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Effects of Methanol Extract of Green Tea on Biofilm Formation of *Staphylococcus aureus*

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Abstract

Background and Objectives: The aim of this study was to investigate the effect of ethanol extract of green tea on biofilm formation of *Staphylococcus aureus*.
Methods: *Staphylococcus aureus* samples were obtained from hospital sources and plant extracts; green tea was made using the rotary. The minimum inhibitory concentration was determined using the microdilution method. Inhibition of biofilm formation was determined by using a microplate.
Results: The results of this study showed that the greatest resistance was to the antibiotics penicillin (88.2%) and erythromycin (82.4%) and the least resistance to the antibiotic Amikacin (76/11%), respectively. The results of the effect of green tea extract showed the lowest inhibitory concentration (MIC) is equal to 0.3 milligrams per milliliter, which is a strain on the inhibitory concentration, and the highest concentration (MBC) MBC resistant *Staphylococcus aureus* equal to 20 milligrams per milliliter, which is controlled unidirectional. The results of green tea extract effects on biofilm showed that with increasing concentrations of less biofilm formation the biofilm formation has been much lower concentration of the extract.
Conclusions: The results of this study indicate good effects of green tea extract to inhibit biofilm formation of *Staphylococcus aureus*, which can be used to treat infections caused by these bacteria's.

Keywords: Biofilm, Extract plant, *Staphylococcus aureus*

1. Background

Antibiotics are the main cornerstones of treatment in antibiotic infections. However, due to the ever-growing resistance of bacteria to antibiotics and the side effects of the drugs, use of a complimentary method for treating infections has been focused. Nowadays, many researchers have focused on using plant extracts for treating bacterial infections.

Green tea is full of antioxidant, anti-inflation and anticancer compounds. Flavones and ethers are amongst the other compounds of green tea. When green tea is directly heated, catchins such as epicatchins, epigallocatchins, epicatchin gallate and epigallocatchin gallate. In Asia, were tea is widely used, it draws with indirect heat of water steam. This way, epigallocatchin is preserved. Catchin is a very powerful antioxidant, which blocks the growth of cancer cells (1). In the past, tea was used as a drug to treat kidney diseases, fever and inspiratory infections. Tea has appeasing and pleasant epiphenols, such as theaflavin and thearubijin. Drawn tea has caffeine, oxidized polyphenols, proteins and carbon hydrates, which are not found in ordinary foods.

Staphylococcus aureus has been long regarded as an effective human pathogen. The infections caused by this bacterium can repeatedly affect the hospital patients and can leave severe effects even after being treated by antibiotics (2). *Staphylococcus aureus* is a warm positive bacteria growing in the skin. It exists in the front part of the noses of 25 to 30% of people without showing any specific symptom. This part is the main origin of bacterial infection. The bacterium can cause a wide range of diseases including endocarditis, esteomilite, pneumonia, shock syndrome, toxic, pustules and abscess (3).

Populations of one or more bacteria geneses that can stick to each other or to live surfaces in an environment of extracellular polysaccharides are called biofilms. The spread of biofilms is a complicated process, which can be explained based on collective patterns of bacteria. Collec-

tive life has many benefits for bacteria compared to individual life (4). Biofilm formation includes initial revertible or irreversible attachment to surfaces, formation of micro-colonies and finally maturation of micro-colonies with formation of exopolysaccharides (5). Biofilm can be formed on different surfaces. Due to its various ways of attachment to surfaces, it has many applications (6).

2. Objectives

The aim of this study was to investigate the effect of ethanol extract of green tea on biofilm formation of *Staphylococcus aureus*.

3. Methods

3.1. Separation of Staphylococcus aureus

The different samples of *Staphylococcus aureus* used in this study were gathered from urine samples of Zabol patients and were planted on special planting environments of Manitol salt agar and blood agar. The pure samples obtained from artificial environment were detected by catalase test. The *Staphylococcus aureus* genus was recognized by using the Guacolase test and investigating the formation of Agglutination. The other geneses were detected by other methods.

3.2. Half-McFarland Suspension Preparation

For preparation of microbial suspension, bacteria were transferred from the reservoir to the agar planting environment (German Merc). After growth of bacteria colonies, planting surface was washed by normal saline and thick microbial suspension was prepared. Then, some of the bacterial suspension was poured into the sterile pipe containing normal saline and its darkness was measured using spectrophotometer at a wavelength of 630 nm. The solution was diluted with normal saline until the darkness of solution equaled that of half-McFarland Solution. As a result, bacterial suspension with concentration of 10^8 cfu/mL was obtained.

