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Anti-Bacterial and Anti-Biofilm Activity of *Glycyrrhiza glabra*, *Rosmarinus officinalis* and *Saponaria officinalis* Extracts on Important Food Pathogens

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Abstract

Background: Biofilms are communities of microorganisms embedded in a self-produced extracellular polymeric matrix. Bacterial cells are protected from antimicrobial agents in biofilm structure.
Objectives: This article aims at exploring the anti-bacterial anti-biofilm effects of the ethanol extracts of *Glycyrrhiza glabra*, *Rosmarinus officinalis*, and *Saponaria officinalis* on food pathogens.
Methods: Methanol extracts of *Glycyrrhiza glabra*, *Rosmarinus officinalis*, and *Saponaria officinalis* were prepared by rotary device. The bacterium was purchased in standard form. Finally, the antimicrobial activities of the extracts on the bacteria were measured by the microdilution method.
Results: The results of this study showed that the lowest inhibitory concentration of G. garlic extract of methanol extract was 0.62 mg/mL, which was inhibited by *L. monocytogenes*, while *B. cereus* was eliminated in all concentrations of the extract. The lowest inhibitory concentration of rosemary extract was 0.62 mg/mL, which was inhibited by *S. dysenterae* and *V. cholera*, while the highest inhibitory concentration was 2.5 mg/mL, which *B. cereus* is restrained. The results of the study showed that the biofilm formation rate decreases with increasing concentrations of the extracts
Conclusions: The result of the study showed that plant extracts have antimicrobial and anti-biofilm effects on food pathogens. Herbal extracts can be used to control food borne pathogens.

Keywords: Anti-Biofilm Effects, *Glycyrrhiza glabra*, *Rosmarinus officinalis*, *Saponaria officinalis* Food Pathogens

1. Background

Bacterial bio film in patients using external devices is a two-stage process that involves binding to surfaces, growing, propagating, and spreading bacteria in layers (1). The formation of biofilms leads to the development of recurrent infections that are resistant to antimicrobial therapies and increase the cost of treatment (2).

Biofilm are organized microbial communities that are enclosed within the matrix of extracellular water-based polymers and form on living or non-living surfaces. In addition to natural surfaces, biofilm structures can also be found on the level of industrial equipment, such as levels involved in food preparation, distribution systems and water storage tanks, air conditioning systems, and oil reservoirs. The formation of these structures by pathogen organisms on the levels of medical equipment, implantation tools, and artificial organs has also caused many clinical problems (3).

In recent decades, the spread of drug resistance among pathogen microorganisms on one hand, and the harmful effects of antibiotics on the other, has led to many studies to achieve anti-inflammatory compounds (4). In this regard, the biological compounds and in particular, the medicinal plants, have been of great interest to the researchers. Food-borne diseases have always been a threat to human health and are considered as an emergency and important issue. Many prevalence have been associated with biofilms. In addition, it has been shown that biofilm is a problem in the food industry due to quick antibacterial resistance. The formation of biofilm is one of the properties of the pathogens such as Salmonella; especially in the food industry, which allows bacteria to bind to differ-

ent levels.

The long history of the use of medicinal herbs in traditional medicine and the identification of many of their therapeutic properties over the years, as well as general acceptability, easy access, low cost of production.

Rosemary is a plant of the mint family, which is a small, durable shrub with aromatic leaves and tiny blue flowers, which blossom at the beginning of spring and end of winter. Its height is 50 cm to 1 meter. It is woody, the leaves of this green plant are permanent, reciprocal, with narrow, long, sharp, and fairly rough edges (5). Rosemary essential oil is one of the compounds whose antimicrobial and antioxidant properties have been proven in many cases, and antimicrobial compounds such as phenolic compounds are found to be abundant. Rosemary essential oils are used in cosmetics (6). It contains essential oils, oleoresins, and tannins. Rosemary essential oil contains one and eight cinnamal, pinen, camfer, boronyl acetate, - D limonene, boronol, micron, terpinole, camphon, linalool, caryophyllene, and rasmarn. Other materials in this plant are amyric acid, epi-carnosic, carnosol, cryptomyelonid, epipermenol, isozersemanol, napus, Ramadal, and rosmarinic acid. The leaves contain 5% - 5.5% volatile oil. The main substance of the oil consists of monoterpene hydrocarbons (α and β), pinhenum, camphon, limonene, camfer (20% - 10%), booneol, sinole, linalool, and verbonil. Rosemary actually contains variable amounts of aromatic and volatile matter (7, 8).

G. glabra, commonly called as Licorice, is one of the most important traditional medicinal plants, which grows in various parts of the world and has been used for medicinal purposes for at least 4000 years. The root of this plant has several pharmacological properties such as anti-inflammatory, antiviral, antimicrobial, and anticancer activities, in addition to immunomodulatory, hepatoprotective, and cardioprotective effects (9).

