

## Antimicrobial Activity of Combined Extracts of *Trachyspermum*, *Thymus* and *Pistachio* against Some Pathogenic Bacteria

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### Abstract

**Background:** Microbial biofilms are responsible for many human infections and increase of antibiotic resistant bacteria. Therefore, finding an efficient way to prevent infection and biofilm formation of bacteria is essential. Medicinal plants are among suitable candidates to inhibit biofilm formation of bacteria. The aim of this study was to evaluate the antimicrobial effects of *Trachyspermum*, *Thymus* and *Pistachio* (T.T.P) combined extracts (methanolic and ethanolic) against six pathogenic bacteria in planktonic and biofilm forms.

**Methods:** The antibacterial activities of T.T.P combined extracts against planktonic form of pathogenic bacteria were evaluated by disc diffusion and macro-broth dilution methods. Also, anti-biofilm activity of the combined extracts was evaluated by microtiter plate method.

**Results:** The results of disc diffusion analysis (MBC and MIC) showed that both methanolic and ethanolic extracts of T.T.P combined extracts were efficient for inhibition of planktonic forms of bacteria, although ethanol extract was more effective compared to the methanolic extract. The T.T.P combined extracts had inhibitory effect against biofilm formation of pathogenic bacteria. Also, these extracts efficiently demolished biofilm formation and prevent metabolic activity of bacteria in biofilm structures. The concentration of each extract had a direct relationship with its inhibitory effect. The maximum inhibitory effects of T.T.P combined extracts on biofilm formation, demolish of biofilm structure and inhibition of the metabolic activity of bacteria in the biofilm were 95.3% for *P. aeruginosa*, 97.2% for *P. aeruginosa* and 81.16% for *E. coli*.

**Conclusion:** The results of this research demonstrated that T.T.P combined extracts can be used as an alternative component with inhibitory effect against pathogenic bacteria in planktonic and biofilm forms.

developed in the left eye. Two patients had no family history suspicious for keratoconus.

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### Introduction

Infectious diseases are common widespread diseases in the world which impose expenses on human societies. The rate of death resulting from these diseases is growing. Antibiotics are one of the main medicines to treat infectious

diseases. Nowadays, due to the vast use of antibiotics, the resistance of bacteria to antibiotics has been increased (1-3).

One of the reasons for antibiotic-resistant bacteria is forming the biofilm and it has been converted to one of the serious problems in infections. Biofilms are attached organizations of

microbial cells with strong adherence to surfaces and they are protected by an extracellular matrix consisting of exopolysaccharides, proteins and DNA.

This structure prevents the penetration and also the proper function of antimicrobial compounds so that some researchers claim that antibiotic resistance of biofilms is thousand times higher than that of planktonic bacteria (4-8). Therefore, in recent years, due to the importance of biofilms in the development of diseases, increase of microbial resistance and side effects of antibiotics, researchers have been interested in using natural medications such as medicinal plants extracts.

Low cost of production, fewer side effects and lack of environmental problems are among the advantages which have converted herbal drugs into suitable candidates to inhibit the antibiotic-resistance bacteria (2, 9). Medicinal plants have effective materials such as secondary metabolites, which have anti-bacterial and anti-fungal effects (10-12). The plants that have been studied in this research have anti-bacterial, anti-inflammatory and antioxidant properties and they increase digestibility and nutrient absorption (13, 14, 15).

Iran has a large variety of medicinal plants that is the basis of traditional medicine in this country. In this study, antimicrobial effects of three plants including *Thymus*, *Trachyspermum ammi* and *Pistachio* were evaluated (). *Thymus* is a plant belonging to the Lamiaceae family. This plant has some medicinal applications in preventing urinary infection, decreasing cholesterol, preventing atherosclerosis, controlling bleeding and treating diarrhea. The most important substances in this medicinal plant are phenolic monoterpenes, thymol, carvacrol, and paracyamol (16, 17).

*T. ammi* belongs to Apiaceae family and the seeds of this plant are used in some part of the world (India, Pakistan, Egypt and Southeast of Iran) as a spice and flavouring. *T. ammi* seeds are rich in fiber, minerals, vitamins, and

antioxidants. The most important chemical substance of this plant is thyme that has anti-bacterial, anti-spasmodic and anti-fungal activities (18-20).

