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Phenolic Compounds from Thymus Vulgaris, Artemisia Annua Extracts and Pure Thymol were tested against Twenty Pseudomonas spp. Strains for Antibacterial and Anti-Biofilm Activities

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***Abstract*---**The widespread use of broad-spectrum antibiotics has resulted in antibiotic resistance for many human bacterial infections, making multi-drug resistance a significant challenge in the treatment of infectious diseases. The antibacterial impact of phenolic plant extracts has been studied in new and different ways. The phenolic extract of *Thymus vulgaris*, *Artemisia annua* and pure thymol were used to study bacterial biofilm formation. In addition, the experiments were carried out to determine the lowest inhibitory concentrations in order to evaluate the antibacterial impact (MICs) against 20 isolations of *Pseudomonas* species. In this report, the plant extract was analyzed by HPLC techniques to determine the Thymol value. Although the *Thymus vulgaris* and *Artemisia annua* phenolic plant extracts and pure thymol concentrations (50, 25, 12.5, 6.25, 3.125, 1.250 and 0.006 mg/ml) (W/V)) were used through antibiotic and biofilm inhibition assay. The best results of MICs of *T. vulgaris*, *A. annua* extract and pure Thymol against bacteria isolates were 5.000 mg/ml, 1.250 mg/ml and 0.006 mg/ml, respectively. Stimulatory, the effect of *T. vulgaris*, *A. annua* extract and pure Thymol on biofilm formation were gave 10 mg/ml, 5mg/ml and 0.006 mg/ml respectively. In conclusion, Thymol fraction exhibited stronger antibacterial activity and was effective against all bacterial isolations tested. As a result, more research is needed to verify their action against additional harmful bacteria and fungi, as well as to investigate the prospect of drug companies using these active components.

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Keywords---*A. annua* extract, antibacterial activity, biofilm, phenolic extracts, Pure Thymol, *T. vulgaris.*

Introduction

Microbial contagion is one of the leading causes of sever disease and death in human. Major factors of these infectious diseases are bacterium and fungi, as well as an increase in antibiotic resistance in the microbial community. Secondary metabolites compounds found in plants, such as alkaloids, flavonoids, terpenoids, and tannins, have been shown in vitro to have antimicrobial effect (Cowan, 1999). Monoterpenes are being used as antimicrobial agents and resistance modulators in natural medicine (Zarrini et al., 2010; Rafii & Shahverdi, 2007).

Thymus vulgaris L. (thyme) refers to the Lamiaceae family of aromatic spice plants. They are native to the Mediterranean region and nearby countries (Murillo-Amador et al., 2013), and have been used in folk medicine to treat respiratory illnesses (e.g., coughs and bronchitis), dyspepsia, and urinary tract issues (Netopilova et al., 2021; Javed et al., 2013; Mohammed & Al-Shahwany, 2020). Several studies have aimed to check the chemical construction and biological properties of the *T. vulgaris* essential oil. According to the European Pharmacopoeia 5.0 (Borugă et al., 2014), the chemical makeup varies, with carvacrol, thymol, linalool, gamma-terpineol, geraniol, and trans-thujan-4-ol/terpinen-4-ol being the most common (Rota et al., 2008).

Artemisia annua L., a member of the Asteraceae family, is related to China but currently grows wild in many countries. Artemisinin is a sesquiterpene endoperoxide lactone that comes from this plant. It has been the subject of numerous investigations, the majority of which have focused on its antimicrobial potential. The EO is heavy in monoterpenes and sesquiterpenes, which could be additional source of commercial value (Bilia et al., 2014). Carvacrol and thymol EO are isomeric. Thymol is a phenolic monoterpene derivative of cymene that is found in nature (C10H14O). Thyme oil is white crystalline oil manufactured from thyme herb and a variety of other plants (Clarke, 2009).

