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The Role of *Melaleuca alternifolia* (Tea Tree) Oil and Casein Polymer Against Pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

One of the most dangerous problems facing burn and wound patients is the resistance of bacteria to antibiotics, which requires replacing them with more effective antimicrobial agents such as essential oils. In this study, tea tree oil was used as an antibacterial agent against bacteria from burns and wounds infections such as Staphylococcus *aureus* and *Pseudomonas aeruginosa*; polymerizing tea tree oil with casein compound and finding the minimum inhibitory concentration values for the casein polymer agent against the bacteria. The bacteria were isolated and identified using the vitek2 compact system. The sensitivity rates of the bacteria to antibiotics were found through disc agar diffusion and measuring the diameters of inhibition. Minimum inhibitory concentrations were determined using the agar dilution method. The study showed that bacterial isolates numbered 4, 10, 23, 26, 27, and 44 were the most resistant among the *S. aureus* isolates, while 3, 4, 5, 7, 13, and 14 were the most resistant among the *P. aeruginosa* isolates. The effect of oil and casein polymer on resistant isolates was tested, and MIC values were obtained, which was 5000 ppm for both types of bacteria, while the MIC ratios of casein polymer varied between 10000 to 20000 ppm for both types of bacteria compared to control.

Keywords: tea tree oil, *S. aureus*, *P.aeruginosa*, TTO polymerization.

دور زيت شجرة الشاي و بوليمر الكزايين المضاد للبكتريا المسببة للامراض

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الخلاصة

واحدة من اكبر المشاكل التي تجتاح مستشفيات الحروق و الجروح هي مقاومة البكتريا للمضادات الحيوية والتي يستوجب ابدالها بعوامل مضادة اكثر فعالية مثل الزيوت العطرية. في هذه الدراسة تم استخدام زيت شجرة الشاي كمضاد بكتيري مع امكانية بلمرته مع مركب الكزايين الفعال لدراسة مدى امكانية استعماله كمضاد لبكتريا الجروح و الحروق *Staphylococcus aureus* و *Pseudomonas aeruginosa* من خلال

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ايجاد قيم اصغر تركيز مثبط لكلا العاملين ضد البكتريا. تم عزل البكتريا و تشخيصها من خلال استعمال vitik2و ايجاد نسب حساسية البكتريا للمضادات من خلال discs agar diffusion و قياس اقطار التثبيط. اوضحت الدراسة بان العزلات 4, 10, 23, 26, 27 44 and, هي الاكثر مقاومة من بين عزلات *S. aureus* في حين كانت 3, 4, 5, 7, 13 14 and, هي اششلاكثر مقاومة من بين عزلات *P. aeruginosa*. تم تجربة تاثير الزيت و بوليمر الكزايين على العزلات المقاومة و الحصول على قيم MIC التي كانت 5000 ppm لكلا نوعي البكتريا في حين كانت نسب MIC لبوليمر الكزايين متباينة ما بين 10000 الى 20000 ppm لكلا النوعين البكتيرية مقارنة مع الكونترول

1. Introduction

One of the major and unintentional problems in developed countries is skin burns in our daily lives. Recently, the rates of burns and their resulting deaths have decreased due to the widespread use of medical treatments and following special medical bulletins, which led to a decrease in the incidence of burns, their severity and the length of the patient's stay in the hospital all over the world [1, 2]. However, mortality rates continue to increase, particularly in developing countries, according to the World Health Organization statistics [3]. Burns can have a negative effect on the skin and other organs, leading to death, disability, serious emotional and psychological complications, and increased financial burdens for families [4, 5]. It is estimated that globally, approximately 6 million people lose their lives from accidents and injuries annually. Based on the total causes of death worldwide, accidents and injuries account for 9%. Every year, burns and injuries have become one of the major problems causing mortality and morbidity. In 2004, burn injuries alone caused approximately 265,000 deaths. The mortality rate was as high as 75%, but these deaths were hundred percent preventable if proper care was taken during the first week of the injury. The incidence of hospitalization ranged from 36% to 90% annually, but a significant number of victims did not receive proper treatment at the hospital. The survivors are often left with grave consequences ranging from severe burns to permanent disability, which would drastically reduce their quality of life. In summary, both the social and economic costs to the nations of the world are enormous. The incidence of accidents and injuries in these developing countries, including burns, has been underreported and improperly addressed; hence, no effective intervention has been established [6].

