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Biotechnological Journal of Environmental Microorganisms(BJEM) 1(2) 2022 100-106

Effects of *Pimpinella saxifraga* L. Extracts on Environmental Isolates of *Enterococcus faecalis* and HeLa Cell Line

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Received: 4 October 2022/ Revised: 1 February 2023 / Accepted: 1 February 2023

Abstract

Pimpinella saxifraga L. is a genus of aromatic plants native to northern Iran. Some of its medicinal benefits are already known. The purpose of this study is to investigate the effect of *Pimpinella saxifraga* L. plant extract on some environmental isolates of *Enterococcus faecalis* and the HeLa cell line. After collecting the plant and drying it, methanolic and aqueous extracts were prepared. Since phenolic compounds have multiple effects, the number of phenolic compounds in the methanolic extract was measured by the Folin-Ciocalteu method. Antibacterial activity against *E. faecalis* was first measured by disk diffusion, and then MIC and MBC were measured by the microdilution method. Finally, the anti-proliferative activity of plant extracts on the cervical cancer cell line (HeLa) was investigated using the MTT method. The volume of phenolic compounds in the methanolic extract was 968.33 mg/ml. The investigation of the antimicrobial effect showed that the methanolic extract has significant effects against *E. faecalis*. The MIC and MBC of this extract were 25 and 100 mg/ml, respectively, and the growth of the cervical cancer cell line (HeLa) was inhibited by about 13% by using the MIC concentration. The results of the experiments demonstrated that the methanolic extract of *P. saxifraga* L. has an inhibitory effect on the growth and proliferation of *Enterococcus faecalis* and the HeLa cell line.

Key words: Antibacterial effect, *Enterococcus faecalis*, HeLa cell line, Methanolic extracts, *Pimpinella saxifraga* L.

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Introduction

Plant extracts are rich in bioactive molecules based on the diversity of their chemical components, such as flavonoids, polyphenols, and alkaloids, which play a highly important role in drug discovery and development (Somaida et al., 2020). At present, 80% of the world's population uses medicinal plants to treat basic diseases, frequently as extracts or isolated active compounds. For many years, medicinal plants have been used to treat diverse infections. Today, these plants have attracted the attention of researchers due to their important properties, including strong antibacterial properties, cheapness, and availability. Therefore, the detection and advancement of new antimicrobial drugs with novel modes of action are necessary to control the appearance of multi-drug-resistant pathogens (Perera et al., 2022). Consistent with the World Health Organization (WHO) report, approximately 80% of the world's population relies on plants or derived products for their treatment. In this research, we investigated the antibacterial activity of the methanol extracts of *Pimpinella saxifraga* (Tchinda et al., 2017).

Pimpinella is a member of the Apiaceae, which includes approximately 150 species distributed in the northern hemisphere. Pimpinella is represented in Turkey by 23 species (5 endemic), 2 subspecies, and 2 varieties, for a total of 27. *Pimpinella saxifraga* is well-known in Turkey as "Teke maydonozu" (goat parsley) or "tas maydonozu" (rock parsley), and its roots are used as a sedative, stomachic, expectorant, and tonic. The fresh leaves of the indigenous *P. anisetum* species known as "Ezeltere" in Turkey are used locally in desserts. Its fruits are preserved using the pickling method. The cultivation of this species is done locally (Baser et al., 2007).

People mostly living in Turkey, China, Korea, Iran, Egypt, Palestine, Lebanon, and European countries (the United Kingdom and Italy) have long used *Pimpinella* species, usually in the treatment of numerous illnesses. Plants belonging to this family have economic significance, and they have long been used as stuffing or vegetables, as well as having traditional remedial values. Pimpinella species were found among the main sources of natural composites having an extensive spectrum of biological actions such as antimicrobial, antioxidant, enzyme inhibitory and/or activating, anti-inflammatory, anti-diabetic, anti-convulsing, etc. (Tepe & Tepe, 2015).

