



Biotechnology

DOI: 10.48402/IMIST.PRSM/jasab-v2i2.23840

Antibacterial activity of the Essential oils against multiresistant bacterial strains isolated from hospital

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ARTICLE INFO

Received November 16th, 2020

Received in revised form December 25th, 2020

Accepted December 26th, 2020

Keywords:

Essential oils,
MIC,
MBC,
T. ritarum,
R. officinalis,
antibacterial activity.

ABSTRACT

Due to increasing bacterial resistance, the research for antimicrobials of plant origin has been an important alternative. Thus, this study aimed to determine the antibacterial effect of essential oils from *T. ritarum*, and *R. officinalis* against 17 bacterial pathogens responsible for nosocomial infections isolated from hospitalized patients. The antibacterial activity was assessed by means of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microdilution method. The essential oils of *R. officinalis* produced high inhibition zones against *Micrococaceae* especially towards *Staphylococcus aureus* strains (25 mm and 30 mm), in comparison, *T. ritarum* essential oils presented a moderate effect with inhibition diameters ranging from 9 mm to 15 mm. Overall, the *T. ritarum* and *R. officinalis* E.Os posses a significant antibacterial activity against 17 bacterial pathogens representing the most common pathogenic microorganisms, particularly, *A. baumannii* and *staphylococcus* strains which have been the most sensitive to *T. ritarum* oils, on the other hand, the most sensitive strains towards *R. officinalis* have been Gram (+) *streptococci* and *E. coli* for Gram (-) bacteria. This study emphasizes the high beneficial effects of the essential oils of *T. ritarum* and *R. officinalis* in inhibiting the growth of bacteria responsible for nosocomial infections. These EOs can be used to develop new therapeutic protocols for resistant infectious diseases.

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1. Introduction:

Antibiotics are critical tools for fighting life-threatening infections among humans and animals, unfortunately, they are losing their effectiveness because of the emergence and spread of antibiotic resistance. The resistance of antibiotics is a serious problem worldwide. Antibiotic resistance limits treatment options and increase therapeutic failure [1]. Currently, people around the world, including the most developed countries, are dying from infections for which, we could not respond to available treatment. In America, according to a report from the Centers for Disease Control and Prevention (CDC), more than 35,000 people die each year by a serious infection caused by antibiotic-resistant bacteria [2]. A similar study in the UK on antimicrobial resistance (AMR) estimates that 700,000 people die each year worldwide from the same infections [3]. Thus, AMR is considered to be a serious and worsening to health and one of the greatest global public health challenges of our time [1]. To address this threat and to save lives, it is necessary, in addition to the improving

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infection prevention and antibiotic use, to develop new antibiotics to treat highly resistant bacterial infections and to bring medicine to the patients who need it [4]. New antibiotics are an important piece of the fight against antibiotic resistance, but finding new therapies to overcome these pathogens is particularly challenging, so, it is judicious to improve use of the effective antibiotics available today and enhance molecules we have at our disposal.

Aromatic and medicinal plants have been occupying a major role in treatment of diseases caused by bacterial infections. In fact, the use of such material for healing purposes has been reported to be a fundamental component in traditional medical practices from prehistoric times, the dark ages, all the way to modern times [5]. A key factor in traditional therapy involves the use of essential oils, they are considered to be a key ingredient allocating the ability to medicinal plants to combating bacterial infections [6]. Essential oils (E.Os) are volatile components which can be derived from different parts of plant material such as flowers, leaves, seeds [7]. Previous studies reported the antimicrobial, antifungal, anti-inflammatory, anti-parasitic, and insecticidal activities of E.Os [8-13]. However, their antibacterial properties remains to be at the forefront of the growing interest E.Os has known in the past years [14].

The antibacterial activity of E.Os is attributed to their diverse molecular structure. The presence of an assortment of compounds belonging to different chemical classes (Hydrocarbons, Lactones, Alcohols, Phenols and Aldehydes) make E.Os capable of attacking bacterial cells on different sites, in fact several studies have reported the antimicrobial mode of action of major essential oil components on multidrug-resistant bacteria, for instance Das et al. [15], illustrated how Eugenol, a common phenylpropanoid found in essential oils can penetrate the outer membrane of multidrug resistant *Staphylococcus aureus* and disrupt its morphology by creating fragments and inducing membrane permeability. Therefore, causing a severe leakage and damage to membrane transport chains. Lambert et al. [16] proved that in addition to causing membrane impairment, thymol and carvacrol are also capable of dissipating the intracellular PH levels of *Pseudomonas aeruginosa* and *Staphylococcus aureus* as well as resulting in inorganic ions leakage. Alves et al. [17] reported that the antibacterial effect of linalool against *Acinetobacter baumannii* is associated with its ability to control biofilm formation, prevent bacterial adhesion to surfaces and inhibit Quorum Sensing (QS). It has also been reported that other essential oil components such as cinnamaldehyde, Linalylacetat and (+)-Menthol can interfere with critical sites for antibacterial activity and inhibit QS in *Escherichia coli* [18,19] whilst allicin can penetrate to the intracellular space of *Salmonella typhimurium* and interact with thiols along with inhibiting RNA synthesis [20,21]. A very considerable number of medicinal and aromatic plants are recognized to be producing species of these bioactive compounds, among these species we find: *Thymus riatarum* [22] as well as *Rosmarinus officinalis* [23].