Among the top 10 public health problems facing humanity are multi-drug resistant bacteria (MDR) and antimicrobial resistance (AMR) [7]. 78% of 147 burn patients had bacteria persistently detected in 359 specimens. The most frequently detected bacteria were *Acinetobacter baumannii* (29%), *Staphylococcus aureus* (26%), and *Pseudomonas aeruginosa* (12%). Resistance rates were as high as 92% for meropenem and 67% for imipenem. In 28 critically ill burn patients, 74 infected specimens were analysed. All *A. baumannii* were carbapenem-resistant; *S. aureus* was mecA positive in 93%, and *P. aeruginosa* was resistant to imipenem in 85% of cases, emphasizing the importance of ongoing surveillance and ultimately the consideration of regional guidelines [8]. Approximately 57% of open wounds are colonized by highly resistant bacteria during the first 21 days of a patient's hospital stay. In the United States, Pseudomonas and MRSA are the most common bacteria colonized in burns, while in Singapore and other tropical areas, the *A. baumannii* is most common due to the humid conditions [9].

Certain plants synthesize and secrete hydrophobic fluid extracts that help them adapt to their environmental factors and, hence, are therapeutically helpful. These compounds are termed essential oils [10]. Essential oils play an essential role in plants, such as defence and attraction. For instance, the potent aroma and acidity of essential oils from citrus fruits help defend plants from predation by deterring herbivores and acting as antibacterial agents. Basil

essential oil is known for its insecticidal activity. Aromas that encourage pollination are found in essential oils extracted from blooming flowers. In addition to their antimicrobial and antipredator activity, lavender and tea tree essential oils are also thought to attract bees and other pollinators. Some plants make chemicals that function as insect repellents or insecticides in the essential oil. Two isomeric thymol compounds are present in the Lamiaceae essential oils. High levels of thymol are found in the essential oils of certain species, such as those of thyme and oregano [11]. These oils also possess a range of beneficial properties, such as analgesic, anti-inflammatory, anti-protozoal, anti-carcinogenic, and gastroprotective effects [12]. Furthermore, essential oils have gained attention in the food industry for their ability to control food-borne microorganisms, prompting a growing interest in plant extracts as alternatives to conventional antimicrobials.

Oil from *Melaleuca alternifolia*, popularly known as tea tree oil (TTO), is one of the most studied and addressed essential oils. The discovery of essential oil from the tea tree was made by European settlers in 1770 [13]. *M. alternifolia* essential oil has multiple compounds that have pharmacologically active effects. However, the composition and concentration of these compounds may fluctuate between both intra-species and inter-species levels. The most extensively studied compounds in *M. alternifolia* essential oil are terpinen-4-ol, gamma-terpinen, alpha-terpineol, alpha-terpinene, and other important compounds. Moreover, the method of extraction, whether physical extraction or steam distillation, of *M. alternifolia* essential oil, may result in essential oil with different compound compositions and concentrations [14]. *Melaleuca* essential oils displayed significant amounts of phenolic alcohols, including 22 components in *M. alternifolia*: terpinen-4-ol; α-terpinene; terpinolene, based on different ripening stages of the plants from the same plot. Additionally, numerous studies have reported that *M. alternifolia* extracts have demonstrated a broad range of pharmacological properties, including antimicrobial, skin-penetrating, antipruritic, anti-inflammatory, and analgesic effects. In support of the folk uses different studies have reported improvements in diaper dermatitis, dandruff, and acne vulgaris after the therapeutic uses of *M. alternifolia* [15]. In Iraq specifically, due to the recent environmental and climatic changes, the growth and resistance of many bacteria have become adapted to the environment, which makes it urgent to discover natural and effective antibacterial agents. This study aims to investigate the effect of a polymerized alternatives from the tea tree oil active compound as antibacterial agents and to decrease the oil toxicity and its disadvantages.

2. Materials and method

2.1 Collection and extraction of tea tree plant

The plant specimens used in this study consisted of the above-ground portions of *M. alternifolia* (commonly known as tea tree), which were obtained from a local market and then classified by the classification team at the Department of Biology/ College of Science/ University of Baghdad; Following collection, the plants underwent a thorough cleaning process, including rinsing with tap water, and were subsequently air-dried at room temperature. They were then stored in pristine conditions until they were ready for use.

Dried plant parts (250 g) underwent steam distillation (Clevenger method) to obtain the essential oil. The plant material, along with 1.2L of distilled water, was brought to a boil and simmered for 3 hours [16]. Subsequently, the essential oil was stored at a temperature of 4 ºC until it was ready for utilization. To create the stock solution, the concentrated oil extract was combined with Dimethyl sulfoxide (DMSO) and diluted. Subsequently, various concentrations (40000 ppm, 20000 ppm, and 10000 ppm) were prepared by mixing specific volumes of the stock solution with specific volumes of DMSO.