The widespread consumption of antibiotics has unpleasant effects on hosts, including high sensitivity, suppression of the immune system, and allergic reactions. It also makes bacteria resistant. The problems raised by resistant bacteria have initiated several challenges in the therapeutic system. Currently, the spread of drug-resistant gram-negative bacteria in hospitals has become a big problem and is increasing in many countries (Zeinali Aghdam et al., 2019).

Enterococcus faecalis is one of the important pathogens causing hospital infections that can show resistance to a wide range of antimicrobial agents (Castilho et al., 2013). In research, the antibacterial activity of Pimpinella saxifrage extract was tested in vitro against *E. facaelis*. In this study, we investigate the effect of *Pimpinella saxifraga* plant extract on *Enterococcus faecalis* bacteria (causing endocarditis, sepsis, meningitis, and urinary and genital tract infections), along with evaluating HeLa cell viability in the presence of the extract.

Material and Methods

Plant collection

Pimpinella saxifraga was collected in spring from the northern regions of Iran (Langarud, Guilan). The leaves of the plant were separated to extract methanolic and aqueous extracts, washed with distilled water, and dried at 40 °C. It was then crushed and used to prepare methanolic and aqueous extracts.

Bacterial strains

The standard strain of *E. faecalis* PTCC 1778 was purchased from the Persian Type Culture Collection (PTCC, Tehran, Iran). The environmental strains obtained from a study performed by Kalantari et al. (2022) and used in this study.

Preparation of plant extracts

The Soxhlet method was used to prepare plant extracts. The amount of 30 grams of the plant was weighed with a scale and placed in a Soxhlet extractor containing 300 mL of each solvent

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(methanol or aqueous) for 4 hours, and the cycle was repeated three times. Then the obtained extracts were concentrated in a rotatory evaporator. The extracts were stored in the refrigerator at 4°C until further use (Hassan & Ullah, 2019).

Determination of total phenol volume

The most active dietary antioxidants belong to the family of phenols and polyphenols. A photometric method based on the color reaction of phenolic compounds with Folin-Ciocalteu reagent (due to its simplicity and reliability) was widely used to determine the volume of total polyphenols. 20 µl of plant extract was added to 100 µl of 2N Folin-Ciocalteu reagent. The final volume of the mixture using distilled water reached 1600 microliters. In the last step, 300 µl of sodium carbonate solution (0.2 mg/ml) was added to the reaction mixture and incubated at 37 °C for 45 min. The optical density of the solution was measured at 760 nm (Shams Moattar et al., 2015).

Determination of antibacterial activity

Disc diffusion and well diffusion agar methods were used to evaluate the antibacterial properties of the plant extract. Several colonies of the bacteria were taken using a sterile loop and inoculated into a test tube containing 5 ml of Müller-Hinton Broth medium, and then the medium was incubated at 37 °C for 1 hour.

In the disc diffusion method, cell suspension 0.5 of the McFarland bacterium, *Enterococcus faecalis*, was cultured with a sterile swab on plates containing Müller-Hinton agar. Paper disks (diameter 6 mm) were inoculated with different concentrations of plant extract (3.1, 6.25, 12.5, 25, 50, 100, and 200 mg/ml) on agar surfaces. The plates were stored at 37 °C for 48 hours. All experiments were repeated three times. Antimicrobial activity is expressed as the mean inhibitory diameters (in millimeters) produced by the extract, and the data were evaluated at least twice.

The agar-well diffusion method was performed to determine the minimum inhibitory concentration (MIC) of plant extract on the desired bacteria. The broth microdilution method was used for this purpose. Extracts were poured from ten microplate wells in 3-1 blank wells, positive control, and negative control, and 4-10 wells of different concentrations (3.1–200 mg/ml) were incubated at 35 °C for 18 h. The results were read with a microplate reader at 630–425 nm (Anantaworasakul et al., 2017).

