification with voucher

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Figure 1. *Acroptilon repens* (L.) DC (Asteraceae).

No: (Tbz-Fph-4041) was carried out at the Herbarium of Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Plant Division and Preparation

The air-dried and powdered aerial parts (100 g) of *A. repens* were extracted with ethanol by maceration at room temperature for 72 hours. The solvent was removed under the vacuum at a temperature below 40°C (9). According to Zanatta et al (10) with some modification. The solvents have been chosen in the order of polarity. To get fractions, the chloroform, ethyl group acetate, and last remaining liquid fractions are gaseous below vacuum at 40°C.

Antibacterial Effects

Based on the National Committee for Clinical Laboratory Standards recommendations, the antibacterial effects of *Acroptilon* extract and re-fraction were analyzed (11). The *Staphylococcus aureus* ATCC 29213 and *Staphylococcus epidermidis* ATCC 35984 (as gram-positive antibiotic resistance bacteria) strains were used for the test in this research.

Disk Diffusion and Well Diffusion Method

We used disk diffusion and well diffusion methods to screen antimicrobial activity in the extract and polar and non-polar fractions of *A. repens*. Bacterial suspensions were adjusted to match the turbidity of a 0.5 McFarland standard, yielding approximately 1.5×10^8 CFU/mL. To prepare bacterial culture on Muller-Hinton Agar, each bacterium was spread with a sterile swab by moistening in bacterial suspension. In the disk diffusion method, we used some standard blank disks impregnated with 50 λ of each extract and fractions and placed on the surface of an agar plate previously inoculated with a standard amount of *S. aureus* and *S. epidermidis* separately. The disks were placed on the plate using sterile forceps at well-spaced intervals from each other (12).

Then, the plates were incubated at 37°C for 24 hours. When incubation time, the plates were tested for the presence of an inhibition zone around the disks. Subsequently, in agar well diffusion, wells of 6-mm

Key Messages

- ▶ *Acroptilon repens* has antibacterial effects.

diameter were punched into the agar medium and crammed with 100 μ L of plant extract and fractions. Then, the plates were incubated in the upright position at 37°C for 24 hours. Wells containing an equal volume of DMSO served as a negative control. The diameter of the inhibition zones around wells was measured supported millimeter after 24 hours.

MIC and MBC Test

The extracts' minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined in triplicate for every strain victimization of the micro-dilute broth technique represented by National Committee for Clinical Laboratory Standards. Dilutions of the extracted series and ethanolic fractions were ready in numerous concentrations in tiny dilution tubes, counting on each extract and fraction. Microorganism suspensions are adjusted to satisfy the quality McFarland 0.5 murkiness and turn out about 1.58 F 1.5 CFU/mL. A similar quantity of bacterium was added to the check tubes. The tubes were then incubated at 37°C for 24 hours. Growing tubes were compared with controls. MBC values were determined by sampling all macroscopically clear tubes when the primary opaque tube within the series. A dilution below the MIC was used for the amount assessed in the MBC method (13).

Antibiofilm Activity

The effect of extract and fraction on biofilm formation was investigated using the microtiter plate method (14). To the 50 μ L of nutrient, the broth was inoculated, and 10 μ L of bacteria measuring 0.5 McFarland was placed in each healthy plate and cultured in a concentration lower than the previously determined MBC. Wells containing bacteria-free medium were used as empty. The incubation time of the plates was 24 hours at 37°C. The supernatant was then removed, and each well was thoroughly washed three times with PBS buffer to remove free-floating cells. For 2 minutes at room temperature, it was stained with 200 μ L of Fuchsin biofilm stain. Excess stains were removed by washing the plate with sterile distilled water, and finally, the dye attached to the cells was dissolved by adding 200 μ L of alcohol acetone to each well and shaken for 2 minutes. Absorption was measured at a wavelength of 492 nm using a microplate reader. The following equation was used to estimate the antibiofilm activity of the extract and the given fractions: Antibiofilm activity (%) = $[1 - (OD_{\text{Test sample}} - OD_{\text{Blank}}) / (OD_{\text{Untreated sample}} - OD_{\text{Blank}})] \times 100$.

Cytotoxic Activity Cell Culture

Vero cells have been purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cells have

been grown in RPMI-1640 medium (Baharafshan, Iran) supplemented with 10% fetal bovine serum (FBS) (Gibco, U.K), 100 U/mL penicillin, and 100 µg/mL streptomycin (Biosera, France). The cells were incubated in a humidified incubator containing 5% CO₂ at 37°C.