2.2 Investigate the phytochemical compounds of tea tree oil using GC-MS technique

The chemical analysis was conducted in the BPC- Analysis Center (Baghdad-Adhamiyah near al Nu'man Teaching Hospital) using Gas Chromatography-Mass (GC-MS) analysis on *M. alternifolia* essential oil using a Shimadzu gas chromatograph. The procedure for this analysis was performed in the same manner as outlined by Tawfeeq *et al.,*[17].

2.3 Polymerization of tea tree oil with casein

2.3.1 Synthesis of compound Z1 (terpene-diallyl maleate adduct)

To create the Z1 compound, 12 ml of TTO and 4g of maleic anhydride with 40 ml of DMF were combined in a 250 ml four-necked flask. This flask should be equipped with an electric agitator, a thermometer, a reflux condenser, and a dropping funnel. The mixture was gently heated until the maleic anhydride melted and then continued heating at 145°C. Next, 0.96 g of p-toluene sulfonic acid catalyst was added to the flask. The reaction is considered complete after heating at 149°C for 2 hours. To obtain the pure terpene maleic adduct, extract the mixture with distilled water and separate the compound using a vacuum to concentrate the mixture into a powdered polymer [18].

2.3.2 Synthesis of Z2 polymer (terpene-diallyl-grafted- maleate adduct anhydride) with casein

Ten ml of Z1 polymer was added to 3.3 g of casein compound with 46 ml of DMF. The mixture was refluxed for 6 hours and then re-crystallized using vacuum at 40 ºC [19].

Casein demonstrated potential as a disinfectant for wounds and may also promote healing, as evidenced by results from research involving dog wounds where casein was utilized as a hydrogel. Casein-based hydrogels (casein gels) offer beneficial characteristics such as mechanical strength, stability, biocompatibility, adhesion, conductivity, sensing abilities, and controlled drug release. These advantages stem from their gelation processes and the functionalization with various polymers. In the food industry, particularly in dairy and functional foods, as well as in biological and medical applications, casein gels serve as a vital protein-based material for delivering bioactive and sensitive drugs, facilitating wound healing, and creating flexible sensors and wearable devices [20].

2.4 Analyses the polymerized compound using FT-IR

The Shimadzu Fourier Transform Infrared Spectrophotometry (FTIR) device was utilized to examine the unadulterated crystals of acrylamide, casein, and polyvinyl alcohol polymers. These specific polymers were chosen to construct a calibration curve that illustrates the intensity (absorbance) of the vibrational modes associated with the distinctive bonds present in each polymer [21].

2.5 Isolation and identification of S. aureus and P. aeruginosa using VITEK2 system

Sterilized cotton swab sticks were utilized to collect samples in private clinics in Baghdad, Iraq. Before sample collection, an ethical approval letter numbered (CSEC/1124/0096) dated (8 November 2024) was obtained from the biomedical research ethics committees in the Department of Biology at the College of Science, University of Baghdad. The sampling period extended from February 2023 to February 2024, during which 150 swab samples were obtained from patients suffering from wound and burn infections; only 80 samples showed bacterial growth. These samples were then transported to the laboratory under sterile and cooled conditions. To identify the isolates, they were cultured on various agar media and incubated for 18-24 hours at 37 °C in aerobic conditions. The primary screened isolates underwent morphological examination for identification purposes.

Mannitol salt agar medium was utilized for the isolation, purification, and identification of Staphylococci, as well as determining their ability to ferment mannitol. This medium is

considered selective, discriminatory, and differential due to its composition, which includes 7.5% NaCl, inhibiting the growth of bacteria other than staphylococci. It contains mannitol and phenol red as pH indicators, allowing differentiation between *S. aureus* and *S. epidermidis* [22]. For the isolation, purification, and identification of *P.aeruginosa*, the bacterial isolates were inculcated into Cetrimide agar medium. This culture medium was used to isolate and identify *P. aeruginosa* as it is a selective medium for them [23].

To determine the identification and antibiotic susceptibility of a specific number of bacterial isolates, the Vitek2 system was utilized. It utilizes specific biochemical processes and newly developed substrates to measure the utilization of carbon sources, inhibition and resistance, and enzymatic activity [24].

2.6 Antibiotic sensitivity test

The standardized antimicrobial susceptibility singular disk procedure, known as the disc diffusion assay, was employed to conduct the antimicrobial susceptibility test. Commonly used antibiotics for treating burn and wound infections were utilized to test bacterial isolates. Under sterile conditions, pure colonies of bacterial isolates were carefully selected from the original culture plates using a sterile wire loop. These colonies were then suspended in a sterile glass test tube with 5 mL of physiological normal saline. To achieve a bacterial concentration estimate of 1.5 to 2 × 10^8 CFU/mL, the suspension was modified to a 0.5 McFarland standard. Using a sterile cotton swab, the prepared bacterial suspension was then inoculated onto the surface of a Mueller-Hinton agar medium. Care was taken to ensure that there was no excess moisture by streaking the swab in four directions, covering the entire plate surface, and achieving evenly distributed inoculums. To ensure complete contact with the agar medium, six antibiotic discs, which are: Azithromycin, Carpencillin, Amoxiclave, Cefixime, Levofloxacin, and Ceftazidime discs, were gently pressed down into each Petri dish using sterilized forceps. The dishes were then left undisturbed at room temperature for approximately 5-10 minutes. Afterwards, the petri dishes were inverted and incubated for 16-24 hours [25].