Several mechanisms of action and biological activities could be obtained by using herbs or their products, like affecting feed efficiency, nutrient absorption, and enhancing digestive secretion, and activities of antiviral, antioxidant, immunomodulator, antimicrobial, anti-inflammatory, coccidiostat, and antispasmodic, besides endocrine and immune stimulation Using herbs or their products, you can gain anti-microbial, anti-viral, immunomodulator, anti-oxidant, anti-inflammatory, coccidiostat, and anti-spasmodic properties, as well as nutritional absorption, feed efficiency, and improving the secretion of the digestive system (Alagawany et al., 2021).

A series of previous research has shown that essential oil of thyme, and it is the main constituent's thymol has been EO of thyme, and it is the major constituent thymol, has been described to have an anti-microbial activity in vivo and in vitro against numerous pathogenic bacteria, according to previous research (Nieto,

2020). The opportunistic bacteria *Pseudomonas aeruginosa (P. aeruginosa)* causes life-threatening infections and septic mortality in burn patients, especially when acquired nosocomially (Norbury et al., 2016).

In a preliminary work have shown previously that the ethanol extract of leaves from *T. vulgaris* and *A. annua* the antibacterial potential of phenolic extract and purified essential oil components including pure thymol against resistant strain *P. aeruginosa* isolated from patients. The goal of this study was to extend on previous research by evaluating the activity of plant extracts and pure thymol in order to determine the appropriate MIC and biofilm concentrations.

Material and Methods

Extraction

Plant samples included T. vulgaris and A. annua lieves were obtained from local herbarium market in Baghdad city. After the plants were air derided and powdered, it kept at 4°C until further investigations. The finely ground sample (50 g) of plants leaves was extracted with ethanol using Soxhlet apparatus for 5 hr. A rotary evaporator was being used to filter and dehydrate the mixture (Ng et al., 2014). The final dried materials were kept in labelled sterile bottles.

Crystallization of Thymol:

Pure Thymol was obtained from the drug testing laboratories of the Iraqi Ministry of Health, national center for drug control and research. Thymol is often obtained from natural varieties, mainly Thymus species. Steam distillation or hydro distillation are used to extract the essential oils. After extraction, the aqueous and organic layers are separated in this process. To obtain pure thymol, distillation column chromatography or crystallization procedures might be applied (Dheer et al., 2019).

Antimicrobial activity

Previous study has demonstrated that the antibacterial and antifungal activity of essential oils of thyme and *A annua* has been the focus of numerous studies. There have been various studies to confirm the broad antibacterial impact of thymol in vivo or in vitro, using both the complete phenolic extract and its major components such as thymol (Nieddu et al., 2014).

Microorganisms

The bacterial strains of Pseudomonas spp. obtained from (Laboratories of the Department of Biology, University of Baghdad), Later the bacteria was isolated clinically from hospitalized infected burn patients. The cultures were subcultured onto cetrimide agar plates and incubated for 24 hours at 37 degrees Celsius. Single colonies were transferred from the plates and injected into tubes containing brain heart broth after incubation. Before being employed, the cultures were incubated for 24 hours at 37°C. (Kaya et al., 2020).

Micro-well dilution assay:

As previously mentioned, the minimum inhibitory doses of pure menthol were established using a microdilution method in 96 multi-well microtiter plates (Al-Bayati, 2009). Pseudomonas isolates were grown overnight on brain heart broth at 37°C and adjusted to a final concentration before being employed as an inoculum. In a concentration range of 200 to 1.56 mg/ml, serial two-fold dilutions were prepared. A column with just bacterial solution was used as a positive control in each microtiter plate, whereas a column with only plant extract was utilized as a negative control. Using of the color of resazurin sodium which acts as an indicator of bacteria growth where bacterial growth was inhibited, the solution in the well gave pink color

Minimum Inhibitory Concentration

The MIC was determined by calculating the minimum amount of extract (*Thymus* extract, *Artimisia* extract, mixture of these extracts, and pure thymol with various concentrations) required to inhibit the development of a test microorganism (Andrews, 2001).