In Vitro Assay for Cytotoxicity Activity (MTT Test)

The cells were washed with 0.5% PBS/EDTA and picked up from 25 cm² flasks victimization 0.25% enzyme/EDTA answer (Biosera, France). The cells were then civilized on 96-well plates (Biofil, Australia using MTT reagent). [3-(4,5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide] (Roche medical specialty GmbH, Germany). The toxicity of ethanolic extract and elements of *A. repens* in Vero cells) was investigated. This methodology will offer living cells for the metabolism of MTT yellow tetrazolium salt to purple crystals of Formazan by mitochondrial dehydrogenases. Cells were ingrained in 96-well plates with a density of 104 cells/200 µL and incubated at 37°C and 5% greenhouse gas for 24 hours. Cells were treated with different concentrations of solvent extract and sections and DMSO (Merck, Germany) as a negative control. Once 24 hours of treatment, 10 µL of MTT chemical agent was supplemental to every well and agitated for 10 minutes. The plates were incubated at 37°C and 5% CO₂ for 4 hours. Then, 100 µL of the dissolved answer was added to each well, shaken for 15 minutes, and dissolved by shaking the Formazan crystals. Finally, the absorption was scan victimization by an assay screen reader (DANA, Model-DA3200, Iran) at 570 nm (14). The subsequent equation was accustomed to estimate the cell viability of the extract and elements of the equation:

$$\text{Cell viability (\%)} = \frac{(OD_{\text{Test sample}} - OD_{\text{Blank}})}{(OD_{\text{sample while not treatment}} - OD_{\text{Blank}})}$$

Data Analysis

In this study, the results were analyzed by SPSS software. The ANOVA test and independent *t* test were used for statistical analysis of antibacterial, antibiofilm, and cytotoxicity activity results. Then, we used Tukey's post hoc tests to compare pairs.

Results

Antibacterial Effects

All the various fractions and extracts of *A. repens* showed antibacterial activity. The disk diffusion and well diffusion methods are shown in (Table 1). The *A. repens* showed stronger inhibitory activity against *S. epidermidis* and *S. aureus*, with inhibition zones of 30 and 26 mm, respectively. The ethyl acetate fraction showed the least inhibitory activity against *S. epidermidis* and *S. aureus*, with inhibition zones of 20 and 19 mm, respectively (Figure 2). The MIC and MBC of ethanolic extract and fractions of *A. repens* are listed in Table 1. The most MIC and MBC were related to the ethanolic extract, and the lowest MIC and MBC were related to water fraction in both *S. epidermidis* and *S. aureus*. For statistical analysis of the results, as the first step, the normalization of antibacterial effects was evaluated by the Shapiro-Wilk test, which confirmed the normal values. The disk diffusion and well diffusion methods of ethanolic extract and fractions were evaluated by a one-way ANOVA test, which showed a significant difference between the antimicrobial effect of the extract and fractions ($P < 0.0001$). To investigate the antibacterial

Table 1. Antibacterial Activity of the Extract and Fractions of *A. repens* Against two Gram-Positive Bacteria

Bacteria	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>
Ethanol extract		
Inhibition zone in well diffusion	26 mm	30 mm
Disk	24 mm	22 mm
MIC	7.8 µg/mL	15.6 µg/mL
MBC	15.6 µg/mL	31.2 µg/mL
Chloroform fraction		
Well	21 mm	21 mm
Disk	19 mm	18 mm
MIC	7.8 µg/mL	7.8 µg/mL
MBC	15.6 µg/mL	15.6 µg/mL
Ethyl acetate fraction		
Well	19 mm	20 mm
Disk	13 mm	15 mm
MIC	3.9 µg/mL	3.9 µg/mL
MBC	7.8 µg/mL	7.8 µg/mL
Water fraction		
Well	25 mm	25 mm
Disk	16 mm	15 mm
MIC	1.25 µg/mL	1.25 µg/mL
MBC	2.5 µg/mL	2.5 µg/mL

