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Study of the Synergistic Effect of Three Volatile Oils on Antibiotic-Resistant Bacteria Isolated from Burns

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ABSTRACT

Background: Recent years increasing antibiotic resistance, the possibility of novel plant derived antibiotics is very important as alternative compounds for antibiotics. Plant extracts involves antibacterial properties.

Objective: The aim of the current study was to evaluate the efficacy of 3 essential oils extracted from 3 plants against pathogenic bacterial isolated from wounds and burns. **Materials and methods:** The essential oils of *A. citrodora, R. officinalis* and *T. vulgaris* were extracted and prepared in serial dilution concentrations. A total of 150 bacterial isolates from wounds and burns were isolated and identified. **Results:** The results showed that *Pseudomonas* was the most frequent as 51 (34%) isolates, while *Enterococcus* and *Rhizopium* were the least as 1 (0.67%) isolate. A susceptibility test was conducted by using 15 different antibiotic discs. Trimethoprim was the most resistant antibiotic for bacterial isolates (79.33%), while Imipenem was the least (40%). Moreover, the susceptibility test was revealed the most resistant was *Rhizopium* with 86.67% and *Enterococcus* was least with 20%. The highest antibiotic resistant isolates 22 were selected for determining the synergistic effect of volatile oils that inhibited of bacterial growth, the concentration of significant synergistic effect was 156.25, 156.25 and 39.0625 ppm of *A. citrodora, R. officinalis* and *T. vulgaris* respectively. **Conclusions:** Little attention has been paid to evaluate the efficacy of Eos as alternative treatment instead of conventional remedy, thus this study tested the activity of 2 EOs against pathogenic bacteria.

Keywords: Susceptibility test, essential oils, antimicrobial-resistant, Experimental study, University of Baghdad.

INTRODUCTION

Main health problem throughout the world is burn and wound injuries. The burn wound appears as a favourable region for opportunistic colonization of microorganisms with endogenous and exogenous source. Burn wound patients are incubator for different of aerobic bacteria and average of isolation of these organisms increase with rise in total body surface area [(1)]. Wounds are open injuries on surface of body; which stay in nexus with environment. Numbers and size of burn injuries plays very significant role in colonization and so in infection by different of microorganisms those grow and reduplicate in aerobic condition. Spacious burn injury deemed as infected wound and immunity of burn patients deemed as suppressed immunity, as well as isolated bacteria must have deemed as agents of infective. Infection is the major cause of death after sever burn injuries, approximately 42–65% of deaths in burn sufferers are attributable to infection, although advancements in burn care at hospitals over the last 50 years [(2)]. As a result, burn patients with infections have more than twice the mortality average of uninfected patients. The kinds of bacterial species that are isolated from patients is related with longitude of hospitalization after a burn injury. Last decades, burn wounds have been among the most destructive type of injuries. Grave thermal injuries reason high mortality and morbidity. Each year 300,000 die due to burns out of 11 million people require medical treatment in the world [(3)].

Result of wound infection by bacteria more than 75% of deaths reasoned by burns. The happening of burn injure infection it associated with both the depth and the size of the wound and the chances of higher on infection are correlated with the longer the wound remains open. Since the late 1800s have been reported some microorganism's growth were inhibited by other microorganism's growth. Which led to finding of natural antibacterial from these reported of antibiosis between microorganisms [(4)].

Some researchers difference between antimicrobial and antibiotic, antimicrobial is utilized in antiseptics materials, whereas antimicrobial is utilized as medicine materials. The World Health Organization was assorted antibacterial resistance as a " dangerous impendence is no extender a prognosis for the future, it is occurring right now in any area of the world and has the possibility to influence anyone, of each age, in each country" [(5)]. Antibiotics can be classified to different types, depending on the pharmacological characteristics were classified to: effect on microbe, mechanism of action and mode of spectrum. Generally, antibiotics act on: cell wall, ribosomal chain and inhibited the synthesis of bacterial RNA or DNA and thus inhibitor the normal cellular processes and protein synthesis [(6)].

Antibiotics caused side-effects, before approve for clinical use, it tested for any negative effects and are considered safe and well for used. Adverse side effects have been a wide ranging extent from moderate to extremely severe, that depending on antibiotic type utilized, microorganism targeted, and the patient [(7)].

In the antibiotic treatment the common phenomenon is bacterial resistance emergence to antibiotics. The resistance emergence reflects development procedure that occur during antibiotic remedy. Due to infections sourced by bacterial resistant, in the European Union, estimated about 25 thousand patients die each year, while, in the United States, about 77 thousand patients die each year [(8)]. Decreasing numeral of novel antibiotics actually being led to expansion of bacterial disease therapy strategies which are alternatives to traditional antibacterial due to excess in bacterial strains which are resistant to common

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antibacterial remedies [9]. Antimicrobial resistance is very important health problem It induced attention and interesting about action of medicinal plants developed. Recent years increasing antibiotic resistance, the possibility of novel plant derived antibiotics is very important as alternative compounds for antibiotics. Plant extracts involves antioxidant and contain phytochemicals demonstrate in vitro antibacterial properties, it can modify bacterial membrane surface hydrophobicity, damage microbial membrane structures and inhibit peptidoglycan synthesis [10]. Plants are an important effective source of natural substances that can be used to treat diseases, eliminate of pathogens and improve health, this feature originates from ability plants to synthesize a large and different group of secondary metabolites. Many of these compound are bioactive matters which plants utilized as protection molecules [11].