Effect on adherence and biofilm formation

On polystyrene flat-bottomed microtitre plates, the effect of different doses of plant extracts on adhesion and biofilm-forming abilities was studied, with some changes, as described by Cramton et al. (1999). Overnight cultures were diluted in trypton soya broth supplemented with 1% (w/v) glucose (1:100). The culture (200µl) was then transferred to the wells of a 96-well polystyrene microliter plate and incubated overnight at 37oC. After incubation, the supernatants were taken from each well, and the plate was gently washed twice with normal saline, dried, and fixed for 1 hour at 65oC. therefore, all the plates were stained with 0.1% (w/v) crystal violet for 10 min, gently washed and the quantitative analysis of biofilm was performed by adding 200 µl of 95% ethanol for 10 min. Finally, in the presence of methylene blue in the de-staining solution, the biofilm was measured at 630 nm using a microplate reader (Amaral et al., 2005; Shahwany et al., 2016).

HPLC:

In an LC-20AD instrument, plant extracts and pure Thymol were quantified using a reversed-phase HPLC-UV technique (Shimadzu, Kyoto). UV detection was carried out at a wavelength of 278 nm in a 40°C oven. Without the use of safe guards, two columns were evaluated: Symmetry C18 (250x4.6 mm i.d., total) (A:B) Sulfuric acid (0.5 mL of 2.5M) in 500 mL of acetonitrile and Sulfuric acid (0.5 mL of 2.5M) in water (500ml). To get the best peak resolution, the flow rates were evaluated.

Statistical Analysis

The experimental design was Complete Randomized Design (CRD). The effect of different plant extracts on some bacterial isolates was studied using statistical analysis. When $P \leq 0.05$ was used to compare the significant difference between

means at dillerences, the least significant difference (LSD) was used (Mason et al., 2003).

Results and Discussion

Analysis of Thymol by HPLC:

The results showed that the plant extracts of *T. vulgaris* and *Artemisia annua* content high amount of thymol substance by using the HPLC method comparing with thymol pure. in addition, HPLC peaks of these compounds for each plant showed in the figures (1&2)

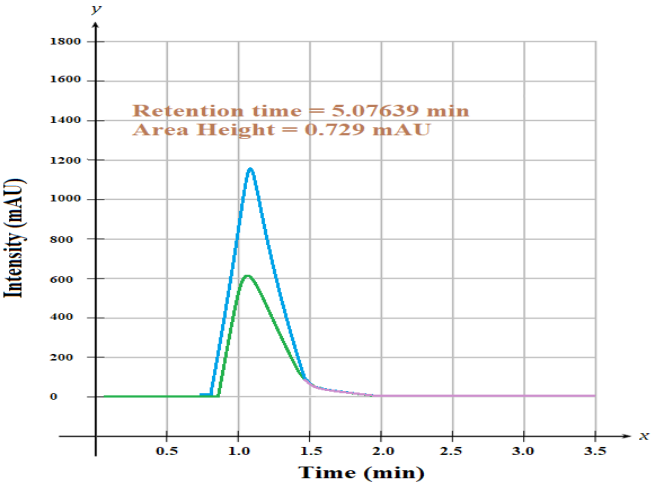

Figure 1. HPLC of standard (green) and Artemisia (blue)