Following the incubation period, bacterial growth inhibition zones surrounding the disc, including the disc itself, were measured and recorded. The diameters of the inhibition zones were assessed using a light source and ruler, with measurements given in millimeters (mm). The results of the inhibition zone diameters were then compared to the standards set for each antibiotic and bacteria, which categorized them as sensitive (S), intermediate (I), or resistant (R).

2.7 Minimum inhibitory concentration of tea tree oil and its polymer against resistance bacteria

The agar dilution method was employed to determine the minimal inhibitory concentration of TTO and its polymer against *P. aeruginosa* and *S. aureus*, as recommended by the Clinical and Laboratory Standards Institute CLSI (2020). The dilution range for the oil and its polymers was set at 40000 to 10000 ppm. The stock solution (500,000 ppm) was prepared first for each treatment. To create the stock of tea tree oil, 5 ml of oil was dissolved in 5 ml of DMSO. As for the polymers, 10 g were dissolved in 5 ml of DMSO separately. Three concentrations of oil and polymer were prepared from the stock solution [26].

To achieve equilibrium, the treatment solution was carefully poured into sterilized universal containers filled with Muller Hinton agar solution. These containers were then placed in a water bath and allowed to reach a temperature of 45 to 50 ºC before being dispensed into Petri dishes. The bacterial culture was standardized to a 0.5 McFarland turbidity (equivalent to 1.5×108 CFU/ml), and 0.2 µL of this inoculum was delicately applied to the surface of the agar using a micropipette. The Petri dishes were inverted and placed in an incubator set at 37 ºC for 18 to 24 hours. The minimal inhibitory concentration, which

corresponds to the lowest concentration of antimicrobial agent that completely halts bacterial growth, was determined based on the absence of any visible growth on the agar surface.

2.8 Statistical Analysis

Present data were programmed by SPSS v. 20.0. Ordinal data were detected by the Pearson-Chi-square test. Scale data showed at Mean±St. Error. The differences among means were detected by the Duncan test (ANOVA). $P \leq 0.05$ was dependent on differences calculation.

3. Results

3.1 GC-MS and FT-IR results

The analysis of the essential oil compounds of tea tree using GC-MS showed that the oil contains a spectrum of active compounds distributed in the form of 49 peaks in the analysis report, as shown in Table 1 and Figure 1.

Table 1: Compounds present in the oil extract of *M. alternifolia* using GC-MS analysis.

Peak no	RT (min)	Area%	Name	%
1	4.248	0.01	Bicyclo[2.2.1]hept-2-ene, 2,7,7-trimethyl-	95
2	4.575	0.05	Tricyclene	97
3	5.057	5.79	.ALPHA.-PINENE	93
4	5.223	1.19	Camphene	97
5	5.747	2.54	Sabinene	94
6	6.416	0.02	.ALPHA.-PINENE	91
7	6.593	0.90	(+)-2-CARENE	97
8	7.283	14.10	Cymene	86
9	8.004	6.69	.gamma.-Terpinene	97
10	8.092	0.06	Cyclononene, 3-methylene-	50
11	8.518	2.33	.ALPHA.-TERPINOLENE	98
12	8.627	0.02	.alpha.-Pinene oxide	94
13	8.699	0.02	2-Methyl-1-(methylamino)-1-cyanopropene	52
14	8.886	0.02	1,3,8-PARA-MENTHATRIENE	90
15	9.26	0.01	NEO-ALLO-OCIMENE	96
16	9.41	0.01	(2-Methylprop-1-enyl)-cyclohexa-1,5-diene	50
17	9.649	0.02	Camphor	55
18	11.522	22.45	4-Terpineol	98
19	11.771	3.48	alpha.-Terpineol	96
20	11.999	0.02	Divinyldimethylsilane	47
21	12.513	0.26	2-Cyclopenten-1-one, 3,4,4-trimethyl-	22
22	12.773	0.01	Cyclohexanol, 3,3,5-trimethyl-	47
23	12.84	0.01	3-Hexyne-2,5-diol, 2,5-dimethyl-	53
24	13.058	0.02	Bicyclo[2.2.1]heptane, 2-methoxy-1,7,7-trimethyl-	22
25	13.234	0.03	Dimethylhexynediol	50
26	13.468	0.02	Spiro[4.4]nonane, 1-methylene-	60
27	14.796	0.04	Ledene	59
28	15.528	0.11	Longifolene	99
29	15.808	0.08	trans-Caryophyllene	99
30	16.348	0.01	Benzene, nonyl-	72