These compounds interact with specific targets cells to act many biological activities. Production of secondary metabolites are warranty of existence of protection molecules of specific structures which are useful to pharmaceutical development. Released of essential oils can be done by heat and pressure, that found in diverse plant parts, such as bark, leaves, flowers, fruit, grass, gums, roots, and wood [12], extraction process are done by different methods, include: aqueous infusion, cold or hot pressing, effleurage, hydro-distillation, phytonic process, steam, steam/water distillation, solvent extraction and supercritical fluid extraction [13].

Essential oil of *Aloysia citrodora* owns antimicrobial characteristics whether anti-bacterial or anti-fungal. Conventionally, it is used as folk medicine to therapy in remediation of cold, fever and spasms [14]. For many years, lemon verbena has been popularly used to remedy of anti-inflammatory, anxiety, asthma, colic, diarrhoea, flatulence, indigestion and insomnia [15]. *Rosmarinus officinalis* L. is ordinarily known as rosemary, belongs to the family: Lamiaceae, it is growing wild and considered one of medicinal plant. Rosemary extracts extend expiration date of foods spoilage, due to natural antioxidant activity therefore used extracts obtained from it for this purpose [16]. Chemical composition of the T. vulgaris essential oil included of: 56.53% of oxygenated monoterpenes, 28.69% of monoterpene hydrocarbons, 5.04% of sesquiterpene hydrocarbons and 1.84% of oxygenated sesquiterpenes. So far, there is no significant clinical studies, no complete knowledge or sufficient information, and no accurate and precise clinical trials and tests support the use of thyme alone for human treatment use [17].

Despite, the conventional health practices modalities, folkloric traditions and anecdote as well as expert opinion suggest it have been recommended for numerous cases, but these indicators are still historical and theoretical and there is deficiency of enough evidence and suitable proofs. Thyme contains thymol whose one of the main components responsible of thyme activity and numerous compounds of apigenin, borneol, caffeic acid, cineol, cymene, eugenol, flavonoids, gerniol, limonene, lutein [18].

luteolin, myrcen, naringenin, oleanic acid, phenols, pinen, polyphenolic acid, rosmarinic acid, sabinene, tetramethoxylated, thymonin, triterpene and zeaxanthin. Dried herbal material involve about 2.5% essential oil, major ingredients are β-myrcen, carvacrol, glycosides, linalool, p-cymene, terpinen-4-ol, thymol and γ-terpinene [19]. Little attention has been paid to evaluate the efficacy of essential oils as alternative and instant treatment instead of conventional remedy. Thus, the aim of the current study was to evaluate the efficacy of 3 essential oils extracted from 3 plants against pathogenic bacterial isolated from wounds and burns.

MATERIALS AND METHODS

Plant samples: Plant samples include the aerial parts "dry leaves" of *A. citrodora* and *R. officinalis* in addition *T. vulgaris* which were gained from Baghdad local herbarium market. Each leaves sample was dried at room temperature then milled down to fine powder form in an electrical grindery to make it easier to handle in the volatile oil extraction steps. Until use, the samples powder has been preserved and stored in clean and dry conditions.

Extraction of *A. citrodora, R. officinalis* and *T. vulgaris* oil: In a round-bottomed flask, 250g of dried leaves was placed, then subordinated to hydro distillation for 3 hours by heating to 60 ºC by using the Clevenger apparatus, the aqueous stage was divided into two layers: the under layer included water, was neglected, while the toper layer involved the oils gathered, after that stored in a refrigerator in a glass vials were locked and marked until used [20].

Preparation of different concentration of plant extract: The concentrated essential oils extracts were utilized as stock, the stock solution was prepared by utilizing Dimethyl sulfoxide (DMSO) to diluted the essential oils, then different concentrations have been prepared, by mixing a specific volume of stock with a specific volume of DMSO [21].

Collection and isolation of bacterial specimens: Sampling for studied were collected from six hospitals in Baghdad-Iraq, Alkadhmiya Teaching Hospital, Alkarama Teaching Hospital, Alkindy Teaching Hospital, Alyarmuk Teaching Hospital, Baghdad Teaching Hospital and Imam Ali General Hospital. One hundred and fifty swab specimens were collected from patients, suffering from wounds and burns infections, swap specimens were transferred to the laboratory under sterile and cooled conditions, these isolates were inoculated by culturing on different agar medium and incubating for 18-24 hours at 37 °C under aerobic conditions. All the primary screened isolates then subjected to various identification tests involved morphological characteristics and biochemical tests [22].

Bacterial identifications by utilizing Vitek2 system: A certain number of bacterial isolates were chosen to assert the identification and antibiotic susceptibility utilizing the Vitek2 system.

Antimicrobial susceptibility test: This antimicrobial susceptibility test or disc diffusion assay was carried out by utilizing standardized antimicrobial susceptibility singular disk procedure, it was done based on modified Kirby Bauer disc agar diffusion test as well-defined by the World Health Organization [23]. The antibiotics were used in this study are: Amikacin 10, Ampicillin 10, Azithromycin 15, Carbenicillin 25, Cefotaxime 30, Ceftazidime 10, Ceftriaxone 10, Ciprofloxacin 10, Gentamycin 10, Imipenem 10, Levofloxacin 5, Meropenem 10, Piperacillin 10, Ticarcillin 10, and Trimethoprim 10.