*Pistachio* belongs to Anacardiaceae family. This plant is found in the central and western parts of Asia and all over the Mediterranean parts and not only it is counted as a rich source of nutrients but also can be considered as a medicinal drug as well. Some researchers have proved the medicinal properties of this plant. For instance, the fruit of this plant has anti-inflammatory effects, decreases cholesterol, controls body weight and diabetes and also its peel has a protective effect against cardiovascular diseases, cancers as well as bacterial and fungal infections. Polyphenol can be mentioned as one of the most important substances in this plant (21-23).

The objective of the current research was to study the antibacterial and anti-biofilm activities of combined extracts of 3 medicinal plants (*Pistachio*, *Thyme*, and *T. ammi*) against six pathogenic bacteria in planktonic and biofilm forms.

## Materials & Methods

### Plant collection, identification and extract preparation

The fresh plants of *Thymus* (leaf), *T. ammi* (seed) and *Pistachio* (skin) were collected from Kerman province, Iran. The taxonomical identification of the plants was confirmed by the botanical expert of the botany Department of Shahid Bahonar University of Kerman, Iran. Plants were air dried and reduced to coarse powder using a pestle and electric blender (Bosch, Germany). The powdered plants were extracted by the modified macerated method. The powder of each plant was respectively mixed with methanol (96%) and ethanol (80%) to Mass-volume ratio 1:10 and was continuously shaken in an incubator for 18-24 hours at 40 °C. Filter paper (Wathman No. 1) was used to remove the coarse parts of the plant and the resulting solution was transferred to the rotary

evaporator to remove the additional solvent. In order to remove the remaining alcohol (ethanol and methanol) the solution was placed in a 40 °C incubator for 48-72 hours. Dry powder of each extract was kept in a dark glass container and at temperature of 4 °C until it was used (24).

### Bacteria and culture conditions

In this study six bacteria including 2 Gram-positive (*S. aureus* PTCC 1189 and *B. cereus* PTCC 1154) and 4 Gram-negative (*E. coli* PTCC 1397, *P. aeruginosa* PTCC 1310, *A. baumannii* PTCC 1797, *K. pneumoniae* PTCC 1290) were used for antimicrobial. These standard strains were purchased from Iranian Research Organization for Science and Technology (IROST). Muller Hinton Agar (MHA, Merck, Germany) was used for disc diffusion test. Muller Hinton broth was used for determination of minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The tryptic soy broth (TSB, Merck, Germany) medium was used for anti-biofilm assay.

### Disc diffusion assay

Anti-bacterial activity of T.T.P combined extracts (methanolic and ethanolic) was assayed by using Bauer-Kirby standard disc diffusion method. Briefly, after overnight cultures of bacteria, cell concentrations were adjusted to  $10^8$  (CFU/ml) compared to McFarland turbidometer and 500 µl of bacterial suspension was added to each MHA agar plate. Then, the sterile filter paper disc (6 mm) (Tehran, Iran), saturated (100 mg/ml) with plant extract was placed for 1 h and allowed to dry for 30 min at room temperature. The discs prepared in the same condition with only the corresponding volumes of ethanol and methanol were used as controls. Plates incubated at 37°C for 18-24h. The results were recorded by

measuring the zone of inhibition (mm) surrounding the discs (25).

### MIC and MBC determinations

MIC and MBC were determined by a macrobroth dilution method using 96-well microtiter plates as recommended by the Clinical and Laboratory Standards Institute (26). Overnight cultures of bacteria were diluted to yield a final concentration of  $5 \times 10^5$  (CFU/ml). Samples were then added to equivalent volumes of various concentrations of extracts in tubes and prepared from serial two-fold dilutions ranging from 0.05-50 mg/ml. These solutions were prepared by dissolving extract stock concentration (100 mg/ml) in sterile culture medium (MHB). Following incubation for 18 h at 37 °C, the lowest concentration of compound that prevented visible growth was recorded as the MIC. Bacteria and MHB were used as control and vehicle controls (bacteria + MHB + solvent), and media controls (MHB) were included.

The MBC was determined by spreading 150 µl on MHA plate from the sample showing no visible growth and it was further incubated for 18-24 h at 37°C.