31	16.555	0.46	.alpha.-Humulene	99
32	16.949	0.12	γ-Cadinene	99
33	17.178	0.09	.beta.-Selinene	99
34	17.359	0.04	.alpha.-selinene	99
35	17.437	0.05	.alpha.-Muurolene	99
36	17.562	0.07	α-Bulnesene	93
37	17.982	0.91	δ-Cadinene	97
38	18.106	0.06	Naphthalene, 1,2,3,4,6,8a-hexahydro-1-isopropyl-4,7-dimethyl-	98
39	18.195	0.02	(-)-ISOLEDENE	87
40	18.304	0.01	Naphthalene, 1,2-dihydro-1,1,6-trimethyl-	80
41	18.356	0.01	Cadala-1(10),3,8-triene	64
42	18.527	0.20	CAPNELLANE-8-ONE	51
43	18.625	0.03	Spiro[4.5]decane	46
44	18.859	0.04	Caryophyllenyl alcohol	68
45	19.118	0.31	(-)-Caryophyllene oxide	91
46	19.596	0.02	3-Cyclohexen-1-carboxaldehyde, 3,4-dimethyl-	78
47	24.239	36.70	Benzyl benzoate	93
48	25.531	0.19	Toluene-4-sulfonic acid, benzyl ester	55
49	25.708	0.14	p-Tolyl sulfone	59

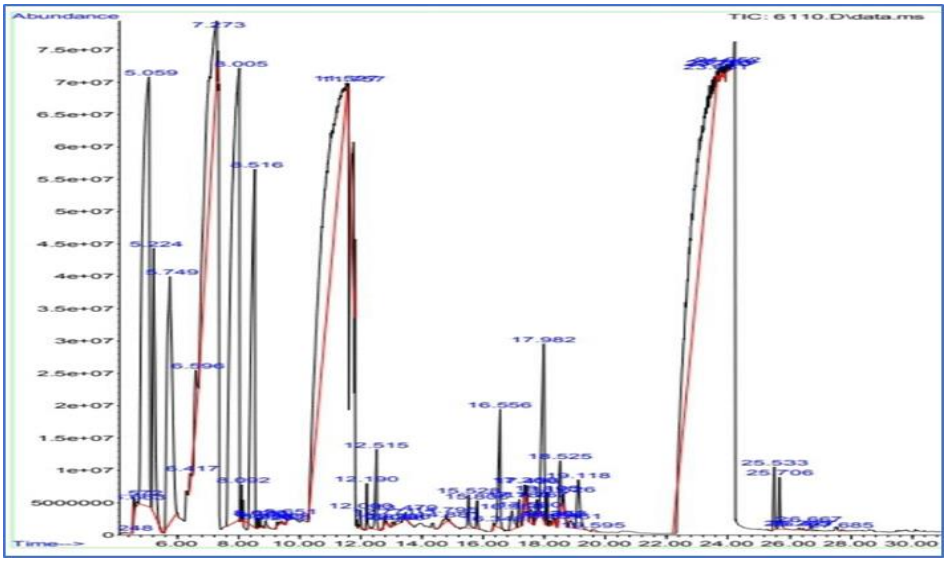

Figure 1: GC-MS profile of *M. alternifolia* oil extract.

The results demonstrated differences in the levels of active compounds found in the tea tree oil, as evidenced by Malik and Upadhyay [27]. This study revealed the presence of the following compounds with higher percentages (4-terpineol 22.45%, cymene 14.10%, γ-terpinene 6.69%, α-pinene 5.79%, α-terpineol 3.48%, sabinene 2.54% and α-terpinolene 2.33%) which indicates differences in the levels of active compounds found in the tea tree oil as reported by Abdoul-Latif *et al.,* [28]. α-Pinene was evaluated for its effectiveness against various strains of both Gram-negative and Gram-positive bacteria, revealing activity against methicillin-resistant *S.aureus* (MRSA) with an IC50 of 68.6 ± 7.9 µg/mL [29]. Additionally, α-pinene showed antifungal properties, as demonstrated by Nóbrega *et al.,* [30], who found it more effective against Candida species than the reference fungicide clotrimazole.

The TTO polymer was prepared using what was mentioned in the material and method by mixing oil with casein compound. After completing their manufacture, samples of the three polymers were sent for analysis using FT-IR technology.