Determination of minimal inhibitory concentration of antibiotic (MIC):

Method of micro-titer plates of 96 wells was utilized according to [24] then isolates most resistant to antibiotics were inoculated in nutrient broth and incubated at 30°C for 24 hours. Muller-Hinton broth was prepared and using as diluent. In this method were used Micro-titer plates of 96 flat wells. All wells of the plate have been winded with 100 µL of Muller-Hinton broth in the first horizontal line excepting the last well which 12th well. Extracts of three plants (volatile oils or essential oil) were used. Extracts of three plants with a serial dilution have range of concentrations 40000, 20000, 10000, 5000, 2500 and 1250 µg/ml, were prepared. Plants extracts as stock solution was prepared. One hundred µL of stock solution was put down in the first well only, and it was mixed well of contents. One hundred µL was taken from the first well and transferred to the second one, then it was mixed well of contents. The other wells from 3 to 10 were submissive to the same process as in previous step. One hundred µL was taken from the 10th well and then careless it, so that the final volume in all tubes was 100 µL. One hundred µL of distilled water was added to the 11th well, mixed well with Muller-Hinton broth, and then 100 µL of it were transferred to the 12th well. Inoculums were ready, by transporting 3-5 colonies into a tube of 5ml of normal saline to gain culture with 1.5 × 108CFU/ml, and modifying of turbidity standard of McFarland 0.5, suspensions were utilized within 30 minute of preparation. Ten µL of bacterial suspension was added to each of the wells from first well and continued until reach the 11th well, except for the negative control wells. The positive control was the eleventh well, whereas the negative control was the twelfth well. Sixteen µL of resazurin pigment was added to all the wells. The microtiter plates was incubated at 37°C for 18–24 hours. The minimal inhibitory concentration (MIC) for each bacterial isolates were defined as the lowest concentration of bacterial agent that showing no visible growth and totally inhibits of bacteria.

Ethical Consideration:

The study was performed with an approval from the Academic Scientific Research Ethics Committee, Department of Biology, College of Science, University of Baghdad.

Statistical Analysis

The collected data were introduced and statistically analysed by utilizing the Statistical Package for Social Sciences (SPSS) version 20 for windows. Qualitative data were defined as numbers and percentages. Quantitative data were described as mean and standard deviation (SD).

RESULTS

Isolation and identification of bacteria:

One hundred fifty of bacterial isolates were isolated and identification from burn wards in Baghdad hospitals, the bacteria isolated were: *Acinetobacter* spp., *Enterobacter* spp., *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella oxytoca*, *Proteus* spp., *Pseudomonas aeruginosa*, *Rhizopium radiobacter*, *Serratia fonticola*, *Staphylococcus sciuri*. *Pseudomonas* spp. were the most frequent as 51 isolates 34%, while *Enterococcus* spp. and *Rhizopium* spp. were the least as 1 isolate 0.67% **(Figure 1).**

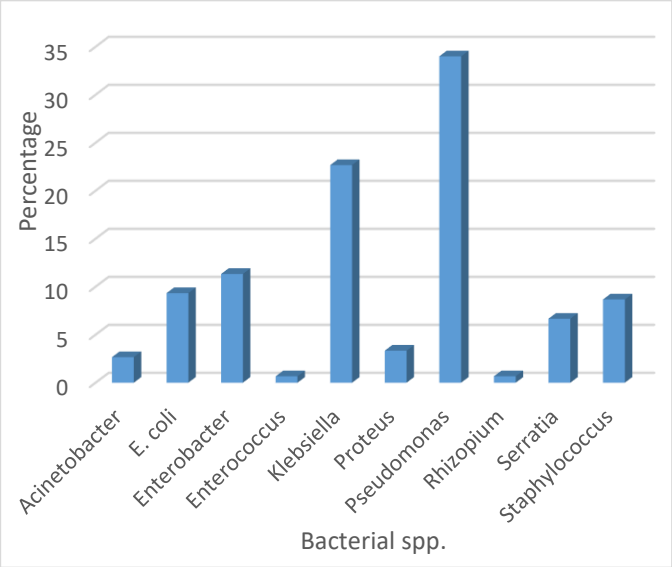


Figure (1): Total percentage of bacterial isolates.

Susceptibility test:

A susceptibility test was conducted for all bacterial isolates; the test was performed by using 15 antibiotics which treat of bacterial wounds and burns infections. The results appeared that most of the bacterial isolates were resistant to most antibiotics.

Trimethoprim was the most resistant antibiotic for bacterial isolates, 119 isolates 79.33%; while Imipenem was the least, 60 isolates 40%. Imipenem was the most susceptible antibiotic for bacterial isolates, 85 isolates 56.67%; while Ampicillin was the least, 23 isolates 15.33 **(Figure 2)**.