### Inhibition of biofilm formation

The effects of extracts on biofilm formation of six pathogenic bacteria were determined by protocol of Jabra-Rizk et al (27) with some modifications. At first, 100 µl of ethanolic and methanolic extracts dilutions (6.25-25 mg/ml) were added to 96-well plate and then, 100 µl of bacterial suspension was added and the plates were incubated for 24 hours at 37°C. Wells containing TSB and sterile water were considered as controls. After incubation, the plate was washed 3 times with PBS and then, 150 µl of 96% methanol was added to fix the attached cells. 200 µl of crystal violet 1% (Merck, Germany) was added to the well and incubated for 30

minutes at 20°C. Finally, 160 µl of acetic acid glacial (33%) was added to the well and absorbed by ELISA reader (India, Biotec, ELX-800) at 630 nm and calculated by the following formula (28):

$$M = 100 \times \{(A-B) - (C-D) / (A-B)\}$$

M: percentage of inhibition of biofilm formation, A: mean of optical absorption of control, B: mean of optical absorption of control medium, C: mean absorbance of the well test, D: mean of absorbance of extract control

### Inhibition of constructed biofilm

The biofilm of bacteria were constructed by adding 100 µl of stationary-phase bacterial cultures in TSB medium to the wells of a 96-well polystyrene microtiter plate and incubated at 37°C for 24 h. After biofilm formation, the medium was aspirated gently, and non-adherent cells were removed by washing the biofilms three times with sterile PBS. The effect of extracts on constructed biofilm, were evaluated by the same procedure except that each extract was added to the wells at selected concentrations (12.5-50 mg/ml) and it was incubated for 24 h at 37°C (29). Further Inhibition of a constructed biofilm was analyzed by crystal violet staining. The percentages of reduction of biofilm structures in the presence of different concentrations of extracts were calculated employing the formula described in the previous section.

### Assessment of biofilm metabolic activity

The effect of the T.T.P combined extracts on metabolic activity of pre-formed biofilm was measured as reported by Hoiby et al. (30) with some modifications. Briefly, pre-formed biofilms were washed twice with PBS and then, the extract (12.5-50 mg/ml) was added and biofilms were incubated for an additional 24 h at 37°C. Afterwards, 50 µl of a Triphenyl

Tetrazolium Chloride (TTC, Merck, Germany) solution was added to each well allowing the reaction to occur in the dark at 37°C for 3 h. Final absorbance was read at 490 nm using ELIZA reader (State Fax 2000). The percentages of reduction of biofilm metabolic activity in the presence of different concentrations of extracts were calculated employing the formula described in the previous section.

### Statistical analysis

All experiments were performed in triplicate and differences for individual parameters between control and treated groups were tested by analysis of variance (ANOVA) and through SPSS Version 18.0 for Windows. Differences were considered significant if the P value was less than 0.05.

## Results

### The inhibitory effects of T.T.P combined extracts against planktonic forms of bacteria

The results of anti-bacterial effect of T.T.P combined extracts (both methanolic and ethanolic extracts) and also MIC and MBC have been presented in Table (1). According to this table *E. coli* and *K. pneumoniae* showed the most and the least sensitivity to these extracts respectively. According to the MIC and MBC results, the MIC of methanol extract for *K. pneumoniae* and *A. baumannii* were 50±0.89 and 50±1.83 (mg/ml) respectively. The MIC of ethanolic extract for *S. aureus*, *E. coli*, *A. baumannii*, *B. cereus*, *K. pneumoniae* and *P. aeruginosa* were 6.25±0.76, 6.25±0.76, 12.5±0.94, 25±0.55, 50±1.01 and 50±1.23 (mg/ml) respectively. The MBC of T.P.P extracts for pathogenic bacteria were between 3.125±0.46 and 50±1.12 (mg/ml). *A. baumannii* and *K. pneumoniae* did not have any MBC values for methanolic extract.