3.1.1 FT-IR analysis of compound Z2 (terpene-diallyl-grafted- maleate adduct anhydride with casein)

This part featured the production of superimposed polymers with the initial stage of the reaction, including the nucleophilic attack of the amine group in casein on the carbon of the carbonyl group in the phthalic anhydride ring. The second stage involves a (co-polymer) of chitosan, phthalic anhydride and casein concerning the infrared spectra of the compound showed the high intensity at the range of (3450) cm-1 vibrations of hydroxyl groups, the stretching vibrations of (3332)cm-1refer to the(N-H), show bands at (2958,2873) cm-1 due to the stretching vibrations of (CH) Alph, band at (1774,1720) cm-1 medium the stretching vibrations of(C=O ester), while at (1600) cm-1 which refers to(C=O amide), (1550,1454) cm-1 which refer to the (C=C) of aromatic ring for these compound as stretching vibrations appeared at (1264) cm-1 of (C-N) and (1219) cm-1 were due to the bending vibrations of (C-O).

The structural characterization was done by FT-IR and showed a broad band of about (3329) cm-1 due to (NH) stretching. (2934, 2866) cm-1 assigned to aliphatic (CH, CH2,) stretching showed peaks at (1797) cm-1 assigned to (C=O) stretching of casein, at (1600) cm-1 assigned to characteristic absorption of (C=O) amide. On the other hand, (1546) cm-1 peak of (C=C), as shown in Figure 2.

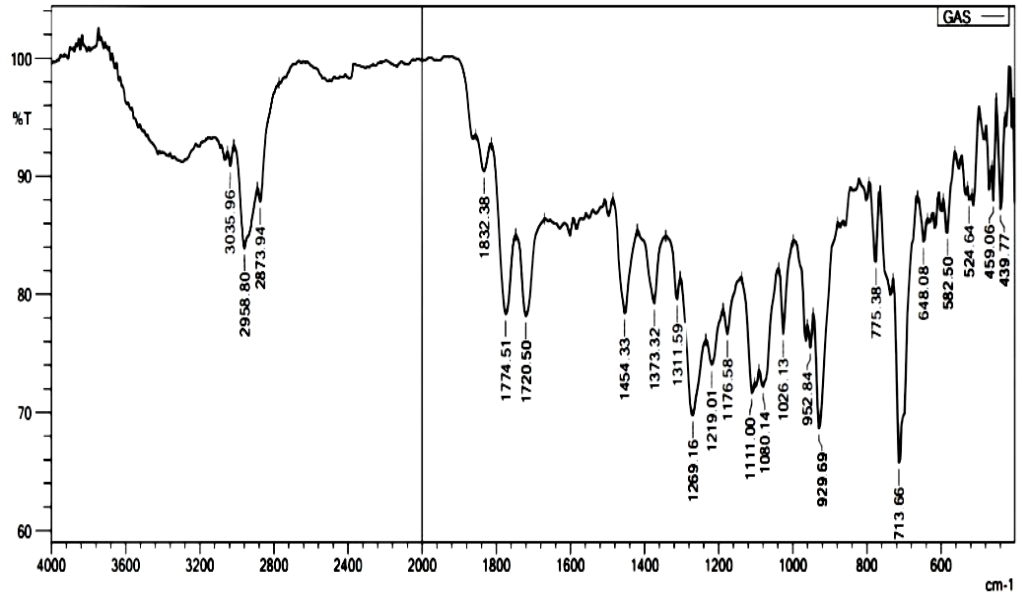

Figure 2: FT-IR result of terpene-diallyl-grafted- maleate adduct anhydride with casein.

3.2. Isolation and identification of resistant bacteria

Out of 80 specimens gathered, 67 (83.7%) demonstrated positive growth following purification and identification, while the other 13 samples (16.3%) showed no growth. Traditional culturing techniques were utilized to identify the various types of bacteria, which involved a visual assessment of both selective and differential culture media. Following this, the identification was confirmed for accuracy using the Vitek 2 system.

The first step in identifying the isolates involved visually assessing the colony morphology on different agar culture media, which were incubated for 24 hours at a temperature of 37 °C. Out of the 67 isolates, 32 were classified as *Pseudomonas* spp, 22 as *Staphylococcus* spp, while the rest were comprised of *Klebsiella* spp. and *Acinetobacter* spp.

The distinct and definitive outcomes related to the resistance or sensitivity of isolated bacteria were achieved through the use of Mueller Hinton agar, a medium specifically designed for antibiotic susceptibility testing [31]. In contrast, nutrient agar is frequently utilized for cultivating and expanding diverse bacterial species, as it supplies the necessary nutrients essential for bacterial growth. As a result, it is a widely adopted medium [32]. Vitik 2 results demonstrate that of 32 *Pseudomonas* spp, only 15 isolates were *P. aeruginosa*, while only 17 isolates from *Staphylococcus* spp were *S. aureus,* which underwent susceptibility testing.

