Study of effect of hydroetanolic extracts thyme, licorice and garlic on biofilm formation of Escherichia coli, Staphylococcus aureus, Bacillus cereus in foodstuff

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Abstract: Biofilms (biolayers) are associations of living bacterial cells that adhere to various surfaces. Formation of biofilms composed of microorganisms on food materials (and surfaces in contact with them) exacerbates health-related problems and economic losses that result from decaying of food materials. Staphylococci, Bacilli, and Escherichia are among the most common bacteria that contaminate foodstuffs. This research studied the effects of thyme, licorice, and garlic extracts on reducing biofilms of these bacteria in a laboratory environment. The method of measuring light absorption by the biofilms in the wells of 96-well plates was used, and results showed the mentioned extracts had antimicrobial properties and reduced biofilm formation by Staphylococcus aureus, Escherichia coli, and Bacillus cereus. Increasing the concentrations of the extracts improved their inhibitory effects (by up to 71 and 64% against E. coli and B. cereus, respectively), but the maximum inhibitory effect on S. aureus was that of the lowest concentration of thyme extracts. Increasing the concentration of garlic extracts improved their inhibitory effects. Moreover, the inhibitory effects of licorice extracts on biofilm formation by Bacillus cereus and Escherichia coli exhibited an ascending trend, but the maximum inhibitory effect on biofilm formation by Staphylococcus aureus was observed at the lowest extraction concentration (0.25). The differences between the inhibitory effects of the three types of extracts on these microorganisms were significant (p<0.05), but the differences between their concentrations were not (p>0.05).

Keywords: biofilm, herbal extract, Bacillus cereus, Staphylococcus aureus, Escherichia

Introduction

Medicinal plants have been used from the distant past. Evidence of the use of these natural resources in Iran is the past history and the documents obtained from it such as Avicenna books in which numerous medical properties of plants are included (Motamedi et al, 2010). In many opportunistic pathogenic bacteria, virulence property is related to the Quorum Sensing (QS) as one of the central regulators. This system uses small messaging molecules to control expression of several virulent factors, including exoproteases, siderophores, exotoxins and some other secondary metabolites. In addition, some behaviors of bacteria such as mobility, reproduction, lighting, biofilm, etc. are under control of this system. Some plants and microorganisms contain compounds which disrupt such behaviors of bacteria including biofilm creation by breaking down the QS messenger molecules. Biofilm is a collection of live bacterial cells that bind tightly to various surfaces. Adhesion of microorganisms to the food and food contact surfaces causes health problems and economic losses due to food spoilage. Today, biofilms are considered by scientists in various fields such as medicine, environment, food processing, etc. Biofilms are one of the causes of food spoilage in food processing factories. Biofilms containing pathogenic bacteria such as Salmonella (Dhir & Dodd, 1995, Humphery et al, 1995, Jones & Bradshow, 1996, Okoye, 2010), Pseudomonas (Brown et al, 1995), Campylobacter and Escherichia coli (Sommer et al, 1994), Bacillus, and Staphylococcus, Listeria (Mafu et al, 1990) have been studied by various researchers. Such biofilms are a source of food contamination which are formed in food-contact surfaces and cause problems. Biofilms can be formed on any surface that is placed in environment containing bacteria. In food processing places, the bacteria along with other organic and inorganic molecules such as meat proteins and milk are absorbed by surfaces and provide the conditions for creation of biofilms.

In addition, the resistance of a microorganism to antimicrobial agents is thousands of times greater than its individual form when this cell enters a biofilm structure. Thus, biofilm is considered as one of the major causes of the spread of drug resistance (Kavanaugh & Ribbec, 2012). As a result, the required concentrations of antibiotics for eradication of bacterial biofilms have exceeded the allowed amount.

This has increased the resistance of bacteria to antibiotics which imposes adverse effects to human health and the environment. Development of drug resistance in human pathogen microorganisms makes research on new antimicrobial agents, including plant sources, inevitable (Castille et al, 2012). One way to control microorganisms is man-made chemical
preservatives. However, using these chemicals in foods has been a human concern and people believe that chemical preservatives endanger their health. That's why using natural ingredients instead of chemicals is of utmost importance and undoubtedly, herbal extracts and essences will be a good alternative to chemicals. Herbal extracts contain ingredients that can be used against many microorganisms. The antimicrobial effects against bacteria, yeasts, and fungi have been proven (Cowan, 1999, Burt, 2004, Thille et al, 2003).