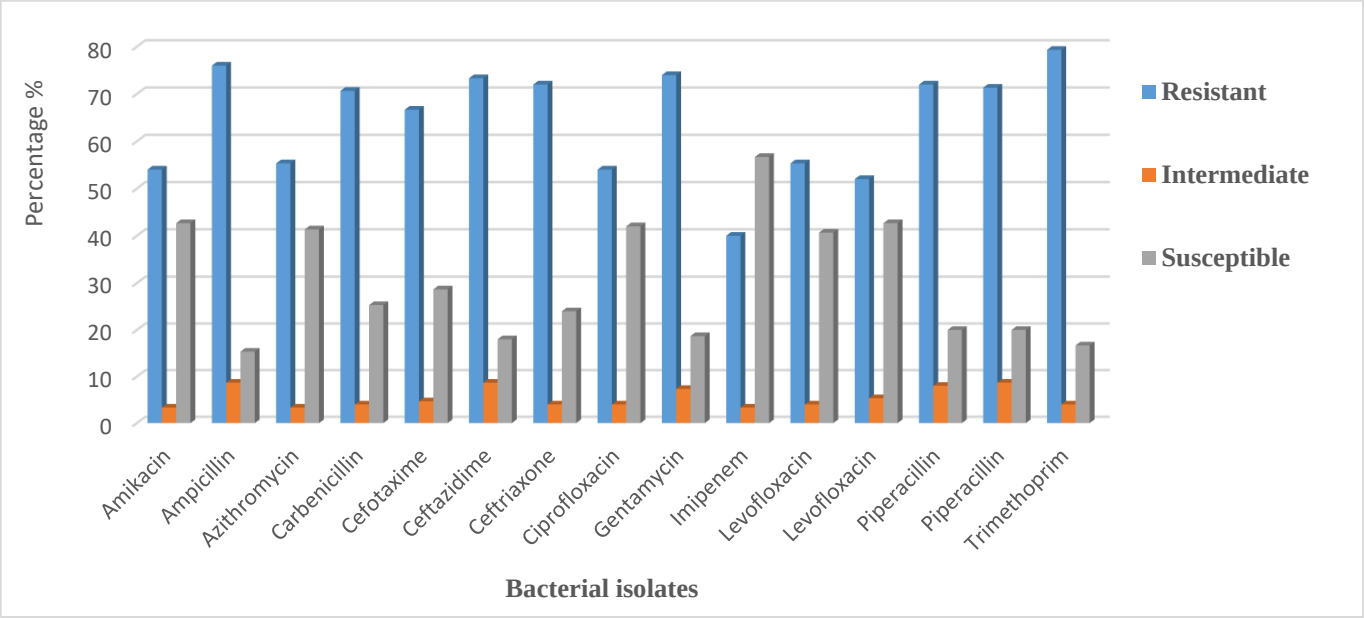


Figure (2): Percentage of resistant, Intermediate, and susceptible bacterial isolates.

The susceptibility test was revealed the most resistant is *Rhizopium* with 86.67% and *Enterococcus* is least with 20%, the most susceptible is *Enterococcus* with 80% and *Rhizopium* is least with 13.33%, the most intermediate is *Acinetobacter* with 8.33% and *Enterococcus* and *Rhizopium* the least with 0% **(Figure 3)**.

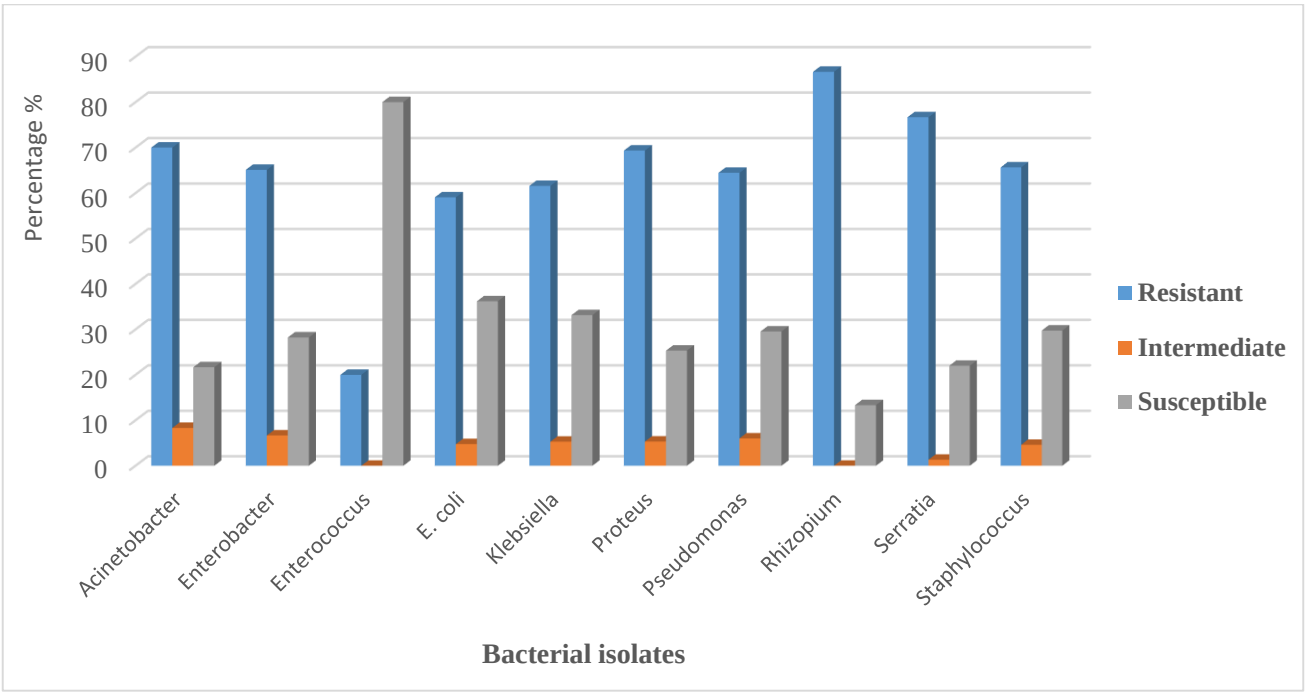
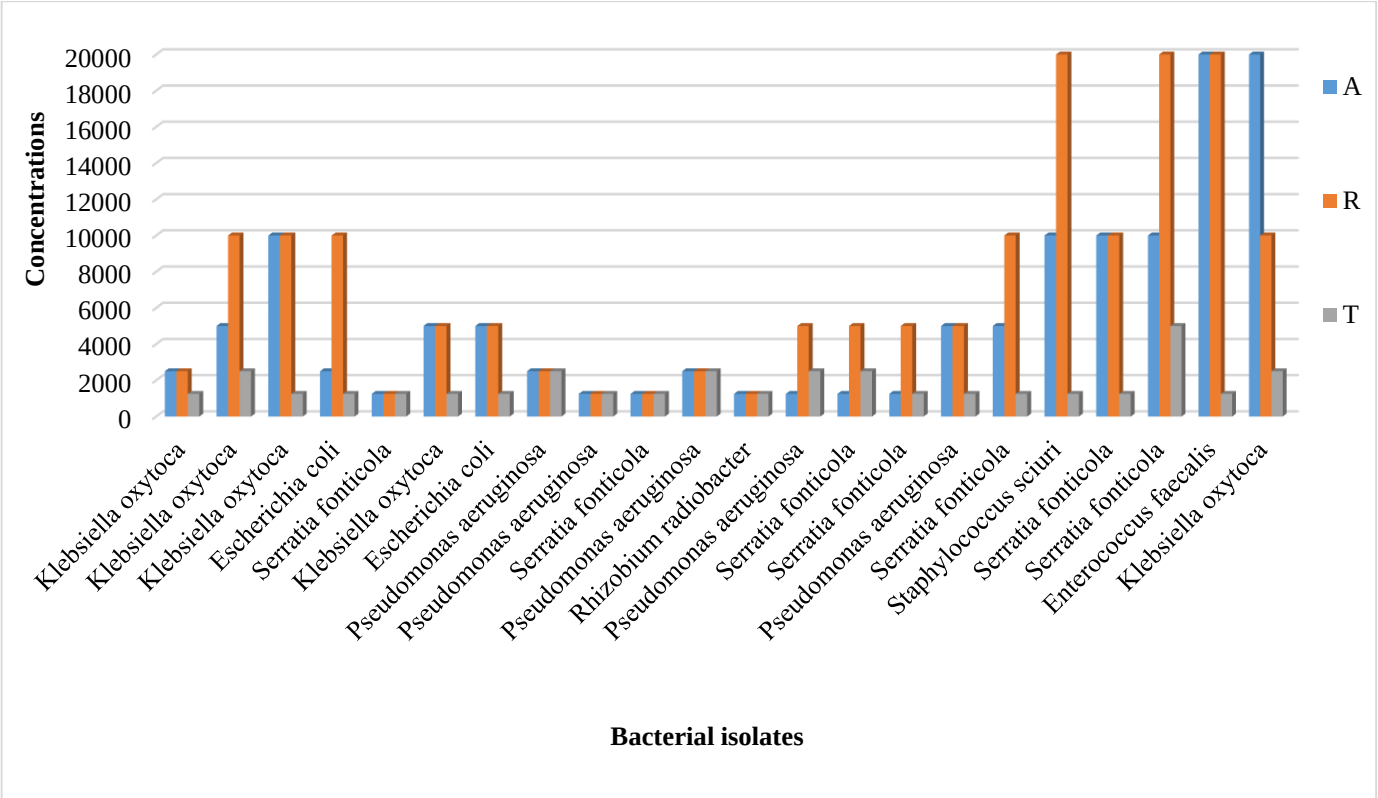