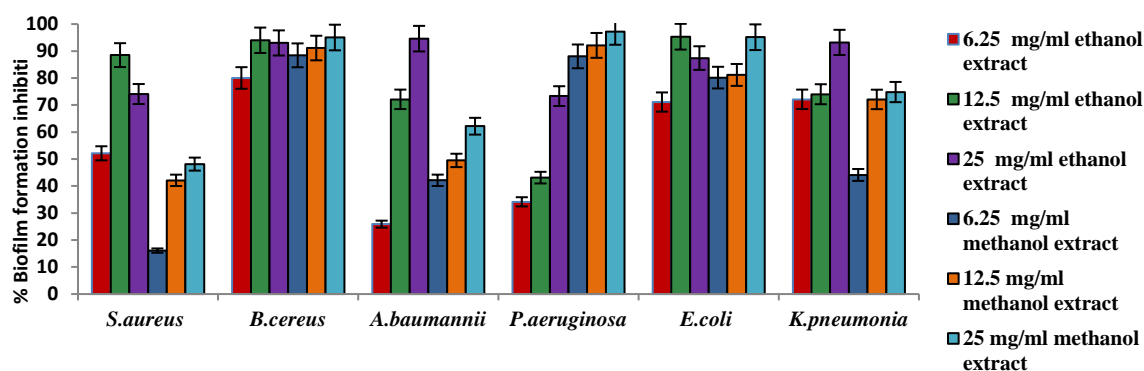
**Table 1.** Antibacterial activity, minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of T.T.P combined extracts against pathogenic bacteria.

Bacteria	Disk Diffusion		MIC		MBC	
	Zone of inhibition (mm)		Methanolic extract (mg/ml)	Ethanol extract (mg/ml)	Methanolic extract (mg/ml)	Ethanol extract (mg/ml)
	Methanolic extract (mg/ml)	Ethanol extract (mg/ml)				
<i>S. aureus</i>	16±0.23	18±0.77	0	6.25±0.76	50±1.08	12.5±0.65
<i>B. cereus</i>	7±0.87	8±1.03	0	25±0.55	50±0.45	50±1.12
<i>P. aeruginosa</i>	15±1.12	16±0.35	0	50±1.23	50±1.76	12.5±1.02
<i>A. baumannii</i>	11±0.76	12±0.67	50±1.83	12.5±0.94	0	3.125±0.46
<i>E. coli</i>	21±1.43	20±1.65	0	6.25±0.76	50±1.54	12.50±1.04
<i>K. pneumoniae</i>	6±0.89	6±1.02	50±0.89	50±1.01	0	25±0.23

### The inhibitory effects of T.T.P combined extracts against Biofilm formation

The efficiency of different concentrations of T.T.P combined extract to inhibit biofilm formation of bacteria has been shown in Figure (1). As shown in this figure, the methanolic extract at concentration of 25mg/ml had the most

efficiency to inhibit biofilms formation of *P. aeruginosa* (95.3 %). This extract had the lowest efficiency to inhibit biofilm formation of *S. aureus* (16.09%) at 6.25 mg/ml concentration. The effects of different concentrations of T.T.P combined extract on inhibiting biofilm formation were significantly different ( $p < 0.05$ ).



**Figure 1.** The efficiency of T.T.P combined extract to inhibit biofilm formation of bacteria at different concentrations

### The inhibitory effects of T.T.P combined extracts for destruction of biofilm structures

The capabilities of various concentrations of T.T.P combined extract in destructing biofilm structures have been shown in Figure (2). As shown in this figure, maximum destruction activity of this extract was related to *P. aeruginosa*

(97.2%) biofilm at 25 mg/ml concentration, however the minimum inhibitory effect was related to *S. aureus* (16.09%) biofilm at 6.25mg/ml concentration. The effects of different concentrations of these extracts on destruction of biofilm were significantly different ( $p < 0.05$ ).