Cells culture

HeLa (cervix adenocarcinoma) cell lines were purchased from the Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% of fetal bovine serum (FBS; Gibco, USA), 100 µg/mL of streptomycin (Gibco, USA), 100 UI/ml of penicillin (Gibco, USA), and 0.25 µg/mL amphotericin B (Gibco, USA), at 37°C in a humidified air atmosphere containing 5% (v/v) CO2.

MTT assay

The MTT assay is a calorie meter that measures the percentage of metabolically active cells. In this study, an MTT colorimetric assay was used to evaluate the anti-proliferative activity of the extract against HeLa cervical cancer cells. In this experiment, HeLa cells were cultured in 96-well plates (1 ×104 cell density per well) for 24 hours. From the absorbance values obtained, percentage cell viabilities were calculated for the various concentrations of the fractions and crude extract using the following formula (Khatibi et al., 2017).

Cell viability (%) = (Live cells) / (Total number of cells) ×100

Results

Total phenol volume of *Pimpinella saxifraga* plant extract

The total phenol volume of *Pimpinella saxifraga* plant extract was measured using the Folin-Ciocalteu colorimetric method and the standard gallic acid curve (in micrograms of gallic acid per gram of dry weight). The total amount of phenol was calculated to be 968.33 mg/ml. The standard curve for gallic acid is shown in Figure 1.

Investigation of antibacterial activity

In this study, the effects of methanol extract against *Enterococcus faecalis* were investigated by disk diffusion and agar-well diffusion methods. The antibacterial effect of different concentrations of methanolic extract of *Pimpinella saxifraga* (3.1, 6.25, 12.5, 25, 50, 100, and 200 mg/ml) against *Enterococcus faecalis* was investigated. As shown in Figure 1 & 3, the highest

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antibacterial effect was related to the concentration of 200 mg/ml methanolic extract of the plant *Pimpinella saxifraga*. Also, according to the results of the antibiogram test, it was found that this bacterium is sensitive to nitrofurantoin antibiotics and resistant to penicillin, rifampicin, kanamycin, amphotericin, and sulfamethoxazole.

Investigation of the MIC and MBC amounts of the methanolic extract of Pimpinella saxifraga

In this part of the study, for methanolic extracts prepared by the Soxhlet extraction method, the minimum inhibitory concentration (MIC) and the maximum lethal concentration (MBC) were determined for Enterococcus faecalis. The concentration of 25 mg/ml of methanolic extract of the plant was considered MIC, and the concentration of 100 mg/ml was considered MBC.

Results of MTT in the HeLa cell line

The viability of HeLa cells at different concentrations (25 to 30 mg/ml) of the methanolic extract was studied using the MTT technique (Figure 2). The results showed that at the highest concentration of the extract (200 mg/ml), there was more than 50% survival. Therefore, the half-maximal inhibitory concentration (IC50) is related to values higher than 200 mg/ml. The inhibitory effect of aqueous plant extract on HeLa cells was almost zero.

