Inhibitory effectiveness of Essential Oil and seeds extracts of *Mentha spicata* (L.) as Antibacterial Activities

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ABSTRACT

This experiment was carried out in the Laboratory of Postgraduate Studies / Biotechnologies / College of Science / University of Kufa / for the period from 10/1/2022 to 1/ 4/2023.This study was conducted to extract the essential oils and the crude extracts from the seeds of the *Mentha spicata* medicinal plant. The essential oils were extracted from the fresh leafs of plant using the Clevenger hydro-distillation method and oils were detected using sulfuric acid reagent. Study the solubility, acidity and the molting point of these essential oils. Also, The basic chemical compounds of the seeds crude extracts were investigated and detected using some chemical reagents for alkaloids, phenols, and glycosides, as well as terpene compounds, and essential oils were detected using the sulfuric acid detector and the filter paper method. Gas chromatography (GC-MS) methods were using to determine chemical compounds of the essential oils and study their biological activities against some bacteria that cause gingivitis isolated from infected male gums. Four concentrations of Oils and alcoholic seeds crude extract were tested 1:1, 2/1, 5/1, 10/1 (v/v) ml of oil /ml of distilled water, in addition to the standard substance as menthol(S1) as a control agent, and Crude Oil as standard (S2) and Kanologe drugs as Positive control (P^*) to evaluate the antibacterial activity that causes gingivitis, using the agar diffusion method, at a rate of three replicates for each concentration and 9 replicates for each treatments . The bacterial samples that cause gingivitis were obtained from Al-Ameen Center for Research and Advanced Biotechnology in Al-Najaf City . The diagnosis of bacteria isolate was also confirmed with the Vitek2 Compact. The results showed that there was a conformity with the ideal specifications of the oils with international standards or close to them, where the solubility 99% in ethanol solvent compare with Methanol , ethers acetone , water was recorded(98, 88,4)% respectively and the boiling point is 98 ° C ,Acidity 2.89n. The results of the

qualitative detection of essential oils were positive with sulfuric acid reagents, and filter paper reagents. Also, The results of the qualitative detection of alcoholic extracts of seeds were positive with the studied chemical reagents, except for the detection of alkaloids, which showed negative results. The number of chemical compounds of essential oils were identified about 31 compounds with different percentages. Menthol was the highest at 21.21%, followed by Thymol at 12.53% and Thujone at 4.01%. The results showed that all the concentrations used gave an area of bacterial inhibition growth rate that increased with increasing oil and seeds extract concentration. The highest inhibition rate of *p.areuginosa* was for oils at a concentration of (1/10) v/v ml Oils: water , with an average inhibition rate of (20)mm , and that the highest inhibition rate was for seeds extracts at a concentration of (1/10) v/v ml extract : water , with an average inhibition rate of (22) mm. While the lowest inhibition rate was for the Oils and seeds extracts at a concentration of (1/1) v/v ml Oils :water with an average inhibition of (00 and 16) mm respectively . However, The highest inhibition rate *Enterococcus fecalis* bacteria was for oils at a concentration of (1/10) v/v ml Oils:water , with an average inhibition rate of (**30**) mm, and that the highest inhibition rate was for seeds extracts at a concentration of (1/10) v/v ml extract : water , with an average inhibition rate of (**20**) mm While the lowest inhibition rate was for the Oils and leafs extracts at a concentration of (1/1) v/v ml Oils :water with an average inhibition of (10 and 20) mm respectively

Keywords: Inhibitory effectiveness, Essential Oil, Seeds extracts, *Mentha spicata* (L.), Antibacterial Activities

INTRODUCTION

Medicinal plants possess a rich source of phytochemicals that are a successful alternative to many manufactured antibiotics that are resistant to pathogenic microorganisms **[3].[21]** indicated that about 80% of the world's population uses traditional herbal preparations as primary treatment. Essential oils are good alternatives that are gaining more attention in the treatment of a wide range of periodontal diseases [18]. Some strategies to combat periodontal disease have been improved mainly through phytochemicals. Gum disease is one of the most common infectious diseases that destroy gum tissue and is an important public health problem. New research and studies aim to discover alternative, effective and natural materials extracted from safe and effective medicinal plants for the prevention and treatment of these diseases. The plant *Mintha spicata* (L) is one of the most important types of medicinal aromatic plants used against bacteria that cause gum disease through its biochemical content**[26].** The plant contains 50% menthol oil and 25% to 28% of tannin, the

chemical compound Punicallin, Granatine C, and Tanic acid, which are potent antimicrobial agents [**25**]. Gum disease is one of the most common diseases caused by gram-negative anaerobic bacteria. Due to the occurrence of periodontitis, the increased resistance of oral bacteria to antibiotics and the negative effects of some antibacterial currently used in dentistry, there is a need to search for safe and effective alternative products, so the current study aims to :