3.2 Antibiotic susceptibility test of resistant bacteria

As shown in Table 2 and Figure 3, the *S. aureus* isolates exhibited the highest levels of multi-drug resistance were 4, 10, 23, 26, 27, and 44, whereas for *P. aeruginosa*, the isolates that were multi-drug resistant include 3, 4, 5, 7, 13, and 14.

Table 2: diameters of inhibition zones of *S. aureus* and *P. aeruginosa* against different antibiotics.

Bacterial type	Isolate no.	Antibiotics					
		AZM	CAZ	LEV	CB	CFM	AMC
P. aeruginosa	1	28	Zero	18	33	Zero	28
	2	32	Zero	17	21	Zero	26
	3	21	10	Zero	6	Zero	Zero
	4	9	7	Zero	Zero	Zero	4
	5	17	Zero	Zero	Z	Zero	7
	6	24	15	22	19	Zero	32
	7	9	Zero	Zero	Zero	Zero	Zero
	8	28	Zero	22	22	Zero	26
	9	26	15	10	19	Zero	32
	10	28	Zero	26	12	Zero	26
	11	29	Zero	32	31	Zero	Zero
	12	31	Zero	32	19	Zero	8
	13	21	Zero	5	10	Zero	10
	14	24	Zero	Zero	Zero	Zero	26
	15	26	Zero	12	21	Zero	28
	16	27	Zero	31	12	Zero	26
	17	34	Zero	19	31	Zero	28
S. aureus	1	19	17	15	21	Zero	9
	4	11	7	6	8	Zero	7
	9	27	21	17	18	Zero	19
	10	13	9	11	15	Zero	10
	12	25	17	16	17	Zero	11
	13	19	14	18	19	Zero	18
	15	22	14	21	25	Zero	12
	19	29	24	11	21	Zero	12
	20	32	16	18	19	Zero	17
	22	31	10	16	11	Zero	17
	23	zero	8	8	12	Zero	Zero
	26	12	11	11	15	Zero	13
	27	16	10	10	13	Zero	9
	28	28	21	11	16	Zero	16
	44	13	9	10	13	Zero	11

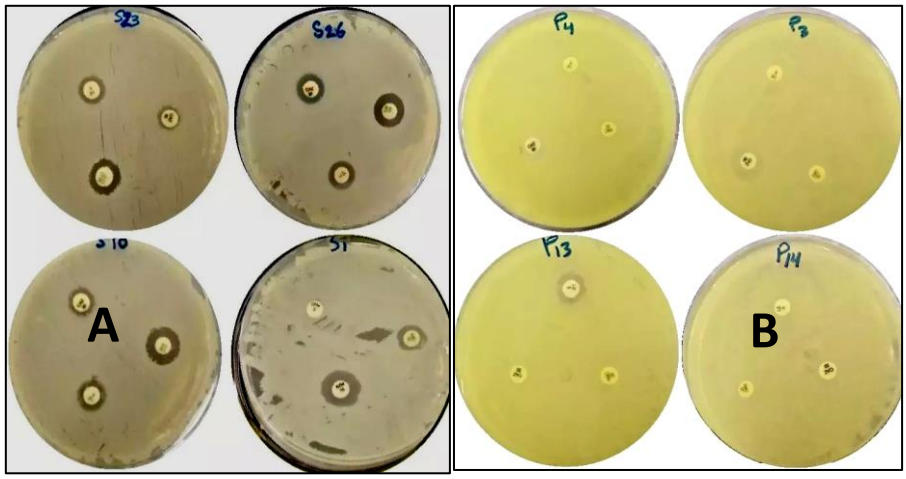

Figure 3: Some of multidrug resistant (A) *S. aureus* isolates, (B) *P. aeruginosa* isolates

Valuable insights into future challenges and suitable treatment approaches can be obtained by analysing the nature of susceptibility. The assessment of bacterial isolates' susceptibility to six different antibiotics revealed that the pathogenic bacteria tested showed different degrees of resistance to these drugs. Each isolate was categorized as resistant, intermediate, or susceptible to a given antibiotic according to the standard inhibition zones. The results demonstrated a significant prevalence of resistance among the bacterial isolates against the antibiotics. Various factors influence the differences in resistance rates, including the antibiotics' mechanisms of action, the specific type of bacteria, the presence of horizontally transferred genes, and genetic mutations [33].