Staphylococcus aureus is the second or the third important cause of the diseases transferred by food (10, 11). Due to its easy growth in different situations, the bacterium can be easily detached from various foods such as milk and its related products, meat products, vegetables, salads, baked foods, and salty foods, especially the ones requiring long-term manipulations (12). Staphylococci food toxicity is caused by consumption of foods polluted to *staphylococcus* Enterotoxin. Among its symptoms are diarrhea, vomiting, saliva increase, stomach-turning, etc. (13).

Bacillus cereus is a gram positive mobile aerobic bacterium with spores, which grow in 1 to 2-micrometer sizes and in some cases 3 - 5 micrometer. It is often chain-like with a width of one micrometer. Although cereus can have saprophyte life chain, it can also act as an opportunistic pathogen in the human body (14).

Despite the wide advances in hygiene, food diseases have turned more widespread. Food and water pollutions are prominent causes of death in the world. This is probably due to pollution of food products and changes in food industries and technologies around the world (15).

Shigella bacterium is a gram negative internal pathogen and a cause of bacilli diarrhea in humans. Its variants are transmitted through fecal oral and enter the human body through polluted water and food. These bacteria are really infectious, thus 10 to 100 bacteria are sufficient for causing infections (16).

The infection caused by this pathogen creates severe disease and intestinal inflation. It is detected by watery diarrhea and dirty secretions because it can attack epithelial cells and enter colon epithelium and exploit the special epithelial cells in lymphatic follicles.

Listeria monocytogenes is a gram-positive, microaerophilic, asporogenic bacillus that has a specific tumbling move among 20°C and 25°C and that produces slight, B-hemolysis on sheep blood agar. It can grow at a variety of temperatures, from 1°C to 45°C, and thus, can thrive in the upkeep of foods at refrigeration temperatures (17).

Cholera, an enteric diarrheal disease caused by the gram-negative bacterium Vibrio cholera continues to be a worldwide health concern.

The pathogenic food microorganisms cause enormous financial and life tolls annually. Further, food decadence due to microorganism growth is still a trouble in the food industry. One method of controlling the growth of bacteria in foods is the use of preservatives and antimicrobial compounds. Chemical preservatives were used for a while for hindering the growth of and eliminating harmful microorganisms. Nowadays, public awareness regarding chemical preservatives and concerns about their side effects has led consumers to favor products without preservatives or with natural ones.

2. Objectives

This paper aims at exploring the anti-biofilm effects of the methanol extracts of *Glycyrrhiza glabra*, *Rosmarinus officinalis*, and *Saponaria officinalis* on food pathogens.

3. Methods

3.1. Bacterial Strains and Culture Condition

To evaluate the antibacterial activity, the plant extracts were investigated using strain of bacteria *Staphylococcus aureus* PTCC1189, *Shigella dysenteriae* PTCC1188, *Listeria monocytogenes* PTCC1298, *Vibrio cholera* PTCC1611, and

Bacillus cereus PTCC1015. The typed cultures of bacterial were sub cultured on nutrient agar and stored at 4oC until required for the study.

3.2. Plant Materials

The plants (*Glycyrrhiza glabra*, *Rosmarinus officinalis*, and *Saponaria officinalis*) were gathered from Zabol, in Southeastern Iran (2016 - 2017), and dried at room temperature. Samples were broken and placed on a glass plate and kept until the extraction process was finished in the laboratory.

3.3. Preparation of Extracts

To prepare the extract, the massage method is used. In this way, each of the 20 g of grinded powders was soaked in 60 mL ethanol 95%, separately for one day (shaking occasionally with a shaker). After one day of dissolving process, materials were filtered (Whatman No. 1 filter paper). Then, the filtrates were evaporated using a rotary evaporator. At last, 0.97 g of dried extracts were obtained and then stored at 4°C in air tight screw-cap tube (18).

3.4. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the extracts was determined according to methods described by CLSI 2009. *G. glabra* extract was diluted in concentration ranging from 100 to 0.78 mg/mL in Mueller Hinton broth. In each dilution tubes, 0.1 mL of the bacterial inoculum was seeded. Control tubes with no bacterial inoculation were simultaneously maintained. Tubes were incubated anaerobically at 37°C for 24 hours. The lowest concentration of the extract, which showed no visible bacterial growth (turbidity) was considered as the MIC (19). To estimate the MIC of the extract more precisely and to confirm the results, a more precise concentration in agar dilution method was used.