Extraction of essential oils from the leaves of the mint plant and qualitative detection of the oils and testing the inhibitory ability of essential oils against the bacteria that cause gingivitis to Production of natural antibiotics that may replace chemicals antibiotic.

MATERIAL AND METHODS

Plant collection and oils extracts preparation

Seeds of *Mintha spicata*, was collected from the growing plants in the home garden in the Najaf city in October 2022 and kept in the laboratory at room temperature even use. seeds crude extracts were done by maceration methods for 24 hours over night. The essential oil was extracted or separated from the leaves according to the method used by **[19],** using the method of hydro distillation using the Apparatus Clevenger by immersing 80g of fresh leaves with distilled water in a 1 liter glass flask.

Solubility of essential oils

The solubility of essential oils was measured by dissolving them with ethanol, methanol, hexane, ether and water in a ratio of (1:1) volume to volume**. [5].** Most of the essential oils are liquid at room temperature, and a few of them either freeze when exposed to low temperatures. (C°5 - C°8) or melted under high temperature conditions (C°17 - C°19), or another type of precipitation may occur in the form of solid blue art when the essential oil is exposed to very low temperatures(5°C) -1°C. **[4].**

Boiling point

The boiling point of essential oils was measured using a boiling water bath. Where the sample is placed in a suitable test tube in which a thermometer is placed to measure the temperature at which the oil begins to boil.

Acidity

Is the number of milligrams of potassium hydroxide (KOH) needed to neutralize the free fatty acids present in one gram of oil or fat. The acidity was calculated as follows: $\text{IA} = \frac{VxNx\ 56.1}{m}$

IA= Acidity V= Volume of KOH N =molarity of KOH solution m= The mass of the oil sample. [8]

Qualitative detection of oils

H2SO4 reagent

Take 21 drops of oil and mixed with 1-3 ml of sulfuric acid to be colorless with peppermint oil.

Filter paper reagent

This test was conducted by adding a little of the clear extract to a filter paper until saturation and exposure to ultraviolet light, the appearance of a gray color indicating the presence of volatile oils**. [15].**

Qualitative detection of chemical compounds in the crude extract

Alkaloid reagents

Dragendorff reagent used for alkaloids detection, ferric chloride 5% for phenolic compounds detection, Benedict's detector for glycosides and sulfuric acid detector for Terpenoids compounds **[17]**

Samples Preparation

To prepare the concentrations used in this study, the volumetric dilution method used by **[2*]** was followed by dissolving a specific volume of oil or crude seeds extracts into a specific volume of water and achieving complete dissolution using 10% DMSO, where concentrations of 1:1, 1/2. 1/5, 1/10 (v/v) ml of distilled water/ ml of oil or leafs extracts were prepared, in addition to the standard substance menthol (S1) , standard of crude oil or leafs extracts (S2) were placed in tightly closed glass tubes, labeled, and kept in the refrigerator at a temperature of 10 °C until subsequent experiments were carried out

Bacteriological tests

The test bacteria included two types of bacteria, one of which were Gram-positive, namely *Pesumonous areuginosa* , and the same were Gram-negative, namely *Enterococcus fecalis*. The cultures were activated on broth nutrient at a temperature of 37˚C for 24 hours, then compared with McFarland's standard solution prepared as mentioned by The diffusion well agar method was used.

RESULTS & DISCUSSIONS

Solubility

The results of the same table also indicate the solubility of essential oils under study in several organic solvents and water. The dissolution process depended on the types of essential oil and the organic solvent used for dissolution. The solubility of essential oils was observed to be highly

soluble in alcoholic organic solvents, and the solubility rate in the alcoholic solvent was 99% ethanol. 94% in methanol and 88% in ether and in water was 4 % insolubility.