In this study, the potential of native medicinal plants was evaluated for analysis of signals effective in QS systems such that we can prevent communications in bacterial populations and expression of their multiple virulent behaviors by disruption in this system. Obviously, in cases of acquisition of such plants, it is possible to produce new drugs or combinations for preserving food against such bacteria. Such compounds have herbal origin and a new antibacterial mechanism with fewer side effects.

Materials and methods

In this study, extracts of thyme, licorice, and garlic were obtained from (Gorgan Pharmaceutical Co). As well, bacterial strains of Escherichia coli, Staphylococcus aureus, and Bacillus cereus were obtained from the (Microbiology Laboratory of Azad University of Damghan). For reading optical absorption, ELISA Reader Model (ELX-800, manufactured by U.S. BioTech Co) was used. First, each of the mentioned bacteria were separately cultured in the medium (LB) and after 16 hours of incubation and shaking at 30 °C, they were diluted at a ratio of 1 to 100. Then, 200 ml of it was added to the microplate.

Then, extracts of different concentrations (0.25, 0.5, 1 mg/ml) were added to the wells. In control treatment, sterile water was used instead of extracts. Each treatment was performed in 3 repetitions and completely randomized design, and the plate was incubated for 16 hours at a temperature of 37 °C without shaking. Then, the contents were emptied and washed three times with distilled water and dried in air. At this stage, 0.2% crystal violet solution was added in each of the wells and emptied after 20 minutes, and the plate was again washed 3 times with distilled water and dried in air. Finally, 200 ml of 98% ethyl alcohol was added to each well and optical density (OD) was read at 600 nm by Elisa reader.

Statistical Analysis

The experimental data was analyzed using SPSS version 21.0 for windows. The differences between groups were examined for statistical significance using analysis of variance (ANOVA) and Duncan's test. Differences were considered significant if P value was less than 0.05. All the experiments were conducted in triplicates. Excel 2010 was used to draw charts.

Results and discussion

The research showed that thyme extract has antibacterial properties and decreases biofilm formation in Escherichia coli, Staphylococcus aureus, Bacillus cereus bacteria. In Table 1, different concentrations of the extract and their inhibitory effects are provided. Inhibitory effect of thyme extract on E. coli and B.cereus biofilm showed an increasing trend. With increasing concentration of the extract, inhibitory effect was also increased. For E. coli and B.cereus bacteria, respective decreases of 67 to 71% and 15 to 64% were observed in biofilm formation compared to the control group. In Staphylococcus aureus bacterium, highest inhibitory effects were for the lowest concentration of thyme such that a 0.25 concentration showed the highest inhibitory effect (92%). In this study, there was a significant difference between micro-organisms (P <0.05), (Diagram1). However, the difference between extract concentrations was not significant (P> 0.05). Regarding the effect of garlic extract on biofilms of E. coli, Staphylococcus aureus, and Bacillus cereus bacteria, it was shown that by increasing extract concentration, the inhibitory effect also increased which was between the ranges of 90.5 to 94.3% for E. coli and between 65.5 to 76.4% for Staphylococcus aureus and 27.5 to 42.7 for Bacillus cereus. The slope of the line in Chart 4 shows this (Table 2). The effects of herbal extracts on biofilms of bacteria were studied statistically and it was shown that there is a significant difference between the extract of licorice and garlic and thyme. However, there was no significant difference between extracts of garlic and thyme (Diagram 2). The effect of licorice extract on decrease of E. coli and Bacillus cereus biofilms showed an increasing trend. However, regarding Staphylococcus aureus, the greatest effect on biofilm was relevant to the lowest concentration of the extract (0.25) equal to 83.1%, and concentrations of 0.5 and 1 had no effect on further reduction of biofilm control. The slope of the line relevant to the extract effect is shown in Figure 5 (Table 3).

![Diagram1](image1.png)

![Diagram2](image2.png)
Diagram 1: mean ± SD effect of extracts on bacterial biofilm formation that different Latin letters on the diagram show a significant difference between the samples (p<0.05).

Diagram 2: mean ± SD effect of inhibitory differential extracts, that different Latin letters on the diagram show a significant difference between the samples.