3.3 Minimum inhibition concentration (MIC) of essential oil casein polymer against resistant P. aeruginosa isolates

A MIC test should be carried out to determine the susceptibility of isolates. The most antibiotic-resistant *P. aeruginosa* isolates were experienced to determine the minimum inhibitory concentration of essential oil and its polymer by utilizing the agar dilution process with serial concentrations of essential oil (40000 ppm, 20000 ppm, 10000 ppm, 5000 ppm, 2500 ppm, and control). In a previous study, *P. areuginosa* bacteria showed little antibacterial activity to tea tree oil when used alone compared to the control but gave a significant positive result in the presence of two outer membrane permeabilizers, EDTA and PMBN [34]. Present findings showed there are significant differences ($p<0.05$) between *P. aeruginosa* isolates growth and concentrations of TTO and casein polymer treatments. All bacterial isolates showed no growth at concentrations (40000, 20000, 10000, and 5000) of TTO treatment, while they were grown at concentrations (2500) compared to the control. Based on casein polymer treatment, all isolates showed no growth at concentration (40000), isolates (3, 4, 5) showed growth at concentration (40000), and all isolates showed growth at concentrations (10000, 5000, and 2500) compared to controls (Table 3).

Table 3: MIC results of (TTO) and casein polymer against the resistant *P. aeruginosa* isolates.

	Conc. (ppm)	Growth of *P. aeruginosa* resistant isolates						P value
		3	4	5	7	13	14	
TTO	40000	-	-	-	-	-	-	P<0.001***
	20000	-	-	-	-	-	-	
	10000	-	-	-	-	-	-	
	5000	-	-	-	-	-	-	
	2500	+	+	+	+	+	+	
Casein polymer	40000	-	-	-	-	-	-	P<0.001***
	20000	+	+	+	-	-	-	
	10000	+	+	+	+	+	+	
	5000	+	+	+	+	+	+	
	2500	+	+	+	+	+	+	
Control (DMSO)	40000	+	+	+	+	+	+	1.00
	20000	+	+	+	+	+	+	
	10000	+	+	+	+	+	+	
	5000	+	+	+	+	+	+	
	2500	+	+	+	+	+	+	

3.4 Minimum inhibition concentration (MIC) of essential oil casein polymer against resistant S. aureus isolates

MIC results of TTO and casein polymer against the multi-drug resistant *S. aureus* isolates were performed by the agar dilution process with serial concentrations (40000 ppm, 20000 ppm, 10000 ppm, 5000 ppm, 2500 ppm, and control); the results showed that the MIC and MBC were respectively 0.1% and 0.4% for the *S. aureus* isolated from foot injuries [35]. The current investigation showed significant differences ($p<0.05$) between resistant *S. aureus* isolates growth and concentrations of TTO treatment. Bacterial isolates (4, 10, 23, 26, and 27) showed no growth at concentrations (40000, 20000, 10000, and 5000) of TTO treatment, while isolate number (44) showed growth at concentration (5000) only, and all isolates showed growth at concentration (2500) compared to control. In contrast, the present study showed no differences ($p>0.05$) between resistant *S. aureus* isolates growth and concentrations of casein, as shown in Table 4.

Table 4: MIC results of TTO and casein polymer against the resistant *S. aureus* isolates

	Conc. (ppm)	Growth of Resistant *S. aureus* isolates						P value
		4	10	23	26	27	44	
TTO	40000	-	-	-	-	-	-	P<0.001***
	20000	-	-	-	-	-	-	
	10000	-	-	-	-	-	-	
	5000	-	-	-	-	-	+	
	2500	+	+	+	+	+	+	
Casein polymer	40000	-	-	-	-	-	-	P>0.05
	20000	+	-	-	-	-	-	
	10000	+	+	+	+	+	+	
	5000	+	+	+	+	+	+	
	2500	+	+	+	+	+	+	
Control (DMSO)	40000	+	+	+	+	+	+	1.00
	20000	+	+	+	+	+	+	
	10000	+	+	+	+	+	+	
	5000	+	+	+	+	+	+	
	2500	+	+	+	+	+	+	

4. Conclusion

Tea tree essential oil shows promising efficacy as an antimicrobial agent against *P. aeruginosa* and *S. aureus*, which are prevalent microbes and significant contributors to infections in surgical patients and those near healthcare equipment. The emergence of bacterial drug resistance necessitates the exploration of new therapeutic options, and plant-derived essential oils may provide solutions to this challenge. Nevertheless, it is important to conduct additional research on cytotoxicity, mechanisms of action, and in vivo analyses. Therefore, polymers have been developed to reduce the cytotoxic effects of oils while enhancing their therapeutic benefits.

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Disclosure and Conflict of Interest

The authors declare that they have no conflicts of interest.

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