Due to their fatty nature, these oils are insoluble in water with very little exception, but they are soluble in the non-polar organic solvents, fatty oils and in high-ether alcoholic beverages **[28].** The complete solubility of essential oils in alcohol is one of The important characteristics that show the purity of the oil and that it does not contain various adulterants **[1]** and that the essential oils extracted in the study were completely soluble in alcohol, indicative of their purity and not containing other materials that cause their insolubility in alcohol.

Essential oils do not generally dissolve in water because they contain hydrocarbon compounds, with the exception of some oxygen compounds that have little solubility in water in limited proportions. They also generally dissolve in organic solvents at a rate of 95% without the occurrence of any turbidity. They also dissolve in vegetable oils and animal fats, except for Volatile oils containing cinnamic aldehyde. **[20]***

Acidity

Results in the same table show that , the pH of the oil extracted from the leafs of the *M. specata* was 2.87. This value indicates that the components of the oil did not partially decompose during distillation. The pH of an essential oil is an important criterion for estimating its quality. **[13]** indicated that the acid number indicates the percentage of free fatty acids in the oil, and whenever the percentage is low in the extracted oil, it is less than pH: (3)indicates that the essential oils are stable and not oxidized.

Boiling point

The results in the same table show that the boiling point of the essential oils extracted from the leaves of the *M.spicata,* about 98 °C. The result was agree with study by **[4]** reported that the boiling point of essential oils is always more than 100 °C, depending on their molecular weights. **[14]** mentioned that the melting point was (143.5 °C) and the boiling point of menthol was (212 °C). **[4]** stated that the boiling point of plant essential oils is always more than 100 C, depending on their molecular weights. Essential oils are highly volatile and lose their properties quickly, when exposed to sunlight, light or heat, they absorb a large amount of oxygen in the air by reabsorption, in At the same time the aroma changes, the boiling point increases and the solubility decreases **[10].**

Table (1): Some physical and chemical properties of essential oils.

Specifications	**Characters**
98^{o}C	**Boiling point oils**

99%	Ethanol	**Solubility**
98%	Methanol	
88%	Ether	
4 %	Water	
2.87		**Acidity**

Figure (1) sulfuric acid reagent

Qualitative Oil tests

H2SO4 reagent

A few drops of the aromatic oil obtained by the distillation in a test tube mixed with 3 ml of concentrated sulfuric acid. It is noted that the light yellow oil turns into a completely colorless transparent oil (Fig. 1). This indicates the high purity of the oils**.[27]**

Filter paper reagent

5 drops of essential oil were placed on Whatman filter paper (1) and left for 2 minutes and examined with the naked eye as all the oil evaporated from the filter paper to indicate the presence of volatile oil extracted from the leaves of the studied plant with a high degree of purity. **[6]** .The essential oils, which are considered hydrocarbons, have the property of volatilization at normal temperature without leaving an effect on the filter paper.

Qualitative Phytochemicals tests

Phytochemical qualitative detection is useful for determining the active plant compounds and may lead to drug discovery later. These tests also facilitate the separation of pharmacologically bioactive compounds **.** Table (2) and (Fig. 2) refer to the chemical detection of phytochemicals in the raw extracts of the seeds of the mint plant, using the Dragendorff reagent to detect alkaloids, the ferric

chloride reagent 5% to detect the phenolic compounds, the Benedict reagent to detect the glycosides, and the sulfuric acid reagent to detect the compounds. The results in the same table show that Dragendorff's reagent gave a positive detection of alkaloids in the crude alcoholic extracts of the seeds, while it did not give a positive result with the oils extracted from the leaves of the plant. Also, ferric chloride reagent 5% showed a positive detection of phenolic compounds, in the crude alcoholic seeds extracts. Benedict's reagent showed positive detection of glycosides in the two types of extracts, and sulfuric acid reagent showed positive detection of terpene compounds in all extracts. The results of this study were agreement with the study of **[16]** the seeds methanolic extract showed a positive detection of alkaloid.