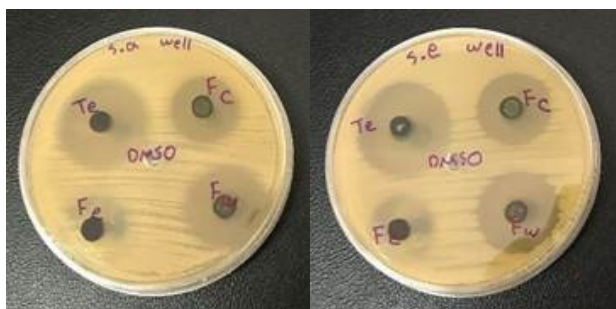


Figure 2. Growth inhibitory zones of ethanolic extract and fractions on *Staphylococcus epidermidis* and *Staphylococcus aureus* by *A. repens* in well diffusion method, Te: Ethanolic extract, Fc: Chloroform fraction, Fe: Ethyl acetate fraction, Fw: Water fraction.

effect of the extract and fractions using Tukey's post hoc tests, the extract and fractions, 2-2 were compared together, where the antibacterial effect of ethanolic extract was found to be higher than water fraction, chloroform fraction, and ethyl acetate fraction, respectively. Also, to evaluate the antibacterial effect, the difference between the extract and fractions on *S. aureus* and *S. epidermidis* was used through a one-way ANOVA test, which showed that the effect of the extract and fractions on the two bacteria was the same and there was no significant difference ($P > 0.05$).

Antibiofilm Effect

The antibiofilm result against *S. aureus* and *S. epidermidis* is shown in Figure 3. The data showed that the biofilm of gram-positive bacteria was sensitive to ethanolic extract and fractions of *A. repens*. The chloroform and ethyl acetate fractions of *A. repens* have the lowest antibiofilm effect, and the ethanolic extract and water fraction of *A. repens* have the most antibiofilm effect on *S. aureus* and *S. epidermidis*. The graphs shows that the percentage of antibiofilm activity in ethanolic extract, ethylacetate, chloroform, and water fractions on *S. aureus* was 84.7%, 27.3%, 14.6%, and 100%, respectively. Similarly, *S. epidermidis* was 94.2%, 11%, 22.8% and 66.1% respectively.

The antibiofilm effect of ethanolic extract and fractions were evaluated by one-way ANOVA. The analysis showed a significant difference between them as the effect of the anti-biofilm of the extract and fractions on the *S. aureus* and *S. epidermidis* is different ($P < 0.05$). Tukey's post hoc tests showed that the ethanolic extract and the water fraction had almost the same antibiofilm effect, and there was no significant difference between them ($P > 0.05$). Nonetheless, they differ significantly compared with chloroform fractions and ethyl acetate fractions ($P < 0.05$). And these results are shown in Figure 3 using the comparison of the overlap of the standard error.

Cytotoxic Effects

The results were expressed based on the percentage of cell viability, normal and cytotoxic Vero cells shown in Figure 4. The results of the MTT test are shown in Figure

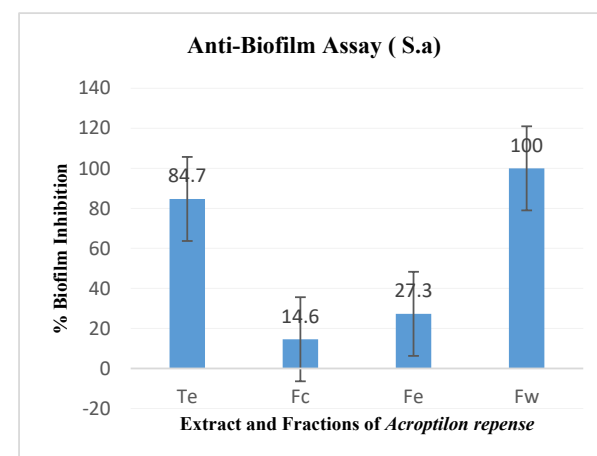
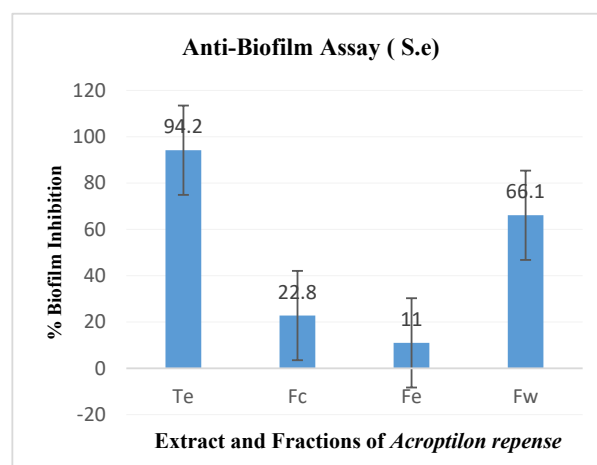


Figure 3. Antibiofilm activity of *A. repens* against *S. aureus* and *S. epidermidis*. Te: Ethanolic extract, Fc: Chloroform fraction, Fe: Ethyl acetate fraction, Fw: Water fraction.