Figure (3): Bacterial species and resistance or susceptibility to antibiotics.

Effect of volatile oils on bacterial growth:

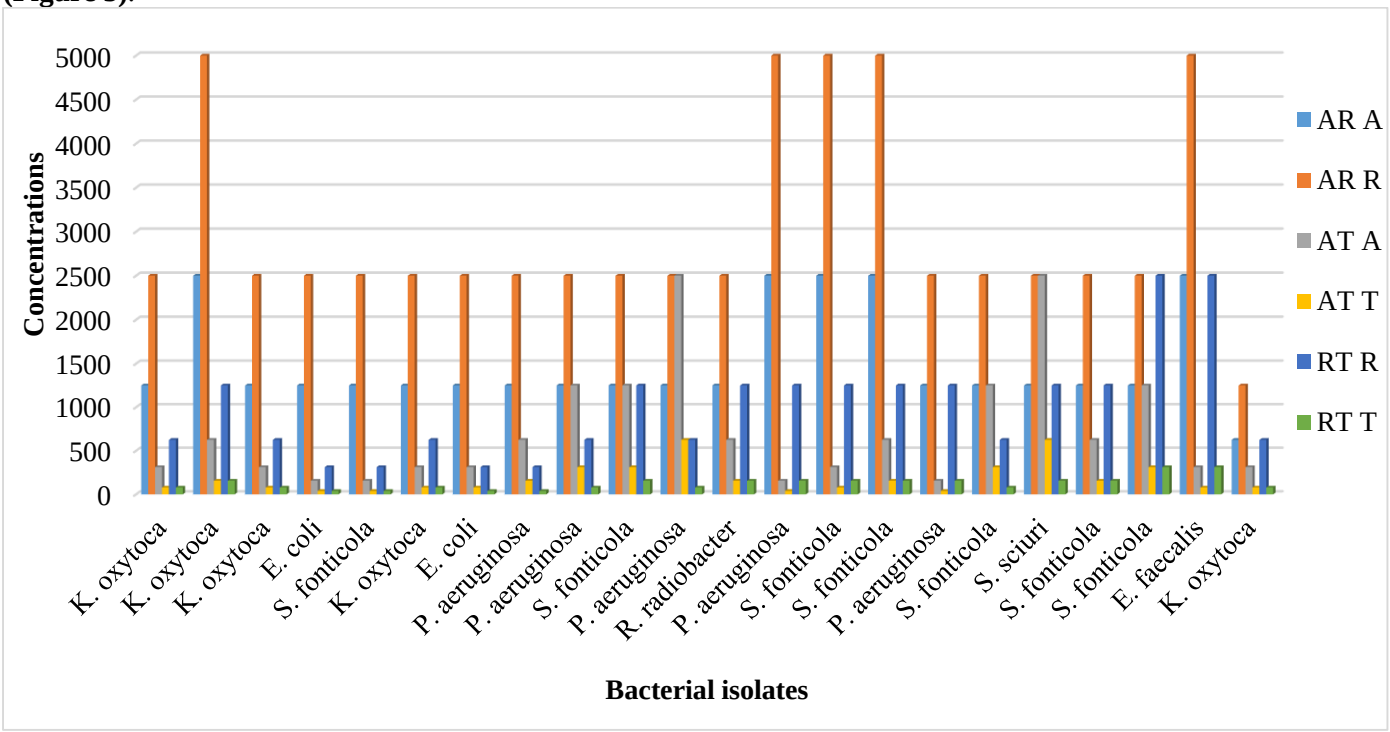
Twenty bacterial isolates which most antibiotic-resistant were selected in addition to two least resistant isolates as control and compared with resistant isolates (bacterial isolate no. 50 and bacterial isolate no. 132). *A. citrodora*, *R. officinalis* and *T. vulgaris* were selected and the essential oil was extracted by Clevenger apparatus, then series of concentrations were prepared to be tested on bacterial isolates that were adjusted by MacFarland, the concentrations prepared were 40000, 20000, 10000, 5000, 2500, 1250 and 625 ppm. The results showed that the concentration 5000, 10000 and 1250 ppm of *A. citrodora*, *R. officinalis* and *T. vulgaris* respectively were most effective **(Figure 4)**.