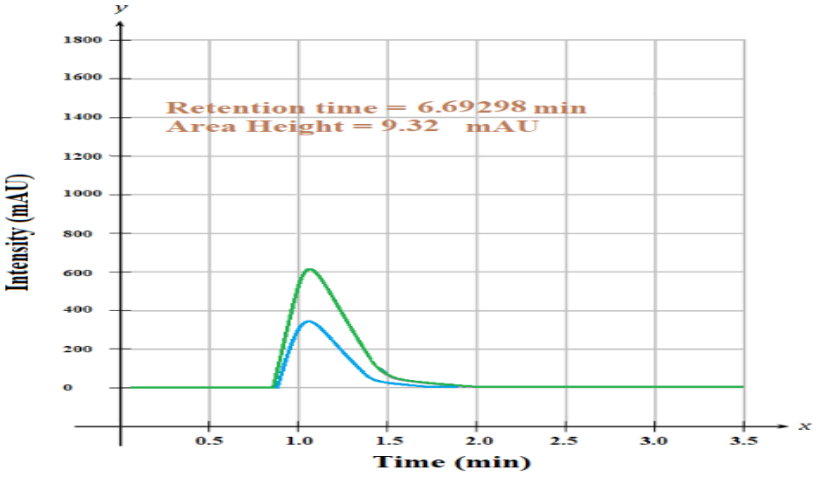

Figure 2. HPLC of standard (green) and Thymus (blue)

Bacterial susceptibility to antibiotics

Bacteria isolates are more resistant to various antimicrobial treatments. Susceptibility tests were summarized in Table 1 *Pseudomonas* isolates to seven different antibiotics by disc diffusion method recommended by CLSI (Cetti et al., 2008). These antibiotics were included in this experiment used for their mode of action inhibits cell wall formation, causing bacterial cell DNA to be released into the environment, inhibition of bacterial protein synthesis, cell wall lysis (Fitzgerald & Newquist, 2013; Smithet al., 2020).

Table 1
Antibiotic susceptibility of Pseudomonas isolates according to CLSI

Sample code	IMI	CAZ	LEV	AK	CIP	CAM	GM
36T	S	S	S	S	S	S	S
P*	I	S	S	S	S	S	S
21P	R	S	S	S	S	S	S
38T	S	R	S	S	R	R	R
51P	S	S	I	S	I	S	R
57T	R	S	S	S	S	S	R
25T	I	S	S	S	S	I	R
77E	I	S	S	S	R	S	R
42E	S	R	I	S	I	S	R
39T	R	R	S	S	R	S	R
20p	R	S	S	S	I	S	R
32E	S	R	1	S	R	S	R
62T	S	R	S	S	I	S	I
35T	S	S	R	S	S	S	S
51E	S	R	S	S	R	S	R
50P	S	R	S	S	S	S	R
2P	S	R	1	S	I	I	R
34E	S	R	S	S	I	S	R
55E	S	S	S	S	S	S	R
76S	R	R	1	S	S	S	R

R: Resistant, I: Intermediate, S: Sensitive, IMI: Imipenem, CAZ: Ceftazidime, LEV: Levofloxacin, AK: amikacin, CIP: ciprofloxacin, CAM: Chloramphenicol, GM: Gentamicin

Table 1 indicate that most isolates were sensitive to Imipenem, amikacin and Chloramphenicol, because of chloramphenicol is lipid soluble, it can pass through bacterial cell membranes. By attaching to the bacterial ribosome (blocking peptidyl transferase) and decreasing protein synthesis, chloramphenicol prevents bacterial growth, also Amikacin, an aminoglycoside, binds to the bacterial ribosome and prevents vulnerable bacteria from producing protein. Bactericidal against Gram-positive and Gram-negative bacteria in vitro (Sizar et al., 2021; Tevyashova, 2021).

Evaluation of MIC values:

The results shown in table 2 indicated that there are differences between the treatments in Minimum Inhibitory Concentration where the pure thymol gave the best result (0.00625 mg\ml), while thymus extract gave the MIC with high concentration about (10 mg\ml) against different species of *Pseudomonas.*

Table 2
Minimum Inhibitory Concentration of plant extracts and pure Thymol against some of Pseudomonas spp. (mg/ml)