5. According to the results, ethanolic extract and fractions with increasing concentrations decreased viability (Figure 5). From the bottom figures, we can easily determine the percentage of cell viability for 4 different concentrations of ethanolic extract and fractions of *A. repens*. Four different concentrations were 60 mg/mL, 30 mg/mL, 15 mg/mL, 7 mg/mL for ethanolic extract, ethyl acetate and chloroform fractions and 4 different concentrations were 5 mg/mL, 2.5 mg/mL, 1.2 mg/mL, 0.6 mg/mL for water fraction, respectively. The ethanolic extract of *A. repens* at 60 mg/mL produces 48% cell viability, 30 mg/mL produces 50% cell viability, 15 mg/mL produces 73% cell viability, 7 mg/mL produces 100% cell viability and the ethyl acetate fraction of *A. repens* at 60 mg/mL produces 40% cell viability, 30 mg/mL produces 47% cell viability, 15 mg/mL produces 72% cell viability, 7 mg/mL produces 80% cell viability and the chloroform fraction of *A. repens* at 60 mg/mL produces 47.3% cell viability, 30 mg/mL produces 66.6% cell viability, 15 mg/mL produces 100% cell viability, 7 mg/mL produces 100% cell viability and the water fraction of *A. repens* at 5 mg/mL produces 40% cell viability, 2.5 mg/mL produces 50% cell viability, 1.2

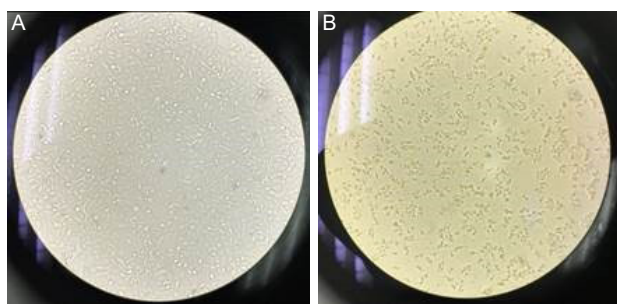


Figure 4. (A) Normal Vero cell and (B) Cytotoxic Effect of *A. repens* in Vero Cell.

mg/mL produces 100% cell viability, 0.6 mg/mL produces 100% cell viability. Extract and different fractions of the *A. repens* exhibited different activity on the Vero cell line. The effect of the samples on the proliferation of Vero cells was expressed as the % cell viability. From the graphs, the concentration of ethanolic extract, ethyl acetate, chloroform, and water fractions yields the value of LC50 (50% mortality) as 30 mg/mL, 30 mg/mL, 60 mg/mL, and 2.5 mg/mL, respectively for *A. repens*.

The cytotoxic effects of ethanolic extract and fractions in four concentrations and control samples were assessed using one-way ANOVA. It showed that the percentage of cell viability at concentration of 0.06 g/mL and 0.03 g/mL of ethanolic extract and fraction chloroform and ethyl acetate have no significant difference compared to each other ($P > 0.05$), while it is significant in comparison with 0.015 g/mL and 0.007 g/mL concentrations ($P < 0.05$). The percentage of cell viability in the concentration of 0.007 g/mL of ethanolic extract and chloroform and ethyl acetate fractions was not significantly different from the control sample ($P > 0.05$). Concentration of 0.005 g/mL in comparison with 0.0025 g/mL concentration of water

fraction also did not differ significantly ($P > 0.05$). The percentage of cell viability in the concentration of 0.0012 g/mL and 0.0006 g/mL of water fraction and control sample were the same and did not show a significant difference when compared ($P > 0.05$). While, concentrations of 0.005 g/mL and 0.0025 g/mL of water fraction were significantly different in comparison with the concentrations of 0.0012 g/mL and 0.0006 g/mL of water fraction and the control sample ($P < 0.05$).

Discussion

Medicative plants contain phytochemicals. Today, these compounds are wont to manufacture drugs. Several phytochemicals from plant sources reminiscent of phenolics and flavonoids are helpful to human health (15).