In conclusion, the high level of heterogeneity in bio-active constituents make essential oils a reservoir of novel potent antibacterial drugs capable of revolutionizing the medical field's response towards the growing number of drug-resistant isolates. Thus, the objective of this study is to elucidate the antibacterial effect of two essential oils extracted from medicinal plants traditionally used in Moroccan folklore medicine: *Thymus riatarum* and *Rosmarinus officinalis*, against bacteria responsible for nosocomial infections isolated from hospitalized patients.

2. Material and methods:

2.1. Plant preparation and essential oil extraction:

The leaves of *T. riatarum* and *R. officinalis* were collected during the period extending from July to November in 2019 from the mountainous Rif region in north Morocco. Plant leaves were left to dry in the dark at room temperature, they were later introduced to a round bottom flask with 700 ml of distilled water and submitted to 4 to 6 hour long hydrodistillation extraction process using a Clevenger-type apparatus. The obtained oils were dried over anhydrous sodium sulfate and, stored in a sealed sample tube at 4°C until further use [24].

2.2. Sample Collection:

17 multidrug-resistant strains of Gram-positive cocci, Enterobacteriaceae and non-fermenting Gram-negative bacilli were isolated from blood, endotracheal aspirations, pus and urine of patients hospitalized in the Mohammed V regional hospital in Tangier, Morocco. These multidrug-resistant strains had been tested against the antibiotics from the families of Quinolones (Ciprofloxacin, Levofloxacin), Cephalosporins of 1st 2nd and 3rd generations (Clindamycin (CL), Cefotaxime (CTX), Ceftriaxone (CRO) Ceftazidime (CTZ) and Cefepime (CEP), Triméthoprim-Sulfaméthoxazole (SXT) and an aminoside (Tobramycin).

2.3. Antibacterial activity:

2.3.1. Evaluation of antibacterial potential of essential oils using the disc diffusion method:

The antibacterial activity of E.Os from *T. riatarum* and *R. officinalis* were assessed against multidrug resistant bacteria using the disc diffusion method, also known as the "Kirby-Bauer method" as a preliminary assay [25]. The protocol consists of introducing 100 µl of an overnight bacterial culture (1.5×10^8 CFU/ml) to the surface of Petri-dishes containing 20ml of Mueller-Hinton agar (MHA), the inoculum is spread along the surface in order to obtain a uniform bacterial lawn

throughout the Petri dish. Sterile filter discs (Whatman No. 1 with 6mm in diameter) containing essential oil (20µl/disc) are added. The plates are then covered immediately in order to prevent E.Os evaporation and incubated at 37°C for 18-24h. After incubation, the efficiency of the E.Os towards the tested microorganisms is expressed as “inhibition zone” around the disc and is measured in mm.

2.3.2. Resazurin-based microdilution method for the determination of minimum inhibitory and bactericidal concentrations:

The resazurin-based microtitration assay was performed on all strains that displayed a sensitivity towards the selected E.Os according to the protocol described by Mann & Markham with modification [26]. Briefly, E.Os were dissolved in DMSO (non lethal dose) to yield a stock solution at an initial concentration of 40µl/ml. Serial Two-fold dilution in sterile BHI (Brain-Heart Infusion broth) was then carried out until the desired concentration series is reached (20µl/ml, 10µl/ml, 5µl/ml, 2.5µl/ml). Aliquots of 180 µl of each dilution is pipetted into wells of a 96-well microtitration plate and supplemented with 20µl of bacterial suspensions (1.5×10^8 CFU/ml). 20 µl of diluted bacterial inoculums in 180 µl of BHI broth, and 200 µl of sterile medium broth were used as controls. After 48h of incubation at 37°C, The MIC is determined by adding 30µl of resazurin sodium salt (0.01%) and further incubating the stained plates for 15 min. The MIC corresponds to the lowest concentration of essential oil that does not produce a change in resazurin staining and therefore corresponds to the absence of any bacterial growth. The MBC is identified as the minimum concentration of the E.Os that does not produce any colony growth. It is determined by directly transferring the content of wells with concentrations above the MIC value into BHI agar [27].

3. Results and discussion:

3.1. Disc diffusion assay:

According to the results obtained, the E.Os from *T. riatarum* and *R. officinalis* revealed a significant effectiveness in inhibiting the growth of all strains at varying spectra. Table1 summarizes the results obtained which are expressed as a mean of inhibition zones ± standard deviation.

R. officinalis E.Os have been mostly active against *Micrococaceae* especially towards *Staphylococcus aureus* strains with inhibition zones reaching as high as 25 mm and 30 mm, in comparison, *T. riatarum* essential oils presented a moderate effect with inhibition diameters ranging from 9 mm to 15 mm.