Pseudomonas Isolations	Treatments			
	Artemisia	Thymus	Combination	Thymol pure
50P	2.500	8.333	0.650	0.006
2P	2.500	10.000	0.208	0.006
34E	2.500	8.333	0.650	0.006
51E	5.000	5.000	0.538	0.006
36T	5.000	10.000	0.313	0.008
25T	1.250	10.000	0.425	0.006
35T	2.500	6.667	0.425	0.006
20P	5.000	5.000	0.260	0.006
55E	2.500	5.000	0.538	0.006
*P	5.000	8.333	0.538	0.006
76S	4.167	8.333	0.850	0.010
62T	2.500	10.000	0.538	0.006
77E	5.000	6.667	0.538	0.013
42E	2.500	8.333	1.050	0.006
38T	2.083	5.000	0.425	0.006
51P	5.000	5.000	0.425	0.006
39T	3.333	8.333	0.425	0.008
51T	3.333	10.000	0.313	0.006
57T	2.083	6.667	0.425	0.006
21P	2.500	6.667	0.234	0.006
L.S.D 5%	0.996**	3.368**	0.307**	0.002**

**: Very significant

The result in (Table 2) indicated that there are a significant interaction between the treatments and Pseudomonas isolation. Thymol gave the best in Minimum Inhibitory Concentration 0.006 mg/ml, while Thymus extract gave 10.000 mg/ml.

The extracts were subjected to the estimate the MIC values were *T. vulgaris* and *A. annua* inhibited 20 isolation of Pseudomonas spp., The result was showed MIC values between 0.01–0.00625 mg/ml, which inducted that thmol have highly effect against bacteria. According to some researchers, thymol acts as an antibacterial agent by increasing the permeability and depolarization of the cytoplasmic membrane (Churklam et al., 2020). Furthermore, thymol-induced proton gradient-related dysfunction and membrane system malfunction have only been described infrequently.

Artemisia extracts was exhibited stronger activity against bacteria, the MIC values between 1.25–5 mg/ml. Some researchers agreed with these findings, and it's possible that this is because the genus Artemisia is one of the most important medical plants due to its antibacterial, insecticidal, antioxidant, and antimalarial qualities, as well as its fragrance chemicals (Haider et al., 2014). *Artemisia* has continued to be of great interest for taxonomists and chemists, It has been reported that a lot of the phytocompounds of *Artemisia* spp. comprise of terpene alcohols, terpenes, 1, 8-cineole, artemisolide, borneol, camphor, bornyl acetate, alkaloids and flavone etc (Kim et al., 2004). In some paper suppose the possible anti-microbial mechanism of the (EO) included synchronous cytomembrane rupture, resulting in intracellular substance leaking (Xiang et al., 2018).

Thymus extract shows activity against some bacterial species, as the MIC showed values between 5-10 mg/ml, Because of its ethnopharmacological significance and high content of bioactive substances, thyme has been extensively studied for its antioxidant and antimicrobial activities with the goal of improving food quality. The goal of this article is to review the natural preservative properties of thyme and their mechanisms of action against pathogens (Nieto, 2020).

Effect of plant extracts on Pseudomonas spp. biofilm formation:

The result indicated that some of plant extract reduced metabolic activity of cell in biofilm on *Pseudomonas spp.* showing an inhibition percentage. Table 3 shown that there are significant differences between *Pseudomonas* Isolations and plant extract concentrations, *Artemisia* plant extract (5 and 2.5 mg/ml) inhibition the formation of biofilm, *Artemisia* extract 5 mg/ml consider more effect than 2.5 mg/ml.

Table 3
Effects of *Artemisia* extract on *Pseudomonas spp.* biofilms formation at 24 hr

Pseudomonas Isolations	Concentrations			Means ISO
	Before	5 mg/ml	2.5 mg/ml	
50P	0.2067	0.0997	0.1190	0.1418
2P	0.1333	0.0700	0.0923	0.0986
34E	0.1910	0.0653	0.0833	0.1132
51E	0.2093	0.0777	0.0923	0.1264
36T	0.1250	0.1010	0.1137	0.1132
25T	0.1610	0.0840	0.0967	0.1139
35T	0.1283	0.0857	0.0960	0.1033
20P	0.1843	0.0803	0.0893	0.1180
55E	0.1337	0.1063	0.1227	0.1209
*P	0.1327	0.0970	0.1153	0.1150
76S	0.1950	0.0620	0.0853	0.1141
62T	0.1250	0.0723	0.0907	0.0960
77E	0.1717	0.0837	0.0923	0.1159
42E	0.2520	0.0640	0.0743	0.1301
38T	0.2170	0.0643	0.0747	0.1187
51P	0.1527	0.0677	0.0793	0.0999
39T	0.1053	0.0527	0.0683	0.0754