3.3. Testing Sensitivity of Microbes to Antibiotics

Sensitivity of *Staphylococcus aureus* samples to the various antibiotics produced in Padtan Teb Company of Iran was measured by the Kerbi-Baer standard disk diffusion method. To this end, half-McFarland of all samples were prepared in Muller Hinton Broth and planted on Agar Muller Hinton environment. Antibiotic disks were positioned on agar Muller Hinton near plate brim. Plates were kept in the incubator at 37 degrees for 24 hours. Diameters of inhibitory clouds were measured to determine the resistance and sensitivity of samples to antibiotics. The results were compared to those of NCCLS standard results.

3.4. Plant Materials

The leaf green tea was collected in the region of Iran (Gorgan, northern Iran), planted at Zabol University, received approval and dried at room temperature during 2014 - 2015. Samples were crashed and transferred into glass containers and preserved until the extraction procedure was performed in the laboratory. The extract was prepared by the maceration method and 50g of the sample was macerated and kept in 96% ethanol for 48 hours. Then, the obtained extract was filtered by filtration paper and was distilled by rotary devices.

3.5. Determining the Extract's Dry Weight

Sensitivity of the bacteria samples having multiple resistances to the plant extract was explored by using the dilution method in the sink. Seven sinks of microtiter plates were added to 100ml of the Muller Hinton Broth. The first sink added 100 mL of the diluted extract solution. After mixture, 100ml of the first sink was added to the second one, and so on. 100 mL of the broth was removed from the last sink and 100 mL of the microbial suspension containing 107 unit/mL (0.5 McFarland) was added to all sinks. The solution was preserved in an incubator at 37 degrees for 24 hours. The first sink inhibiting the growth of bacteria after being positioned in the incubator was considered as MIC. To guarantee the precision, 10 microliters were transferred from bright sinks to the agar Muller Hinton environment. After 4 hours, the first concentration that could remove 99.9% of the bacteria was shown as the minimum removal concentration (7).

3.6. Biofilm Formation

Dilution method was used to investigate the effect of the plant extract of green tea in forming bacteria biofilms. Seven sinks of microtiter plates were added 100 microliter of the nutrient broth nutritive liquid and TSB. The first sink was added 100 mL of the diluted extract (10 mg of the extract in 1 cc of DMSO solvent). After the mixture, 100 mL of the first broth was added to the second sink. The same act was done for all sinks except the witness sink. 100 mL of the planting environment was removed and 100 mL of microbial suspension was added to all sinks. The sinks were covered with cellophane nylon (or para film) and were positioned in the shaker incubators at 37 degrees for 24 hours. Then, the sinks' contents were replaced with sterile physiological serum. Microplates were slowly shaken for cells of weak or no bond to be detached. Once again, sinks were emptied and 300 mL of 96% alcohol was added to the sinks during 15 minutes for the cells to be fixed. After removal of alcohol, sinks were colored with 2% viola crystal during 5 minutes. Then, sinks were rinsed and 33% acetic acid was

added to them. Subsequently, the sinks were positioned in ELISA Reader at a wavelength of 492 nm.

3.7. Statistical Treatment of the Result

The mean values were analyzed with the MINITAB release 13.20 program statistically by the general one-way analysis of variance (ANOVA) to find out the most effective plants and the most sensitive test organisms.

4. Results

The study results showed that the highest resistance was to penicillin antibiotics (88.2%) and Erythromycin (82.4%) and the lowest resistance was to Amikacin (11.76%) (Table 1). Results of investigating the effects of green tea showed that the minimum inhibitory concentration was 0.3 mg/mL in which one sample was inhibited and that the maximum inhibitory concentration was 10 mg/mL in which two samples were inhibited. The highest removal concentration of MBC was against *Staphylococcus aureus* and about 20 mg/mL in which one sample was inhibited (Table 2).

Strains that were exposed to sub MIC and MIC levels of extract exhibited a reduction in the OD492 reading. These results showed that these strains exhibited an impaired ability to form a biofilm compared to the control. The achieved results showed that the bacteria resistant to cephalosporin compared to the sensitive ones are able to form more biofilms (Table 3).

Enhancing the duration has increased the amount of formation. However, as it can be seen in diagrams, different densities of extract have an inhibiting effect on the biofilm formation in comparison with the situation that there is no inhibitor factor in the environment.

5. Discussion

Results of investigating the effects of green tea extract showed that the minimum inhibitory concentration was 0.3 mg/mL in which one sample was inhibited. The maximum inhibitory concentration was 10 mg/mL in which two samples were inhibited. The highest removal concentration of MBC was against *Staphylococcus aureus* and about 20 mg/mL in which one sample was inhibited. Results of the effects of green tea extract on biofilm showed that biofilm formation decreases at higher concentrations and increases at lower concentrations.