3.5. Biofilm Formation Assay in the Presence of the Biocides

To measure the bioavailability of standard bacteria, a 18 to 24-hour culture of each isolate was made in broth at 30°C. One mL of this culture was then added to 10 mL of the sterile TSB medium and its opacity was adjusted by optical absorption readings between 0.88 and 0.1 at 625 nm. This was done with a spectrophotometer. The suspenson's opacity is equivalent to 0.5 McFarland's standard, and contains more than 10^8 bacteria per mL. Subsequently, from this suspension, 250 μL of each microplate was filled with a specific bacterial suspension, in which case approximately 25×10^6 bacteria were present in each well. Control wells only contain a sterile environment. The genus of the microplates is 96 polystyrene wells, each well having a capacity of 300 μL and 50 μL of the extract at a concentration of 10 mg/mL. The surface of the plates was then coated and the incubation was carried out at 37°C for 24 hours. After 24 hours, the nutrient solution and microbial suspension were removed from the wells, and each well was washed three times with sterile physiology 300 μL.

Plates are also shaken to remove planktonic or unplugged cells during shitten. The bacteria were then attached to the wall and bottom of the well, fix with 95% ethanol. After 15 minutes, the contents of the wells were set. Plates were placed in a laboratory for drying in a local area. After drying the plates for five minutes with 200 microlitres, the color of the crystal violet 2%, used for hot dyeing, was stained. After this time, additional colors were washed out by placing the plates on the urban water pipe.

This color is very suitable for biofilm measurements and can also be used to measure biocides on biofilms. After washing the additional colors, the plates were placed at the laboratory temperature to dry. After drying the plates, the biofilms were visible in the purple colored rings on the wells.

Then, quantitative measurement of the production of biofilms in the form of purple colored rings was visible on the well. Then quantitative biofilm production was measured by adding 200 μL of acetic acid 33% to each well. Then, the optical absorption of the crystalline violet color in the solvent was read at 492 nm at a wavelength by ELISA (20).

4. Results

The results of this study showed that the lowest inhibitory concentration of G. garlic extract of methanol extract was 0.62 mg/mL, which was inhibited by *L. monocytogenes*, while *B. cereus* was eliminated in all concentrations of the extract.

The lowest inhibitory concentration of rosemary extract was 0.62 mg/mL, which was inhibited by *Shigella dysenteriae* and V. cholera, while the highest inhibitory concentration was 2.5 mg/mL, which *B. cereus* is restrained.

The lowest inhibitory concentration of *S. officinalis* extract is 1.25 mg/mL, which is inhibited by *S. aureus* and *Shigella dysenteriae* bacteria. The highest inhibitory concentration was 2.5 mg/mL, which *L. monocytogenes*, *V. cholera*, and *B. cereus* bacteria were inhibited in this concentration.

The highest and lowest losses of rosemary extract were 5 and 0.62 mg/mL, and *B. cereus* and V. cholera bacteria were eliminated (Table 1).

The results of the study showed that the biofilm formation rate decreases with increasing concentrations of the extracts (Tables 2 - 4).

5. Discussion

The findings revealed that ethanol extracts of licorice, rosemary, and soap flower could inhibit food pathogens so that the licorice extract showed the highest inhibitory effect on the bacteria and the lowest inhibitory effect was observed for *Glycyrrhiza glabra* against Shigella decenter.

Safety of food against microbes and its safety during preserving needs limiting initial pollution, inhibiting or limiting growth, and eliminating the microbes. Due to growing concerns of consumers and hygiene officials about chemical preservatives and their harms, consumers have favored natural preservatives that have no side effects and give pleasant smell and flavor to foods.

Several studies have explored the effects of various food extracts on important food pathogens. Food extracts are potential sources of antibacterial compounds and that is why they are so effective and useful. Antimicrobial effects of plants and their extracts have been widely studied around the world. Various types of plants have been studied based on the local plants' Flore and people's taste.

Comparison of the results concerning antimicrobial effects of different plants is really difficult. This is mainly due to various laboratory methods of exploring extracts' antimicrobial effects, preparation methods, samples and their sources, plant growth stages, and the used bacteria samples. Antimicrobial effects of the extract have been known for so many years. Nowadays, the general trend among consumers and officials of national and international organizations is to use natural preservatives instead of chemical ones. This trend has led to studies for scientific knowledge of these natural preservatives. Therefore, antimicrobial effects of plant extracts at various concentrations against food pathogens were initially explored in laboratory conditions and were detected in food materials. Studies were conducted using inoculation and these studies can finally be used to predict microbial growth in food materials to ensure human health.

In the study of Shirazi et al., which investigated the antimicrobial activity of sweetness, the results showed that *S. paratyphi* B had the most susceptibility to the concentration of 5% of the extract, while the sweet extract had a little inhibitory effect on *Shigella bacteria* (21).

The results showed that the lowest inhibitory hole diameter of Rosemary extract was against the *B. cereus* bacteria (5 mm) and the highest inhibitory diameter was *S. aureus* (13 mm).