Table(2): phytochemicals detection of the seeds extract

Plant	**Chemicals**	**Reagents**	**Seed**	**Olis**
M. specata	**Alkaloids**	**Dragndorffs**	**+**	**-**
	Phenol	ferric chloride	**+**	+
	glycosides	Benedict's	**+**	+
	Terpens	sulfuric acid	**+**	+

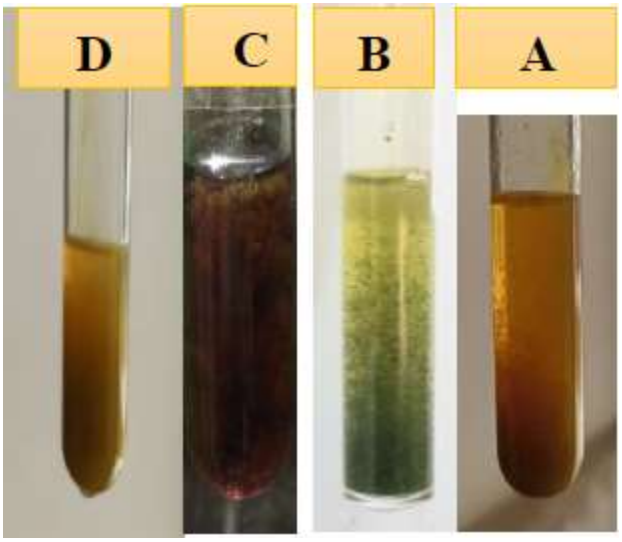

Figure (2): Phytochemical detection of crude alcoholic seeds extracts

A: Alkaloids B: Phenols C: Terpenes D: Glycosides

Phytochemicals detection in Leaf oils of peppermint *M. spectra* by GC-MS chromatography

Gas chromatography - mass spectrometry was used to determine the chemical content of the essential oil studies . The results show in Table (3) and Figure (3) that the chemical constituents that were identified in peppermint oil using GC-MS analysis technique compared to the mass spectra were these phytochemicals and indicate the presence of 31 peaks indicating the main phytochemical contents, including eight Aldehydes and ketones, 18 monoterpenes and 5 sesquiterpenes, 3 monoterpene ketones, 4 alcohols, and 1 compound oxide, including the compound Menthol (21.21%), which belongs to the group of Monoterpenes and has antibacterial properties **[30]** Antifungal [12]**,** Anticancer **[22],** and against antipruritic skin itching **[23],** then a compound of thymol at a rate of 12.53%) and belongs to the Monoterpenoid phenol group, which has antibacterial properties; antifungal **,** followed by β-Humulene, with a ratio of (4.32%) within the sesquiterpene group, which has antibacterial properties[**24];** The fourth component is α-Thujone (4.01%) and belongs to the Monoterpene ketone and has antibacterial properties **[7]** followed by 1,8-Cineole with a percentage of 3.93%) within the group of Monoterpenes and has antibacterial properties **[29],** followed by Camphene (3.20%) Monoterpenes.

Table (3): Detection of phytochemical compounds by GC-MS chromatography in *M.spicata* essential oils

ame	**Formula**	**RT**	**Area (%)**
α-Thujone	monoterpene ketone	10.69	4.01
Limonene	Monoterpenes	19.81	2.91
Camphene	Monoterpenes	21.02	3.20
Menthol	Monoterpenes	30.62	21.21
β -Terpinolene	Monoterpenes	37.20	1.21
α-Acorenol	Sesquiterpene	22.1	0.96
Menthone	Monoterpenes	21.78	3.10
β -Humulene	Sesquiterpene	57.29	4.32
Linalool	Monoterpenes	41.55	1.44
α-Terpinene	Monoterpene alcohol	31.51	0.83
Sabinene	Monoterpenes	21.51	0.71
Carvacrole	Monoterpenoid phenol	52.60	1.88
Terpinen-4-ol	Monoterpene alcohol	42.11	1.01
1,8-Cineole	Monoterpenes	41.23	3.93
Geraniol	Monoterpenes	41.55	2.20
β-pinene	Monoterpenes	8.65	2.10

Pinen-3-ol	Monoterpene ketone	13.74	0.58
Camphor isomer	Monoterpene ketone	12.79	2.13
carveol	Monoterpene alcohol	15.94	1.11
Eucalyptol	Monoterpene oxide	10.39	1.89
Bornyl acetate	Monoterpenes	19.06	0.76
20.31 Carvyl acetate	Monoterpenes	19.91	0.98
Thymol	Monoterpenoid phenol	18.5	12.53
Isoborneol	Monoterpene alcohol	15.19	1.33
Bornyl acetate	Monoterpenes	18.16	0.51
γ-Muurolene	Sesquiterpene	24.35	0.23
Caryophyllene	Sesquiterpene	26.15	1.03
Sclareol	Monoterpenes	13.1	0.84
Bronyl acetate	Monoterpenes	12.9	2.4
γ- Cadinene	Sesquiterpene	26.16	0.71
31			**73.2**