A: A. citrodora, R: R. officinalis, T: T. vulgaris.

Figure (4): MIC of volatile oils that inhibited of bacterial growth.

Synergistic effect of volatile oils extracted from two plants: According to MIC of plant extract, the volatile oils were mixed for two plant species to study the effect on inhibition of bacterial growth. The sequential concentrations were prepared as follows: *A. citrodora*: 5000, 2500. 1250, 625, 312.5, 156.25 and 78.125 ppm; *R. officinalis*: 10000, 5000, 2500. 1250, 625, 312.5 and 156.25 ppm; *T. vulgaris*: 1250, 625, 312.5, 156.25, 78.125, 39.0625 and 19.53125 ppm. The results showed that: 1250 ppm of *A. citrodora* with 2500 ppm of *R. officinalis*, 625 ppm of *A. citrodora* with 156.25 ppm of *T. vulgaris* and 625 ppm of *R. officinalis* with 78.125 ppm of *T. vulgaris* was significant concentration (MIC) **(Figure 5)**.



A: A. citrodora, R: R. officinalis, T: T. vulgaris

Figure (5): MIC of volatile oils for two plants that inhibited of bacterial growth.

Factorial design: To study the synergistic effect of the volatile oils for plants, a factorial design was made, which consists of 3*3*3=27, ten concentrations were selected to study the effect on bacterial growth **(Figure 6)**.

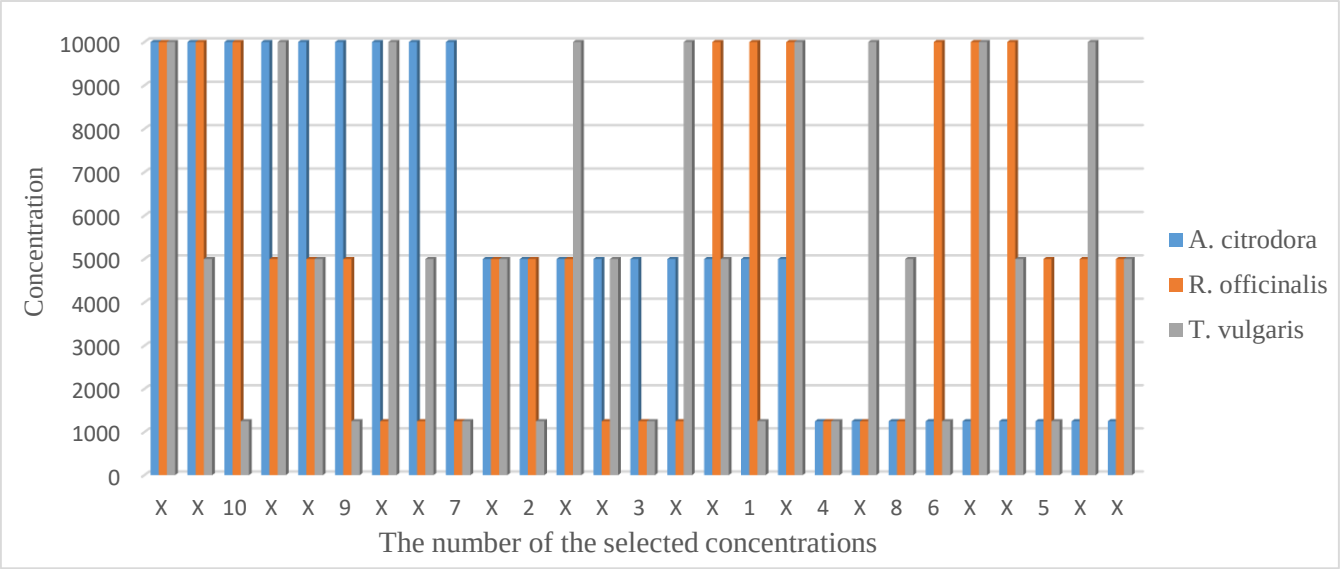


Figure (6): Factorial design and ten concentrations were selected.