Table 1: present effect of thyme extract and O.D absorbance on microorganisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Concentration of extract (mg/ml)</th>
<th>Blank (medium &amp; microorganism)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0/5</td>
</tr>
<tr>
<td>E. coli</td>
<td>71</td>
<td>69</td>
</tr>
<tr>
<td>S. aureus</td>
<td>88</td>
<td>91</td>
</tr>
<tr>
<td>B. cereus</td>
<td>64</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 2: present effect of garlic extract and O.D absorbance on microorganisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Concentration of extract (mg/ml)</th>
<th>Blank (medium &amp; microorganism)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0/5</td>
</tr>
<tr>
<td>E. coli</td>
<td>94.3</td>
<td>92.4</td>
</tr>
<tr>
<td>S. aureus</td>
<td>76.4</td>
<td>72.3</td>
</tr>
<tr>
<td>B. cereus</td>
<td>42.7</td>
<td>35.1</td>
</tr>
</tbody>
</table>

Table 3: present effect of licoric extract and O.D absorbance on microorganisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Concentration of extract (mg/ml)</th>
<th>Blank (medium &amp; microorganism)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0/5</td>
</tr>
<tr>
<td>E. coli</td>
<td>95.2</td>
<td>93.3</td>
</tr>
<tr>
<td>S. aureus</td>
<td>81.1</td>
<td>82.4</td>
</tr>
<tr>
<td>B. cereus</td>
<td>73.3</td>
<td>71.8</td>
</tr>
</tbody>
</table>
Escherichia coli bacteria are considered as the main cause of ulcerative colitis and bloody urine. Infection caused by these bacteria is spread through contaminated food, contaminated water, and humans (Moreira et al, 2005). This bacteria has caused a lot of concern in public and health communities and is found abundantly in food.

Thyme has antiseptic effects. Its essence is sometimes used in antiepileptic and anti-rheumatism drugs, and its hot and spicy taste is used in the food industry. Its antiseptic effects are also used in health industry and scented soaps or in formulations of toothpastes as well as mouthwash solutions (Zargari 1993, and Deanse et al, 1995).

Mohsenpour et al. (2013) studied the antibacterial effects of thyme and garlic on S. aureus bacteria and showed that both methanol and ethanol extracts of thyme reduce biofilm formation by 99 percent. The ability to inhibit biofilm formation was much more in treatment with methanol extract of garlic compared to its ethanol extract. Based on the results of the mentioned study, extracts of such herbs can be used as compounds with the ability to inhibit bacteria in single and biofilm forms. Results of this research are in line with the current study.

Sepahi et al. (2012) investigated the effect of several medicinal plants on Pseudomonas aeruginosa biofilm. The results showed that the aqueous extract of asafoetida, garlic, and thyme lead to decrease in biofilm formation. Sepehri et al. (2014) showed in their study that herbal extracts reduce biofilm formation by S. aureus bacteria. This plays an important role in reducing illnesses and the present research also suggests this.

In the study conducted by Okoye et al (2010), high antimicrobial effect of herbal extracts on E.coli and S. aureus was shown. In the present study, the effects of plant extracts on bacteria biofilm was shown up to 95%.

In another study, Tabak et al. found that aqueous extract of cinnamon and thyme alcoholic extract have antibacterial effects on Helicobacter pylori and also showed that thyme antibacterial effect is greater than aqueous extract of cinnamon because it prevents urease and the growth of bacteria.

The study conducted by Aishima (2014) showed that Hibiscus Sabdariffa extract is an inhibitor of Candida albicans biofilm. In another study, Badrunnisa et al (2011) showed the proper inhibitory effect of E. tereticornis hydro-alcoholic extract on Bacillus cereus, Thuringiensi, and Pseudomonas aeruginosa bacteria, and the inhibitory effect increased with concentration increase.

Here, we studied the antibacterial effects of thyme, garlic, and licorice on biofilms of Staphylococcus aureus, E. coli, and Bacillus cereus bacteria. The greatest effect was observed on Bacillus and E. coli which was in line with the results of other researchers. The mentioned extracts also showed a good effect on reduction of Staphylococcus aureus biofilms. It is suggested that a greater number of medicinal plants as well as problematic bacteria be investigated in the food industry. Some research must also be conducted to identify the active substances in these plants and their effects on
reducing biofilm so that we can commercialize these compounds and substances as natural preservatives in food and remove biofilm in food industry and machinery.

References


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