Effectiveness of essential oils and crude leafs extracts against p.areuginosa and E.facils bacteria

The results show in Figure (4 A1 ,A2) indicate that all concentrations of oils and crude alcoholic extracts of *M. spicata* seeds have antibacterial activity against *p.areuginosa* isolated from infected gums with different inhibition rates, which increases with increasing the concentration. It was found that all the concentrations of *M.spicata* seed extracts gave differences in the inhibition zone between all concentrations, compared with the control group treatment (S1) and (S2), as well as the antibiotic group used kenaloge drugs (P*) against pathogenic bacteria, which recorded an average of (17,18,00) mm, respectively, and the highest average inhibition zone at concentration 10/1 (v/ v) extract / distilled water was (20) mm, compared with the studied concentrations 1/1, 1/2, and 1/5 (v/v) with an average of (0, 15, 16) mm respectively.

[11] mentioned that phenolic compounds are among the largest phytochemical compounds in the mint plant that have clear activity against bacteria that cause gingivitis in humans, and the phenolic compounds cause destruction and damage at the level of the wall of bacteria, which shows an increase in the membrane permeability of potassium protons and ions. Decrease in ATP stock within the cell and cause damage to its cellular proteins.

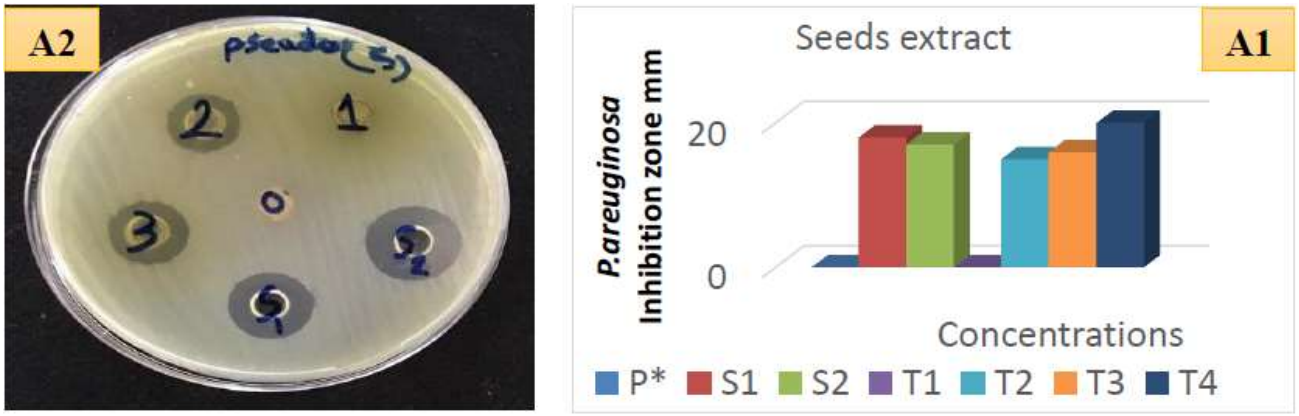

Figure (3A1,A2): Inhibition zone of the growth rate of *p.areuginosa* bacteria using crude alcoholic extract of seeds

Also, the results in Figure (4 B1,B2) indicate that all concentrations of essential oils of *M. spicata* plant have antibacterial activity against *p.areuginosa* isolated from infected gums with different inhibition zone rates, which also increases with increasing concentration. Where it was found that all concentrations gave clear differences in inhibition zone rate between all concentrations, compared with the control group S1 and S2, as well as the antibiotic group used kanalog (P*) against pathogenic bacteria, which reached an average of (17,18,00) mm, respectively, and was higher the average of inhibition zone at concentration 10/1 (v / v) Oil / distilled water is (22) mm compared to the other concentrations studied 1/1, 2/1 and 5/1 (v /v) O/ D.W with an average of (16, 17, 20) mm respectively.