Synergistic effect of volatile oils extracted from three plants:

Ten concentrations of synergistic effect were selected depending on results of MIC for *A. citrodora*, *R. officinalis* and *T. vulgaris*, 156.25, 156.25, 39.0625 it was significant synergistic effect followed by 156.25, 312.5, 39.0625 ppm that inhibited of bacterial growth **(Figure 7 [a], [b])**. The prepared serial concentrations ranged 10000, 5000, 2500. 1250, 625, 312.5, 156.25, 78.125, 39.0625 and 19.53125 ppm, according to the essential oil stock was prepared depending on factorial design.

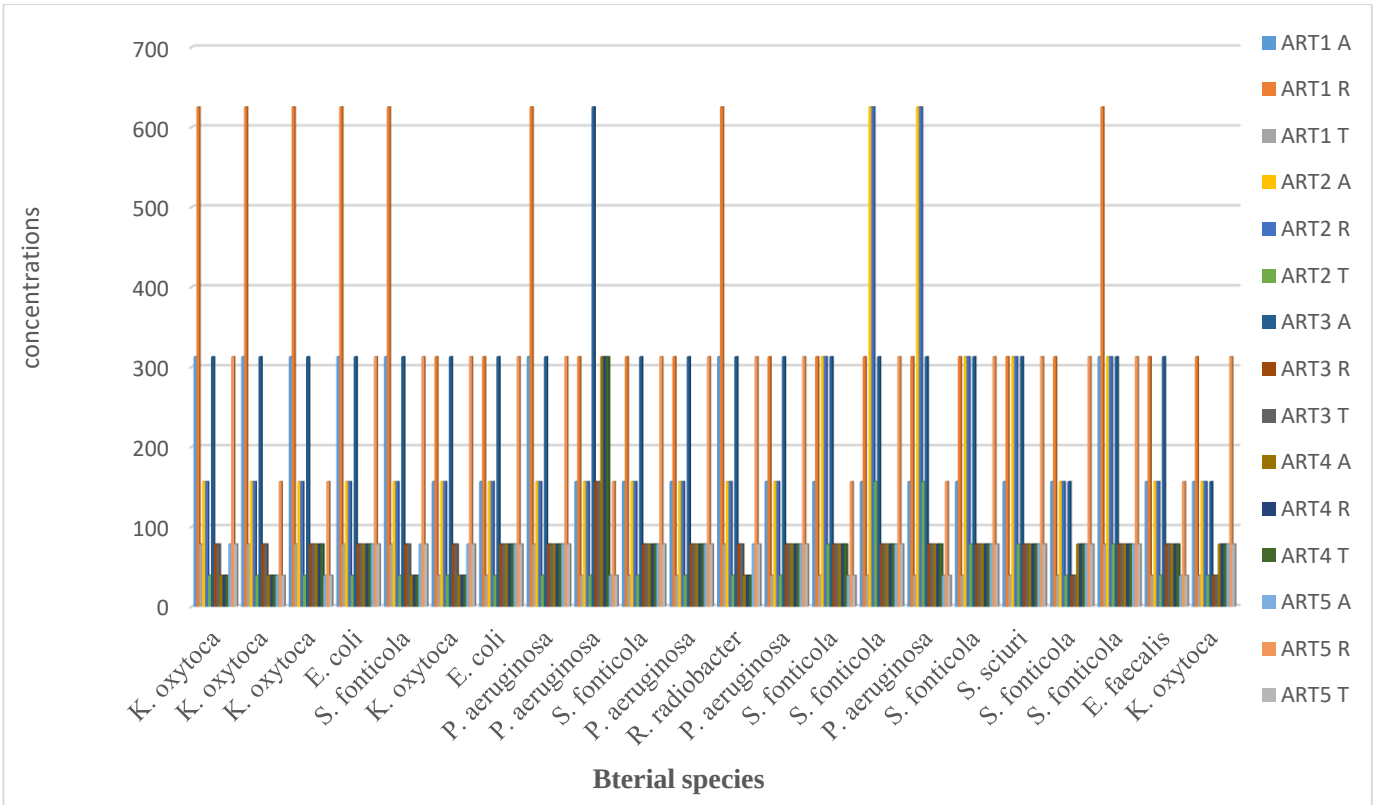


Figure (7 a): MIC of synergistic effect of volatile oils that inhibited of bacterial growth.