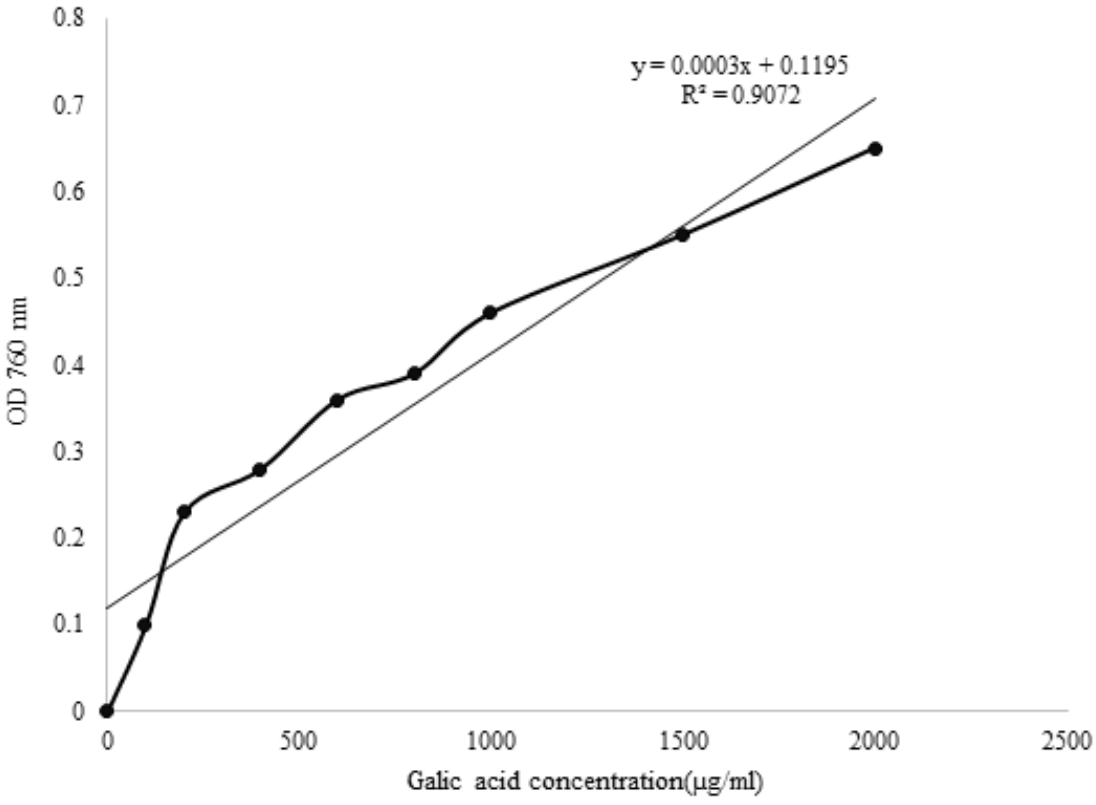

Figure 1. Gallic acid standard curve

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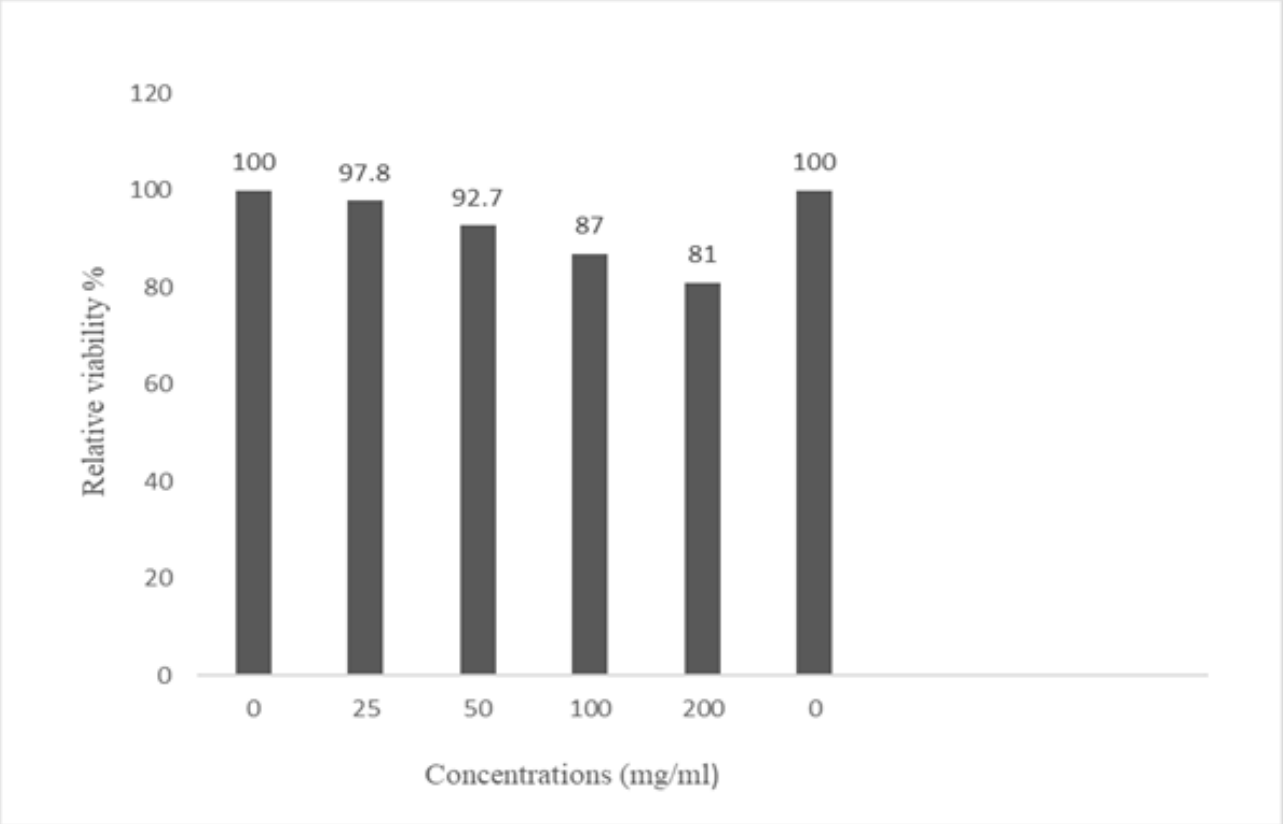