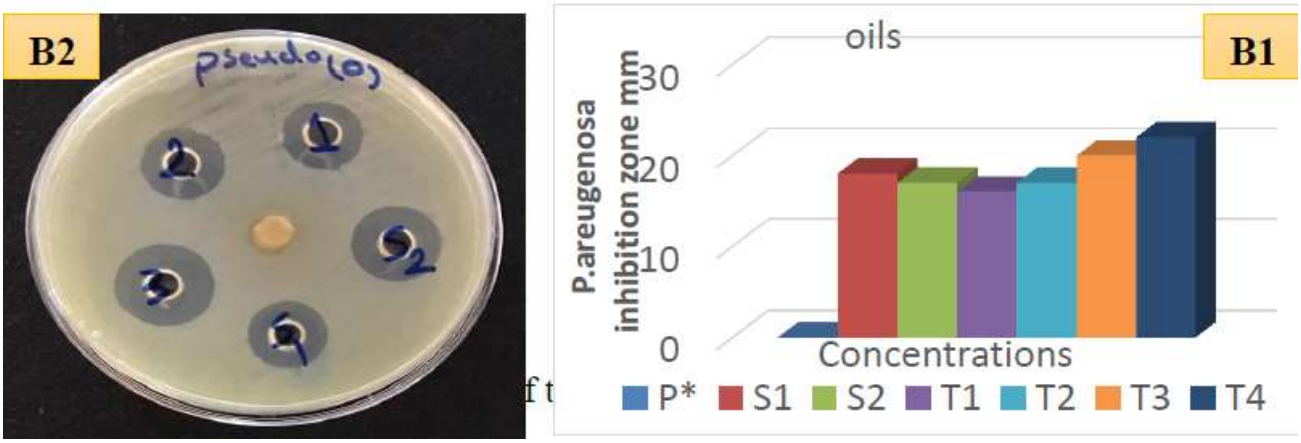

Figure (4B1, B2): Inhibition zone of the growth rate of *p.areuginosa* bacteria using essential oils

Scientific studies have shown that essential oils with MICs ranging from 19 to 100 µg/mL are potent antibacterial agents **[9].** Essential oils (EOs) are composed of volatile compounds, such as terpenes,

terpenoids, phenol-derived aromatic components, and aliphatic components. With strong aromatic molecules, EOs possess several biological properties, including antibacterial activities, **[8].**

Meanwhile, the results showed in Figure (5C1,C2) that all concentrations of crude alcoholic extracts of mint seeds have antibacterial activity against *E.fecalis* bacteria isolated from infected gums with different rates of inhibition. The antibacterial inhibitory also increases with increasing the concentration of the crude extract of *M.spicata*. It was found that all the studied concentrations gave significant effects compared with the control group S1 and S2, which amounted to (17 and 18) mm, respectively, as well as the antibiotic group used kenalog ((P* against bacteria and amounted to (00) mm and the highest average zone of inhibition rate was at concentration 1 /10 (v / v) distilled water / extract is (20) mm compared with the studied concentrations 1/1, 1/2 and 1/5 (v/v) with an average of (10.14 20) mm, respectively. The results from the same table also showed that there were significant differences between the raw concentrations of the oils and the control treatments S1 and S2, which gave the highest rate of inhibition and reached (17 and 18) mm, respectively. The results showed in the study of [9] that the alcoholic extracts of the mint plant *M spicata* is less biologically effective against bacteria than the effectiveness of essential oils.