51T	0.1640	0.0580	0.0680	0.0967
57T	0.1663	0.0587	0.0877	0.1042
21P	0.1593	0.0813	0.0917	0.1108
Mean	0.1657	0.0766	0.0916	
L.S.D 5%		0.0021**		0.0053**
		0.0092**		

**: Very significant

In this research, the anti-inflammatory effect of essential oils from different Artemisia species was already described, with Artemisia suppressing the pro-inflammatory signaling pathways NF-B and MAPK (Abad et al., 2012). Table 4 showed a are significant differences between *Pseudomonas* Isolations and *Thymus* extract concentrations, thymus plant extract (10 and 5 mg/ml), thymus plant extract with 10 mg/ml gave the best result as anti-biofilm.

Table 4
Effects of Thymus extract on Pseudomonas spp. biofilms formation at 24 hr

Pseudomonas Isolations	Concentrations			Means ISO
	Before	10 mg/ml	5 mg/ml	
50P	0.2067	0.0893	0.0993	0.1318
2P	0.1333	0.0817	0.0857	0.1002
34E	0.1910	0.0717	0.0933	0.1187
51E	0.2093	0.0907	0.1117	0.1372
36T	0.1250	0.1027	0.1143	0.1140
25T	0.1610	0.0940	0.1030	0.1193
35T	0.1283	0.0937	0.1043	0.1088
20P	0.1843	0.0850	0.0923	0.1206
55E	0.1337	0.1050	0.1137	0.1174
*P	0.1327	0.1033	0.1203	0.1188
76S	0.1950	0.0787	0.1017	0.1251
62T	0.1250	0.0907	0.0963	0.1040
77E	0.1717	0.1097	0.1330	0.1381
42E	0.2520	0.0750	0.0913	0.1394
38T	0.2170	0.0673	0.0757	0.1200
51P	0.1527	0.0797	0.0850	0.1058
39T	0.1053	0.0967	0.1017	0.1012
51T	0.1640	0.0713	0.0780	0.1044
57T	0.1663	0.0837	0.0983	0.1161
21P	0.1593	0.1190	0.1350	0.1378
Mean	0.1657	0.0894	0.1017	
L.S.D 5%		0.0020**		0.0051**
		0.0087**		

**: Very significant

Thyme essential oil had inhibitory effect on planktonic form of *P. aeruginosa,* and thymus extract decreased the biofilm formation by this bacteria. Some researchers indicated that the inhibitory effect of these plant extracts was higher when these extracts dissolved in ethanol than other solvents because ethanol is

the best choice to thyme extracts to reach with higher anti-microbial efficiency (Gonçalves et al., 2011).

The result of table 5 indicated that there are significant differences between *Pseudomonas* Isolations and plant extract concentrations, the combination of 50% of *Thymys* extract and 50% of *Artemisia* extract (1.25 and 0.650 mg/ml). 1.25 mg/ml concentration inhibition the biofilm formation more than 0.650 mg/ml.