Figure 2. Viability of HeLa cells in the presence of different concentrations of methanolic extract of *Pimpinella saxifraga*L.

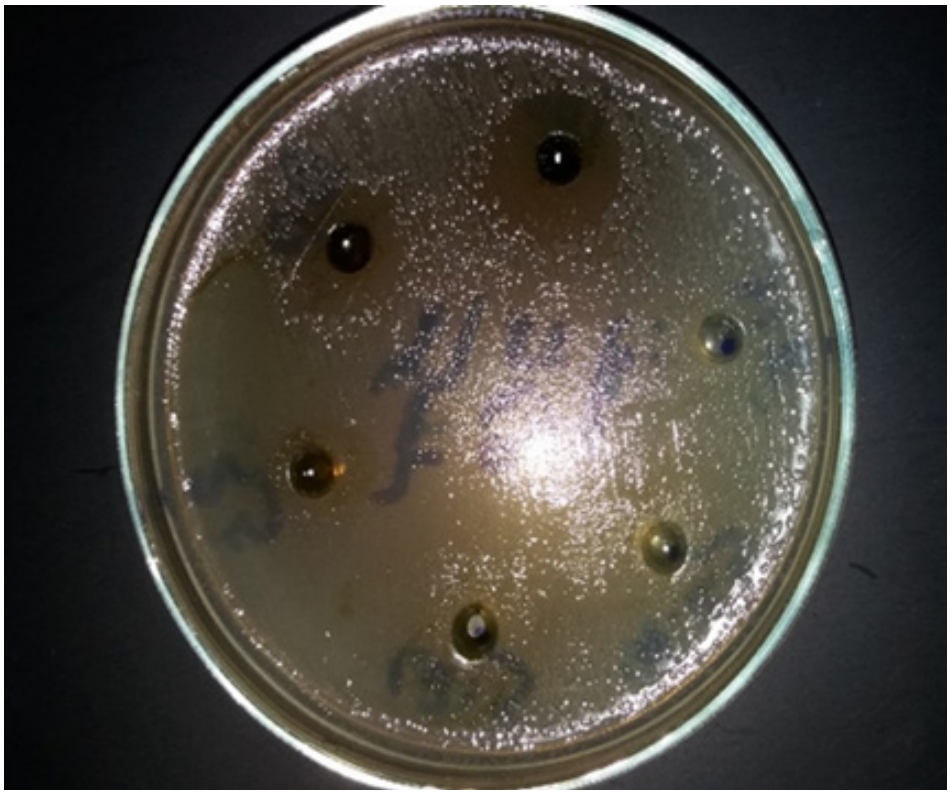

Figure 3. Antibacterial effect of methanolic extract of *Pimpinella saxifraga* L. against environmental strains of Enterococcus *faecalis.*