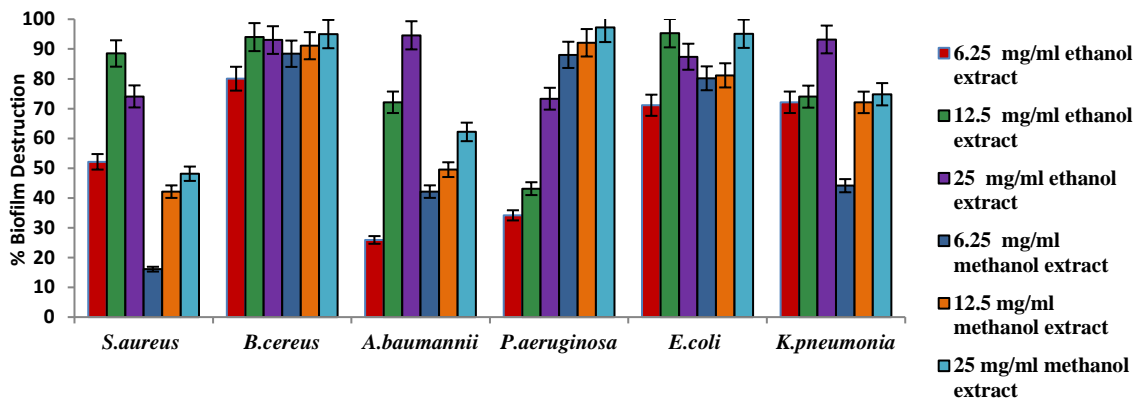


Figure 2. The efficiency of T.T.P combined extract in destruction of pathogenic bacteria biofilm at different concentrations

**The inhibitory effects of T.T.P combined extracts against biofilm metabolic activity**

The efficiency of different concentrations of T.T.P combined extracts on activity of dehydrogenase enzyme in the studied bacteria have been shown in figure (3). As shown in this figure, the highest inhibition of enzyme activity with this

extract (25mg/ml concentration) was related to *S. aureus* (OD=0.6). The lowest inhibitory effect of this extract (6.25mg/ml concentration) against dehydrogenase enzyme activity was related to *K. pneumoniae* (OD=1.9). The effects of different concentrations of this extract on inhibition of enzyme activity were significantly different ( $p < 0.05$ ).

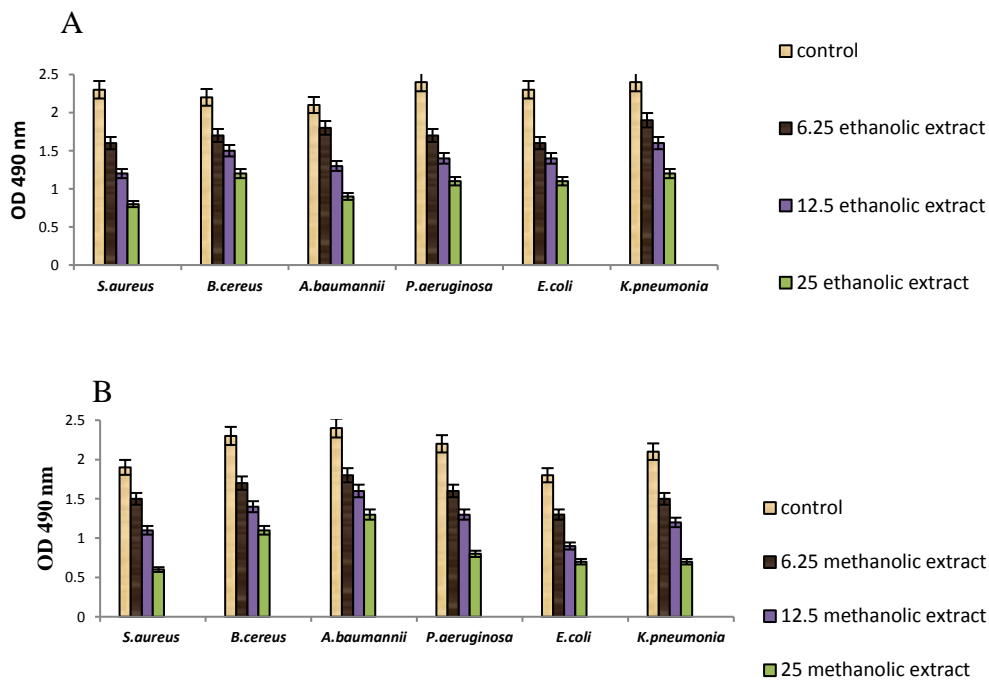


Figure 3. The inhibitory effect of different concentrations of methanolic extract (mg ml<sup>-1</sup>) (A) and ethanol extract (mg ml<sup>-1</sup>) (B) of T.T.P combined extract on dehydrogenase enzyme activity of bacterial biofilm

## Discussion

Nowadays, one of the major problems in fighting against infection diseases is increase of resistance of microorganisms to antibiotics. One of the reasons of antibiotic resistance is formation of biofilm structure and 65% of infections are related to this structure (31-33). Medicinal herbs can play a significant role to control human infectious diseases. Many people living in developed countries use medicinal herbs to treat various diseases (34) and several studies have been done on the antibacterial and antifungal activities of the mentioned herbal extracts, but few studies have been performed on their effects against pathogenic bacteria.