Fernandez-Lopez examined the antimicrobial activity of rosemary extract. Results showed that Rosemary extract inhibited 11 bacteria such as *Lb. lactis* FMRD, *Br. thermosphacta* CRA, *Lb. carnosum*, *Br.* (22).

In a study by Jarrar et al., which examined the antimicrobial activity of rosemary extract against methicillin-resistant *Staphylococcus aureus*, the results showed that the minimum inhibitory concentration of ethanolic extract was in the range of 0.33 - 3.3 mg/mL (23).

Microbial biofilm represents a major virulence factor associated with chronic and recurrent infections.

An emerging approach to face the problem of antimicrobial resistance can be done by encapsulating the antibiotic in a stable nanosystem to improve the drug delivery and localize the drug release at the site of action to decrease the side effects. The results of the study showed that the biofilm formation rate decreases with increasing concentrations of the extracts.

The researchers' efforts to introduce anti-biofilm compounds have led to the recognition of plant compounds that naturally use plants to protect themselves against bacterial deposition. These compounds, which have molecular weights less than 5 kDa, are called Parvome. Among these compounds are alkaloids, terpenoids, flavonoids, coumarins, peptides, glycosides, nucleosides, and polyphenols (24).

In a study, five species of anti-biofilm were introduced. Concentration under MIC of *Rhodiola crenulata*, *Epimedium brevicornum*, and *Polygonum cuspidatum* was able to inhibit bacterial biofilm bacteria acne. The effective ingredient of these plants, which produced anti-biofilm properties, was resvelatrol, icariin, and salidoside, respectively (25).

Plant compounds can inhibit biofilms in a variety of ways; plants whose bacterial growth extracts inhibit or inhibit growth inhibit or reduce bacterial biofilm formation; however, some plant compounds without killing either. Inhibition of bacterial growth affects biofilm. The advantage of these compounds is that bacteria do not resist to them (26).

The ethanol extract of Meliadubia was used as an antibiotic biofilm compound and showed that this extract interferes with and inhibits the system in the *E. coli* bacteria that inhibits the biofilm of this bacterium (27).

5.1. Conclusions

Findings of this study showed that plant extracts have anti-microbial and anti-biofilm effects on food pathogens. It is worth mentioning that further studies on the subject, especially simultaneous use of various plant extracts, and using internal and external effective factors to inhibit pathogens in food models, are necessary.

Table 1. MIC and MBC of Plant Extracts Against Food Pathogens (mg/mL)

Bacteria	MIC, *Glycyrrhiza glabra* (mg/mL)	MBC, *Glycyrrhiza glabra* (mg/mL)	MIC, *Rosemary* (mg/mL)	MBC, *Rosemary* (mg/mL)	MIC, *Saponaria officinalis* (mg/mL)	MBC, *Saponaria officinalis* (mg/mL)
S.aureus	1.25	2.5	1.25	2.5	1.25	2.5
Shigella dysenteriae	1.25	1.25	0.62	1.25	1.25	2.5
L. monocytogenes	0.62	1.25	1.25	2.5	2.5	5
V.cholera	1.25	2.5	0.62	0.62	2.5	5
B. cereus	Complete inhibition		2.5	5	2.5	5

Table 2. Effects of Different Concentrations of *G. glabra* Extract on Biofilm Formation Bacteria

Bacteria	0.62, mg/mL	1.25	2.5	5	10	Control
S.aureus	0.009	0.006	0	0	0	0.012
S.dysenterae	0.007	0.004	0	0	0	0.010
L. monocytogenes	0.004	0	0	0	0	0.006
V.cholera	0.004	0.001	0	0	0	0.005
B. cereus	0	0	0	0	0	0.008

Table 3. Effect of Different Concentrations of *Rosemary* Extract in Biofilm Formation Bacteria

Bacteria	0.62 mg/mL	1.25	2.5	5	10	Control
S.aureus	0.013	0.006	0	0	0	0.017
S.dysenterae	0.004	0	0	0	0	0.008
L. monocytogenes	0.007	0.005	0	0	0	0.010
V.cholera	0.004	0	0	0	0	0.007
B. cereus	0.008	0.005	0.003	0	0	0.010

Table 4. Effects of Different Concentrations of *Saponaria officinalis* Extract on Biofilm Formation Bacteria

Bacteria	0.62 mg/mL	1.25	2.5	5	10	Control
S.aureus	0.015	0.008	0	0	0	0.017
S.dysenterae	0.008	0.005	0	0	0	0.010
L. monocytogenes	0.007	0.004	0.001	0	0	0.007
V.cholera	0.009	0.008	0.005	0	0	0.011
B. cereus	0.010	0.008	0.006	0	0	0.012

Footnotes

Authors' Contribution: All authors had an equal role in study design, work, statistical analysis, and manuscript writing.

Conflict of Interests: The authors declare no conflict of interest.

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