The inhibitory effects of green tea extract reveal the probability that oxidation metabolites decrease the inhibitory and antioxidant effects of green tea leaves during fermentation. Researchers believe that bacriocide catchins can harm two fat layers of the membrance. Although polyphenols are powerful antioxidants, they can act as a pro-oxidant in some specific situations. It seems that tea polyphenols impose their inhibitory effects on bacteria by their pro-oxidant features. There are several reports on the antimicrobial effects of different types of tea and their pure and polyphenols on a range of microbes. A probable cause of such effects is the interactions of tea components with high purity or insufficiency of the antimicrobial components of tea. Researchers believe that plant polyphenols and Tanens leave their inhibitory effects on the growth of microbe cells through oxidation and production of hydrogen peroxide. In some conditions, there might special genes in bacteria that increase antioxidant defense in bacteria and overcome the inhibitory effects of Tanens (8).

Wheeler showed that tea extract can remove or inhibit the growth of bacteria such as *Vibrio cholera*, *Shigella dysenteriae*, *S. epidermidis* and *S. aureus*. He also reported that some concentrations of tea, which are found in a cup (3 mg/mL) can remove the *S. aureus* resistant to Meticiline (9). Tea is also reported to have synergistic effects on antibiotics (10).

Nataro reported that catchin, Epigalocatchin, Epicatchin galate and Epigalocatchin, which are found in green leaves, inhibit the dissemination of Verotoxin from *E. coli* (11).

Lee at al. showed that bacteroids are easily removed by the polyphenols metabolites of tea (12).

Klibanov et al. found that tea extract has effects on *Clostridium*, *Pseudomonas* and plant pathogenic bacteria such as Ervinia (13).

Hamilton reported that green tea is full of antioxidant and anticancer materials (1).

The study done by Bokaeian, showed that the concentrations of 5 and 10 mg/mL are the most restrain in the biofilm formation of the isolated plates (14).

Toda reported that tea extract can remove or inhibit pathogenic bacteria such as *S. aureus*, *S. epidermidis*, *Shigella dysenteriae* and *Vibrio* geneses such as *Vibrio Chrola* (15).

Other experts also revealed that green tea leaves polyphenols can inhibit the growth of *Escherichia coli*, *Streptococcus* and *S. aureus* (16).

Further, researchers found that black tea outperforms green tea in inhibiting biofilm formation. Green tea at concentrations of 4.5 or 5 mg/mL has bacteria-side effects on microorganisms.

In this regard, *Proteus mirabilis* and *Escherichia coli* showed the highest sensitivity to black tea and green tea, respectively. *Klebsiella pneumonia* showed the highest resistance to both extracts (17). The same results were found for

Table 1. Sensitivity Pattern of 17 *Staphylococcus aureus* Samples

Antibiotic	Disk Concentration, m.c.g	Percentage of Studied Bacteria		
		Sensitive	Semi-Sensitive	Resistant
SXT	15/23 + 25/1	4 (23/5)	1 (5/9)	12 (70/6)
AM	10	3 (17/6)	2 (11/8)	12 (70)
GAZ	30	9 (52/9)	3 (17/6)	5 (29/4)
TE	30	5 (29/4)	8 (47/1)	4 (23/5)
E	15	1 (5/9)	2 (11/8)	14 (82/4)
P	10	1 (5/9)	1 (5/9)	15 (88/2)
CRO	30	1 (5/9)	12 (70/6)	4 (23/5)
AN	10	15 (88/23)	0	2 (11/76)
CF	10	2 (11/8)	1 (5/9)	14 (82/4)

Abbreviations: AF, Amicasine; AM, Ampiciline; CRO, Ceftriacson; E, Eritromicine; SF, Seftazidium; SXT, Tri-Metoperium- Sulphametoctazol; TE, Tetraxiline.

Table 2. Inhibition Pattern of *Staphylococcus aureus* Samples at Various Extract Concentrations

Bacterial Code	MIC/MBC Green Tea, mg/mL
1	10/20
2	5/10
3	5/10
4	2.5/5
5	2.5/5
6	10/10
7	1.25/1.25
8	0.62/0.62
9	5/10
10	2.5/5
11	5/10
12	5/10
13	5/10
14	2.5/5
15	0.62/1.25
16	0.3/0.62
17	5/10

Bordetella pertussis (the cause of Pertussis) (18).

Researchers found that tea extracts have effects on Clostridium geneses, plant pathogenic bacteria such as *Ervinia* and *Pseudomonas* geneses.