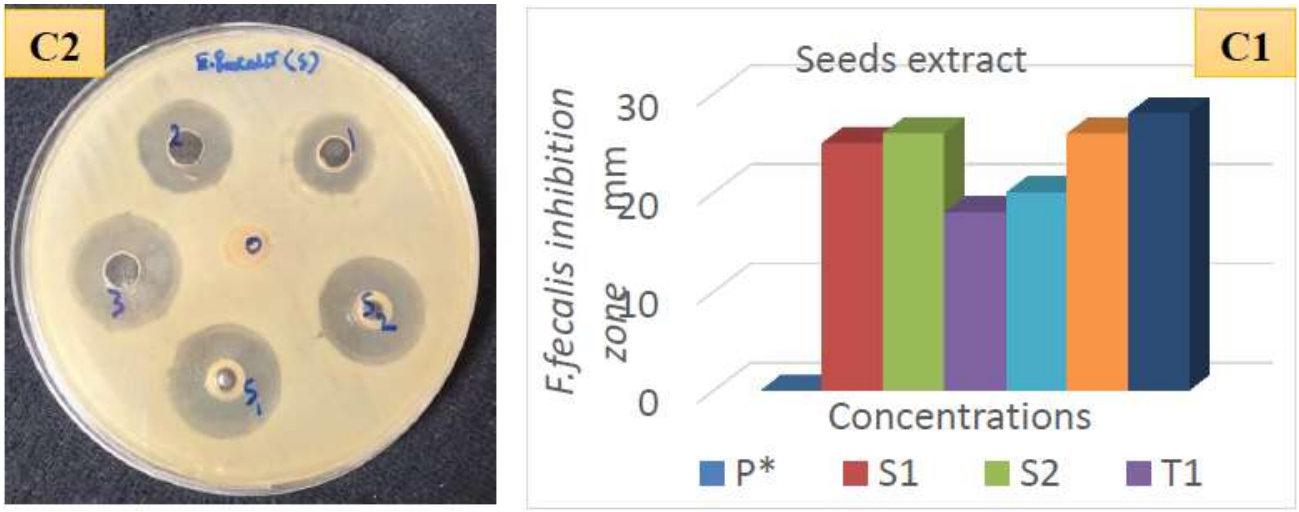

Figure (5C1, C2): Inhibition zone of the growth rate of *E.fecalis* bacteria using crude alcoholic extract of seeds

Meanwhile, the results showed in Figure (6D1,D2) that all concentrations of essential oils have antibacterial activity against *E.fecalis* bacteria isolated from infected gums with different rates of inhibition. The antibacterial inhibitory also increases with increasing the concentration of the essential oils of *M.spicata*. It was found that all the studied concentrations gave significant effects compared with the control group S1 and S2, which amounted to (25 and 26) mm, respectively, as well as the antibiotic group used kenalog ((P* against bacteria and amounted to (00) mm and the

highest average zone of inhibition rate was at concentration 1 /10 (v / v) distilled water / extract is (30) mm compared with the studied concentrations 1/1, 1/2 and 1/5 (v/v) with an average of (20.22,26) mm, respectively. The results from the same table also showed that there were significant differences between the raw concentrations of the oils and the control treatments S1 and S2, which gave the highest rate of inhibition and reached (25 and 26) mm, respectively.

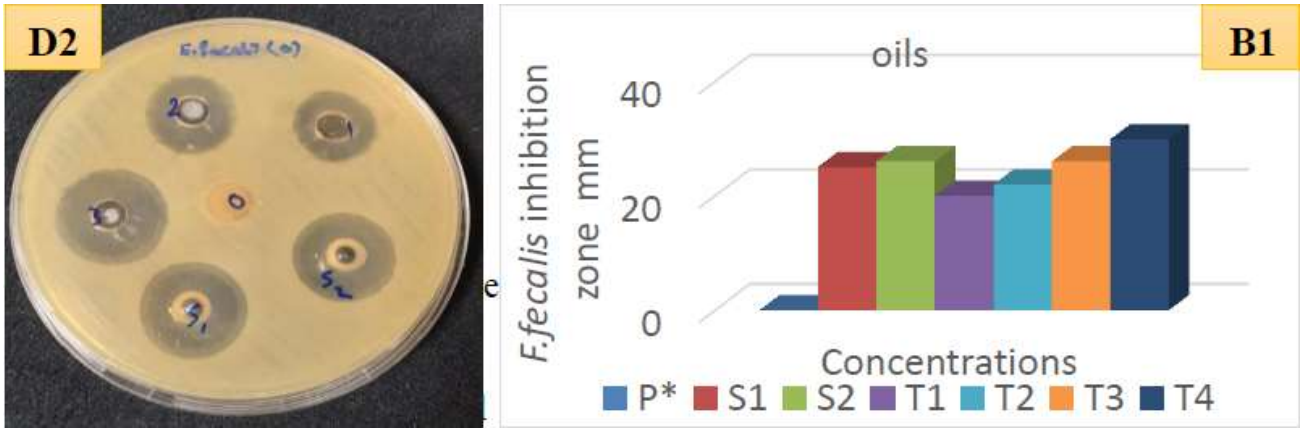

Figure (5D1, D2): Inhibition zone of the growth rate of *E.fecalis* bacteria using essential oils

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