Plant extracts can act through more than one mechanism because they consist of a mixture of active compounds. Hence, potentially they have many beneficial effects. To evaluate antibacterial, anti-biofilm, and cytotoxic effects of *A. repens*, all ethanol extract and chloroform, ethyl acetate, and water fractions of aerial parts were used. In this section, we used extracts and details of the plant that have been dissolved in excessive concentrations in distilled water and did not precipitate after centrifugation. Some cases, which were not soluble in water alone, used DMSO to the allowed extent (0.1%). About fractions, the solvent and material obtained from each faction are different. Therefore, the maximum concentration obtained from each of them is also different. Accordingly, separate charts are drawn. The 10%, 20%, and 40% fractions were fully soluble in water. We used the maximum DMSO in the biological systems to solve the other fractions. The antibacterial effect of *A. repens* was analyzed by the disk diffusion and well diffusion methods.

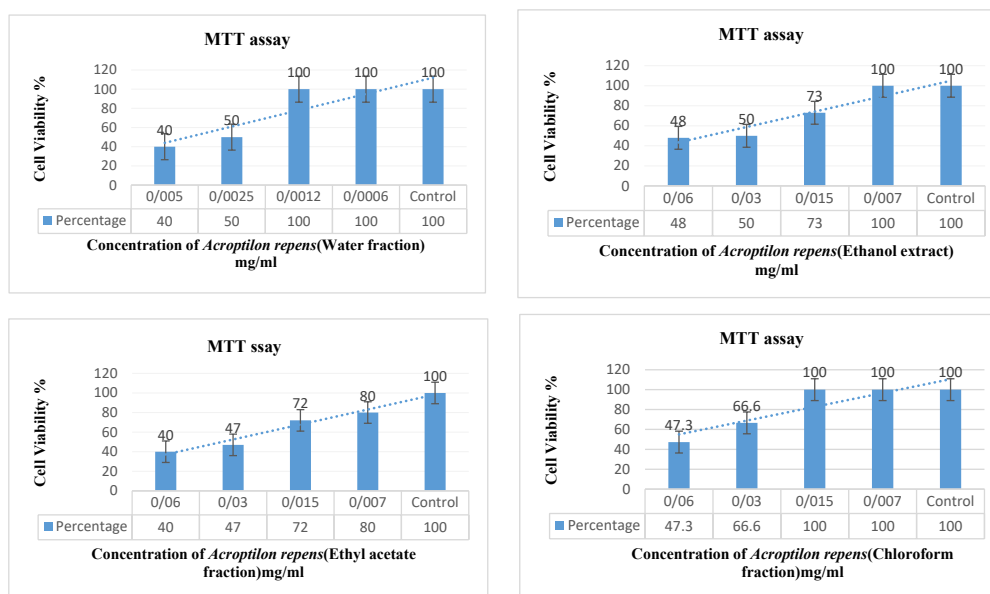


Figure 5. Percentage of Cell Viability of Ethanolic Extract and Fractions of *A. repens* Against Vero Cell Lines.

We observed that the ethanolic extract and chloroform, ethyl acetate, and water Fractions showed antibacterial effects. We screened *A. repens* extract for antimicrobial activity against the two gram-positive bacteria. The results of antimicrobial activities are presented in (Table 1). The evaluation of the antibacterial activity of the extract indicated significant activity against all gram-positive bacteria. The *A. repens* showed stronger inhibitory activity against *S. epidermidis* and *S. aureus*, with inhibition zones of 30 and 26 mm, respectively (Figure 2). The lowest MIC and MBC were related to the water fraction. Antibacterial activity of the extract of *A. repens* was carried out. According to Norouzi-Arasi et al, the essential oil of the aerial parts of *A. repens* has potent antibacterial activity (7). In a biological screening of the essential oil of *A. repens* by Razavi et al, it was indicated that the essential oil exhibited significant phytotoxic activity (8). Due to the increasing role of biofilm in various infections, antibiofilm activity was also considered in the selection of plant extracts.

Biofilm treatment is regarded as one of the main problems of human conditions, despite these plants as a treatment. Diseases have been used for centuries. The results of the study illustrated the antibiotic potential of some extracts. Based on the obtained results, a biofilm of gram-positive bacterium is sensitive to extracts and ethanolic fractions. The extensive production of exopolysaccharide (EPS) causes biofilms, forming a molecular network. The formation of a molecular network causes the initial binding of bacteria, increases the resistance to antimicrobial agents and environmental stress, and on the other hand, causes the formation of micro-colony structures. Quorum-sensing chemical signals act as a protecting barrier for cells by regulating EPS production and increasing biofilm formation.