In the present study, the anti-bacterial properties of T.T.P combined extract against six pathogen bacteria were studied. In disc-diffusion, MIC and MBC methods, our results demonstrated that T.T.P combined extracts (methanolic and ethanolic) have high efficiency to prevent pathogen bacteria. The MIC and MBC values ranged from 3.125 to 50 mg/ml. The efficiency of extract in disc diffusion experiment and liquid medium was different. Since the distribution of plant extract in liquid medium was better than solid medium. Generally, ethanol solvent is more efficient to interact with the components of T.T.P combined extract in comparison to methanol solvent and consequently causes effective materials to exit from the plant increasingly and leads to higher concentration of these materials in T.T.P ethanol extract rather than in T.T.P methanol extract (35-37).

Some researchers have confirmed the antibacterial activity of three plants studied in this research. Hassanshahian et al (2014) studied the antimicrobial activities of *Trachyspermum ammi* essential oil against different kinds of microorganisms by microtiter plate method. They have reported *K.*

*pneumoniae* as the most sensitive bacterium to this plant extract and they did not observe any zone of inhibition for *S. aureus* (38).

Mohsenipour and Hassanshahian (2015) revealed antibacterial activity of *Thymus vulgaris* extracts against six pathogenic bacteria in planktonic and biofilm forms. They concluded that this plant extract could efficiently inhibit biofilm formation of tested bacteria and there was a direct relationship between extract concentration and inhibitory effect. Our results are in agreement with the mentioned study (39).

Bisignano et al (2013) studied antimicrobial activity of pistachio. They concluded Pistachio extracts were active against Gram-positive bacteria with a bactericidal effect observed against *L. monocytogenes*, *S. aureus* and MRSA clinical isolates. Extracts from raw shelled pistachios were more active than those from roasted salted pistachios. Therefore, pistachio extract could be used to control the growth of some microorganisms in foods and to improve their safety and it might find application as a topical treatment for *S. aureus* infections (40).

These herbal plants extracts have phenol compounds with anti-bacterial properties which disturb the cell membrane of bacteria and decrease absorption of necessary metal ions.

. Also, they destroy the cell walls' proteins and interfere with the action of the membrane enzymes and conclusively cause increase in the penetration of cytoplasm membrane to ATP and ATP exit (41-44).

It is necessary to note that T.T.P combined extract is effective against biofilm formation, demolish of biofilm and preventing metabolic activity of bacteria in biofilm structures. Also, the inhibitory effect of the extract is directly related to its concentration.

The efficiency of ethanol extract in inhibiting biofilm formation was more than its efficiency in the destruction of biofilm or prevention of metabolic activity in the bacterial cell in biofilm structure which can be led to the result that the ethanol extract includes a molecule which is related to bacterial biofilm formation, but this extract has little ability to resist against biofilm structure. The difference in our results is possibly due to various chemical materials in different plant species and the difference in extraction methods.

The ability of phenolic compounds in inhibiting initial biofilm formation has already been demonstrated. For example, Carvacrol inhibits the biofilm of *S. aureus* and *S. enterica* and prevents the formation of mature biofilms (45). According to Kouidhi et al. (2015), *Pistacia atlantica* has phenolic compounds that prevent the formation of biofilms of pathogenic bacteria (46). Burt et al. (2007) demonstrated the

inhibition of flagella production by sub-lethal concentrations of carvacrol for *E. coli*, which was the first explanation for the inhibition of biofilm formation and for *E. coli*, the formation of flagella is known to be essential for biofilm formation (42-48).

Based on the results of this study, it can be concluded that the T.T.P combined extract has the potential to inhibit six pathogenic bacteria. Therefore, it can be used as an alternative component with inhibitory effect against pathogenic bacteria in planktonic and biofilm form.

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