There are several reports on antimicrobial effects of various types of tea (19) and its pure polyphenols (20) against a range of microbes. Tea has also been reported to have synergistic effects on antibiotics (21).

The study of Sharifi Mood et al. states that antibacterial activities of Ajowan essential oil (AEO) have been evaluated against two gram negative bacteria; *Klebsiella* and *E. coli* and one gram positive bacteria; *Staphylococcus aureus* (*S. aureus*). The minimum inhibitory concentration (MIC) value was determined against all mentioned bacteria, the antibacterial activity of AEO was assessed against all selected pathogens and different MIC levels were observed. The essential oil was effective for *S. aureus* with MIC of 1.25 mg/mL, followed by *E. coli* with MIC of 2.5 mg/mL and *Klebsiella* with MIC of 5 mg/mL (22).

The study of Jahani et al. was done with an aim to discover the function of some medicine plants on pestiferous *Pseudomonas aeruginosa* and *Escherichia coli* in humans. The results showed that Teucrium polium extracts have the minimum density of inhibitory for *Escherichia coli*, 25 ppm, whereas the maximum of this is for *Peganum harmala* and *Prangos ferulaceae* with 100 ppm. The lowest minimum concentration inhibitory value of extracts *P. harmala*, *T. polium*, *T. pratensis* and Rumex was found in 25 ppm against *P. aeruginosa* (23).

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The study of Javadian stated that the current study was to investigate the antimicrobial activity of an extract of the

Table 3. The Effects of Different Concentrations of Extract Plant on the Biofilm Formation of Bacterial

Bacterial Code	0.3	0.62	1.25	2.5	5	10	20
1	0.010	0.007	0.005	0.004	0.003	0.001	-
2	0.015	0.012	0.008	0.008	0.005	-	-
3	0.017	0.014	0.012	0.009	0.006	-	-
4	0.008	0.005	0.003	0.003	-	-	-
5	0.007	0.006	0.002	0.002	-	-	-
6	0.012	0.011	0.008	0.006	0.006	-	-
7	0.002	0.002	-	-	-	-	-
8	0.001	-	-	-	-	-	-
9	0.005	0.004	0.003	0.003	0.002	-	-
10	0.011	0.007	0.007	0.005	-	-	-
11	0.008	0.008	0.005	0.004	0.003	-	-
12	0.012	0.012	0.010	0.006	0.002	-	-
13	0.013	0.010	0.009	0.008	0.008	-	-
14	0.004	0.003	0.003	0.002	-	-	-
15	0.003	0.001	-	-	-	-	-
16	0.003	-	-	-	-	-	-
17	0.009	0.006	0.006	0.005	0.003	-	-

Peganum harmala flower and *Heracleum persicum* against *Acinetobacter baumannii*. The results show that the levels of MIC extract and essential oil of *Peganum harmala* were observed in ranges from 6.25 ppm to 12.5 ppm and 3.1 ppm to 25 ppm, respectively. The highest MIC value was observed as 12.5 ppm in *A. baumannii*. The levels of MIC extract and essential oil of *Heracleum persicum* were observed in ranges from 5 ppm to 20 ppm and 12.5 ppm to 10 ppm, respectively. The highest level of MIC extract and the highest essential oil value of Heracleum persicum were observed as 20 ppm and 10 ppm, respectively, in *A. baumannii* (24).

The study done by Bokaeian was aimed to detect antibacterial activity of silver nanoparticles produced by *Plantago ovata* seed extract against antibiotic resistant *Staphylococcus aureus*. The silver nanoparticles revealed Gaussian distributions with the average diameter of 13 nm with some deviations. The results showed that the highest and the lowest MIC of *P. ovata* seed extract were 100 and 12.5 mg/mL, respectively (25).

The study of Bokaeian evaluates the effect of *W. somnifera* extracts on drug resistant *E. coli* strains isolated from clinical samples. The results showed that the isolated *E. coli* strains were sensitive to these antibiotics: erythromycin (52.94%), tetracycline (76.47%), ceftazidime (41.17%), cefixime (35.29%), penicillin (76.47%), ampicillin (58.82%) and nalidixic acid (41.17%). Examination of the herbal extracts showed that the highest maximum inhibitory concentration (MIC) against drug resistant *E. coli* was 200 ppm. The lowest MIC was 50 ppm, where three strains of *E. coli* were inhibited at this concentration (26).

Other studies reported that green tea leaves' polyphenols have inhibitory effects on growth of *Escherichia Coli*, *Streptococcus*, *Staphylococci aureus* and *Bordetella pertussis*.

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