Discussion

Infectious diseases and cancers are the main causes of morbidity and mortality worldwide and there is a constant demand for new therapies to treat and prevent these life-threatening diseases. The use of synthetic antibacterial and anticancer drugs has not succeeded within the broad range of clinically relevant bacterial pathogens and cell lines, probably because of their adverse side effects and antimicrobial resistance (Amin et al., 2018). Plants are widespread sources of components with diverse bioactivity, enabling their use for functional food production and medicinal purposes. Nowadays, there is an increasing demand for natural antioxidants due to safety concerns with synthetic antioxidants. Because of their natural antioxidant components, herbs are great sources of antioxidants (Zaïri et al., 2018). There is a correlation between antioxidant activity and total phenolic content (Al-Rimawi et al., 2016). The amount of total phenolics in the extract was determined by the Folin-Ciocalteu procedure, using gallic acid as the standard. The results shown in this study revealed that the methanolic extract of the plant contains an excellent antioxidant.

Cervical carcinoma is the fourth most common cancer in women, and it was estimated that the new cases and deaths for 2020 will be 604,000 and 342,000, respectively. The majority of new cases and deaths appear to have occurred in developing and underdeveloped countries (Jovanović Galović et al., 2017).

In this study, the cytotoxicity effects of the all-methanol extract of *Pimpinella saxifraga* on the HeLa cervical cancer cell line by the MTT method were examined. The extract of *P. saxifraga* showed the highest cytotoxicity at 200 mg/ml. The results showed that the methanolic extract of the plant significantly inhibited the growth of HeLa cells by increasing the concentration (25–200 mg/ml). According to the results, it can be expected that the extract inhibition effect on cell growth, even at 200 mg/ml may increase. The extract half maximal inhibitory concentration (IC50) on HeLa cancer cells was 1.0 ± 1 mg/ml. Rahimifard and his colleagues showed that the Mentha extracts have cytotoxic effects on the Hela cell line (Rahimifard et al., 2010). Unfortunately, no research has been found on the effect of the extract or essential oil of *Pimpinella saxifraga* on the growth and proliferation of HeLa cells. It has been shown in various studies that petroselinic acid is one of the main components of the plant essential oil and that it has an inhibitory effect against bacterial growth (Ksouda et al., 2018; Yoshino et al., 2022).

In previous research, no studies have been performed on the minimum bacterial concentration (MIC) and maximum bacterial concentration (MBC) of *Pimpinella saxifraga* extract. In this investigation, the antibacterial properties of the plant extract against *Enterococcus faecalis* MIC and MBC methanolic extract were determined to be 25 mg/ml and 100 mg/ml, respectively. According to the values of MIC and MBC, it seems that the methanolic extract of this plant is one of the most effective plant compounds against *Enterococcus faecalis*. The results of this study were comparable to those of a study conducted by Najafi et al. (2019). They examined the antibacterial effects of green tea and aloe vera extract on *E. faecalis*. The MIC and MBC for this bacterium were 20 mg/ml and 100 mg/ml, respectively (Najafi et al., 2019). In MBC evaluation, the two methanolic extracts of *Myrtus communis* L. and *Eucalyptus galbie* showed no bactericidal effect on *E. faecalis*. The MICs for both extracts were 12.5 mg/ml (Raoof et al., 2019).

Conclusion

The results of our research showed that the volume of total phenol in the methanolic extract was 968 mg/ml. The investigation of the antimicrobial effect showed that the methanolic extract is more effective. For this reason, it was used for the next steps. The MIC and MBC of this extract were 25 and 100 mg/ml, respectively, and the growth of the cervical cancer cell line (HeLa) was inhibited by about 13% by MBC dilution. However, more studies should be done, and concentrations higher than 200 mg/ml of the methanolic extract of the plant should be evaluated. There is a possibility that the survival of HeLa cells at higher concentrations of 200 mg/ml methanolic extract will reach zero.

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