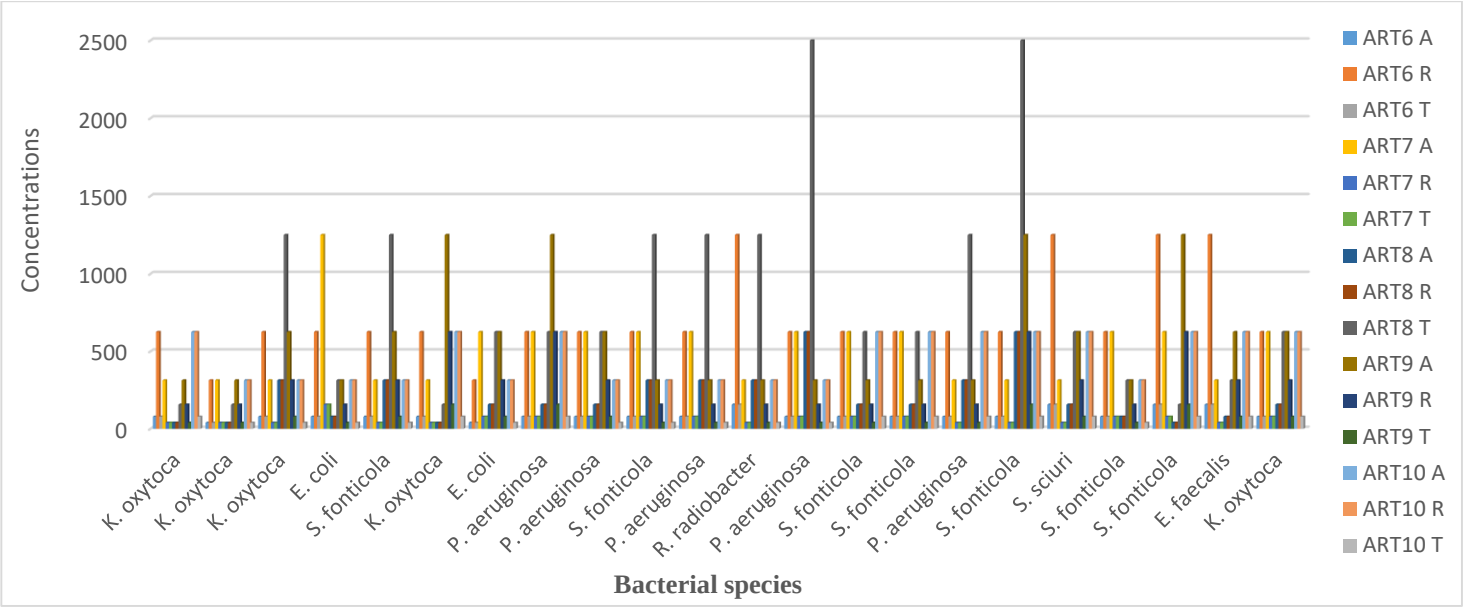


Figure (7 b): MIC of synergistic effect of volatile oils that inhibited of bacterial growth.

DISCUSSION

In this research, 150 isolates of bacteria were isolated from patients burn wards in Baghdad hospitals, that indicates of contamination of wounds and infection. All isolates were tested for susceptibility by using 15 antibiotics, the results showed that all of these isolates are resistant to many antibiotics [25].

This study also found that the synergistic effect of essential oils from three plants mixed were inhibited bacterial growth with a significant difference more than the essential oil of one plant alone or the synergistic effect of two plants mixed. The area of burned is susceptible to infection and invasion of microorganisms especially bacteria, many of pathogenic isolates have antibiotics resistance acquired [26].

Burns infection by bacteria were caused more than 75% of deaths are a result of wound. Knowing the species of bacteria that invade and contaminate wounds and burns is very important [27].

In certain study, the proportions of isolated bacteria were as follows: *A. baumannii* 15.38%, E. coli 8.46%, *K. pneumonia* 13.85%, *P. mirabilis* 4.42%, *Pseudomonas* spp. 18.46%, *S. aureus* 33.85% [28]. While the rates of bacterial resistance to antibiotics were as follows: *S. aureus* for amoxicillin/clavulanic acid 84.09%, ampicillin 88.63%, cefoxitin 56.81%, ciprofloxacin 54.54%, erythromycin 54.54%, fusidic acid 52.27%, ofloxacin 63.63%, penicillin 86.36% [29]. Whereas *P. aeruginosa* for amoxicillin/clavulanic acid 95%, cotrimoxazole 75%, methicillin 86.36%, piperacillin 80%, rifampicin 65%, ticarcillin 100%, ticarcillin/clavulanic acid 100% [30].

While *A. baumannii* for amoxicillin/clavulanic acid, ciprofloxacin, cotrimoxazole, levofloxacin, piperacillin, piperacillin/clavulanic acid, ticarcillin, ticarcillin/clavulanic acid and tobramycin 100% [31]. Whereas *K. pneumoniae* and *Enterobacteriaceae* spp. for amoxicillin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, ceftriaxone, cephalothin, ciprofloxacin, cotrimoxazole and ticarcillin 100%. The risk of bacteria resistance to antibiotics does not mean that the bacteria are resistant to every antibiotic, but the risk remains possible even if the bacteria are resistant to only one antibiotic [32].

Knowing and studying the synergistic effect of volatile oils and identifying its components is very important to determine the maximum use of its antibacterial efficacy and the minimum required concentration that inhibits the growth of pathogens to use for medicinal, therapeutic and health purposes [33]. Depending on experts' opinions, the antibiotic period will end quickly, thus, the benefit of detection of alternatives for antibiotics is important. Safe alternatives of antibiotics for microbial resistance problem, it will be plant extracts, volatile oils particularly, due to it has very low harmful or side effects. The volatile oil of thyme or lemon contains abundant amounts of terpenoids and terpenes. These compounds are highly effective against many species of microorganisms, including severe resistant strains and adaptability in different conditions and environments [34].

CONCLUSION

This research was to test the action of 3 EOs against pathogenic bacteria isolated from wounds and burns, and evaluating the effect of an alternative treatment from these plant extracts as well as resistance to antibiotics.

The essential oils of *A. citrodora, R. officinalis* and *T. vulgaris* were extracted and prepared in serial dilution concentrations. The results showed that *Pseudomonas* spp. were the most frequent as 51

isolates, while *Enterococcus* spp. and *Rhizopium* spp. were the least as 1 isolate.

A susceptibility test was conducted by using 15 different antibiotic discs. Trimethoprim was the most resistant antibiotic for bacterial isolates 79.33%, while Imipenem was the least 40%.

Moreover, the susceptibility test was revealed the most resistant was *Rhizopium* spp. with 86.67% and *Enterococcus* spp. was least with 20%. The highest antibiotic resistant isolates 22 were selected for determining the synergistic effect of volatile oils that inhibited of bacterial growth, the concentration of significant synergistic effect was 156.25, 156.25 and 39.0625 ppm of *A. citrodora, R. officinalis* and *T. vulgaris* respectively.

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Conflict of interest: The authors was advertising no discrepancy of benefit (it is nil).

Author contribution: Authors contributed equally in the study.

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