Table 5
Effects of combination of *Artemisia* and *Thymus* extract on *Pseudomonas spp.* biofilms formation at 24 hr

Pseudomonas Isolations	Concentrations			Means ISO
	Before	1.25 mg/ml	0.650 mg/ml	
50P	0.2067	0.0927	0.1017	0.1337
2P	0.1333	0.0713	0.0920	0.0989
34E	0.1910	0.0753	0.0843	0.1169
51E	0.2093	0.0747	0.0943	0.1261
36T	0.1250	0.0987	0.1117	0.1118
25T	0.1610	0.0660	0.0897	0.1056
35T	0.1283	0.0977	0.1033	0.1098
20P	0.1843	0.1047	0.1123	0.1338
55E	0.1337	0.1200	0.1247	0.1261
*P	0.1327	0.1020	0.1237	0.1194
76S	0.1950	0.0930	0.1383	0.1421
62T	0.1250	0.0577	0.0847	0.0891
77E	0.1717	0.1097	0.1283	0.1366
42E	0.2520	0.0563	0.0733	0.1272
38T	0.2170	0.0670	0.0773	0.1204
51P	0.1527	0.0743	0.0940	0.1070
39T	0.1053	0.0903	0.1003	0.0987
51T	0.1640	0.0903	0.1037	0.1193
57T	0.1663	0.0633	0.0853	0.1050
21P	0.1593	0.0983	0.1187	0.1254
Mean	0.1657	0.0852	0.1021	
L.S.D 5%		0.0020		0.0051
		0.0088		

The result show that plant extracts enhanced effect on biofilm inhibition and inhibited the formation of biofilm. Workers have reported the anti-biofilm action of numerous plant extracts against biofilms of human pathogenic bacteria (Carneiro et al., 2011; Karlapudi et al., 2012).

The results shown in table 6 indicated that there are significant interactions between *Pseudomonas* Isolations and Thymol pure with two concentrations (0.012 and 0.006 mg/ml), Thymol gave best biofilm inhibition with 0.012 mg/ml.

Table 6
Effects of Thymol pure on *Pseudomonas spp.* biofilms formation at 24 hr

Pseudomonas Isolations	Concentrations			Means ISO
	Before	0.012 mg/ml	0.006 mg/ml	
50P	0.2067	0.0853	0.0950	0.1290
2P	0.1333	0.0567	0.0647	0.0849
34E	0.1910	0.0510	0.0660	0.1027
51E	0.2093	0.0650	0.0713	0.1152
36T	0.1250	0.0950	0.1003	0.1068
25T	0.1610	0.0753	0.0870	0.1078
35T	0.1283	0.0787	0.0913	0.0994
20P	0.1843	0.0793	0.0940	0.1192
55E	0.1337	0.0820	0.0960	0.1039
*P	0.1327	0.1027	0.1167	0.1173
76S	0.1950	0.0600	0.0930	0.1160
62T	0.1250	0.0577	0.1050	0.0959
77E	0.1717	0.0847	0.0973	0.1179
42E	0.2520	0.0560	0.0850	0.1310
38T	0.2170	0.0607	0.0740	0.1172
51P	0.1527	0.0563	0.0660	0.0917
39T	0.1053	0.0887	0.1023	0.0988
51T	0.1640	0.0760	0.0860	0.1087
57T	0.1663	0.0643	0.0803	0.1037
21P	0.1593	0.0757	0.0847	0.1066
Mean	0.1657	0.0726	0.0878	
L.S.D 5%		0.0020**		0.0052**
		0.0091**		

**: Very significant

Thymol's impact on bacterial biofilms can disintegrate Gram-negative bacteria's outer membrane, releasing lipopolysaccharides (LPS) and increasing cytoplasmic membrane permeability (Koraichi Saad et al., 2011).

Conclusion

Plants have been shown to have antimicrobial action in the form of essential oils and extracts, *T. vulgaris* and *A. annua* have been shown to have antibacterial potential. Thymol is often obtained from natural varieties, especially Thymus species. Pure thymol gave the best results in all of the experimental studies in this research. On bacteria, thymol exhibited an antibacterial effect. The capacity to permeabilize and depolarize the cytoplasmic membrane was credited with the antibacterial properties. Thymol's anti-inflammatory and antibacterial qualities, according to recent study, make it good for dental health. As a result, more research is needed to see how effective they are against other harmful bacteria.

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