Compounds that can inhibit the sense of quorum can help manage biofilms to some extent. One of these sites involves the inhibition of diguanylate cyclase, which is responsible for the production of cyclic (3'-5')-dimeric guanosine monophosphate (c-di-GMP) in bacterial cells. This molecule controls adhesion biosynthesis and EPSs related to biofilm formation. Phytochemicals such as N-[4-(phenylamino) phenyl] benzamide have such activity. Another potential target site for antibiofilm activity, which targets extracellular polysaccharide degradation, is biofilm dispersion. Phytochemicals such as light spermidine also have this property (14). Based on the results of the present study, for the first time, the antibiotic activity of ethanolic extract and chloroform, ethyl acetate, and *A. repens* water fraction may be against the biofilm created by *S. aureus* and *S. epidermis*. To test the cytotoxicity effect of the extract and fractions, we used the MTT test. The results were expressed based on the percentage of cell viability. According to the results, ethanolic extract and fractions with increasing concentrations decreased viability. Prema et al conducted an in-vitro cytotoxicity study on combined plant extracts and evaluated the

cytotoxicity effects of combined plant extracts and the anticancer effect of ethanol extraction HT-29 cell line. Results showed significant growth suppression in a dose-dependent manner (16).

We investigated the non-polar compounds of a methanolic extract with GC-MS. Based on the study results, compounds such as germacrene D and β -caryophyllene are present in the non-polar part of this extract at about 3% and 0.8%, respectively. These substances are from volatile organic hydrocarbons, especially sesquiterpene, and are commonly produced in some plant species due to their antimicrobial and insecticidal properties. The two prominent molecules are germacrene A and germacrene D and act as insect pheromones (17).

The composition of many essential oils of β -caryophyllene is a natural bicyclic sesquiterpene. The effects of this substance in humans have been studied. The results of a study by Dahham et al showed that β -caryophyllene had a more pronounced antibacterial activity against *S. aureus* and an antifungal activity than kanamycin. On the other hand, β -caryophyllene has selective anti-proliferative effects on colorectal cancer cells (IC₅₀) (18). There are a variety of compounds such as flavonoids, sesquiterpene, and phenylpropanoid glycosides that cause unique effects of this plant extract. Zhao et al examined chemical constituents of the methanolic extract *A. repens*. After separation, *A. repens* ethanolic extract was purified by chromatography. 11 compounds were obtained as 2 alpha, 9 beta-dihydroxy-dehydrocostus lactones, cynaropicrin, apigenin, stigmaterol, 4'-hydroxywogonin, ethyl caffeate, p-methoxy-cinnamic acid, luteolin, daucosterol, apigenin-7-O-beta-glucoside, and syringing were identified (19). As a sample, cynaropicrin is a sesquiterpene lactone. Binding to the bacterial enzyme MurA is mediated by the addition of Michael to the Cys115 thiol group. The side chain's monounsaturated ester mimics the phosphoenolpyruvate substrate. Irreversible inhibition of this enzyme stops the cytoplasmic biosynthesis of peptidoglycan precursor molecules (20). Due to the small amount of these compounds, this extract identification of polar compounds was the subject of further studies. In other studies, these two compounds were identified as part of the essential oils from this plant (21-23).

Limitations of the Study

Our results are only for the native plant of Maragheh region, which may change its effects by changing the environment.

Conclusions

Our study showed that ethanolic extract and chloroform, ethyl acetate, and water fraction *A. repens* (L.) DC has antibacterial and antibiotic effects on *S. aureus* and *S. epidermidis*. This is the first time the antibiofilm activity of ethanolic extract and chloroform, ethyl acetate, and water fraction of *A. repens* has been reported against

biofilm formed by *S. aureus* and *S. epidermidis*. According to the results of the cytotoxic test, ethanolic extract and fractions with increasing concentrations decreased viability. Accordingly, the ethanolic extract and fractions of *A. repens* can be used as a candidate for a new source of medicine for infections caused by *S. aureus* and *S. epidermidis*.

Authors' Contribution

Study conception and design: ZA, RN, MZ, AT. Data collection: ZA and AT. Analysis and interpretation of results: MZ. Draft manuscript preparation: RN. All authors reviewed the results and approved the final version of the manuscript.

Conflict of Interests

Authors have no conflict of interest.

Ethical Issues

This study has been approved by the Ethics Committee of Qom Islamic Azad University with number (No. 15430507961012).

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