Regarding the Gram (-) *Enterobacteriaceae*; *K. pneumoniae 12* has been the most sensitive strain against the actions of *T. riatarum* (35 mm), followed by *K. pneumoniae 8_s* (20mm) and *K. pneumoniae 17_s* (15 mm). As for *Escherichia* strains, *E. coli 15_s* exhibited the highest sensitivity for *R. officinalis* E.Os (25mm). The results for non fermenting Gram (-) bacilli revealed that *T. riatarum* E.Os was the most active towards the tested strains (*A. baumannii* and *P. aeruginosa*) generating a suppressive effect estimated at 23 and 10 mm respectively, which is considered to be a higher inhibition rate compared to *R. officinalis* E.Os (11 mm and 5 mm).

Table 1. Diameters of the inhibition zones of the tested essential oils in (mm).

Type	Family	Strain	<i>T. riatarum</i>	<i>R. officinalis</i>
Gram (+)	<i>Micrococaceae</i>	<i>Staphylococcus aureus 1_s</i>	13	30
		<i>Staphylococcus aureus 2_s</i>	15	30
		<i>Staphylococcus aureus 3_s</i>	11	25
		<i>Streptococcus D10_s</i>	9	5
		<i>Streptococcus D11_s</i>	11	10
		<i>Enterococcus faecalis</i>	11	11
Gram (-) Bacilli	<i>Enterobacterales</i>	<i>Escherichia coli 9_s</i>	16	15
		<i>Escherichia coli 13_s</i>	10	4
		<i>Escherichia coli 14_s</i>	7	3
		<i>Escherichia coli 15_s</i>	10	25
		<i>Escherichia coli 16_s</i>	10	5
		<i>Klebsiella pneumoniae 6_s</i>	11	9
		<i>Klebsiella pneumoniae 12_s</i>	35	10
		<i>Klebsiella pneumoniae 17_s</i>	15	11
		<i>Klebsiella pneumoniae 8_s</i>	20	15
	Non fermenting Gram-negative Bacilli	<i>Acinetobacter baumannii</i>	23	11
<i>Pseudomonas aeruginosa</i>		10	5	

3.2. Microtitration assay:

The MIC and MBC results are presented in Table 2 and Table 3. In direct correlation with the preliminary assay. *T. ritarum* E.Os exhibited a strong effect towards gram-negative bacteria *A. baumannii* with a capability to suppress its growth at 2,5µl/ml, this concentration is the lowest active concentration obtained throughout all the screenings. In most cases, the MBC values of *T. ritarum* E.Os were double or four times their minimum inhibition concentrations. Most *E. coli* strains have been inhibited with 5µl/ml oil concentration whilst it took concentrations of 10µl/ml to 20µl/ml in order to eliminate all bacterial presence. A similar sensitivity profile is observed regarding the gram positive strains *S. aureus* and *E. faecalis* whilst streptococci could only be inhibited by concentrations ranging from 10µl/ml to 20µl/ml.

K. pneumoniae and *P. aeruginosa* have both presented a tolerance to the tested concentrations with the exception of *K. pneumoniae* 17_s (MIC at 10µl/ml and MBC of 20µl/ml), indicating a need for volumes higher than 20µl/ml to eliminate the strains. In regards to *R. Officinalis* E.Os, the tested bacterial strains showed varying responses in the examined concentration range. The most sensitive strains are *Streptococcus* D10_s and *K. pneumoniae* with MICs of 2.5µl/ml followed by *Streptococcus* D11_s, and *E. coli* strains 9_s, 13_s and 15_s with MICs of 5µl/ml, *S. aureus* 2_s, *S. aureus* 3_s and *P. aeruginosa* with MICs of 10 µl/ml.

Table 2. Minimal inhibitory and minimal bactericidal concentrations of *Thymus ritarumin* (µl/ml).

Strain		<i>Thymus ritarum</i>	
		MIC	MBC
Gram-positive cocci	<i>Staphylococcus aureus</i> 1 _s	5	20
	<i>Staphylococcus aureus</i> 2 _s	5	10
	<i>Staphylococcus aureus</i> 3 _s	5	20
	<i>Enterococcus faecalis</i> 4 _s	5	20
	<i>Streptococcus</i> D10 _s	10	20
	<i>Streptococcus</i> D11 _s	20	>20
Gram-negative Bacilli	<i>Pseudomonas aeruginosa</i> 5 _s	> 20	>20
	<i>Klebsiella pneumoniae</i> 6 _s	20	>20
	<i>Klebsiella pneumoniae</i> 12 _s	20	>20
	<i>Klebsiella pneumoniae</i> 17 _s	10	20
	<i>Klebsiella pneumoniae</i> 8 _s	>20	>20
	<i>Escherichia coli</i> 9 _s	5	10
	<i>Escherichia coli</i> 13 _s	5	10
	<i>Escherichia coli</i> 14 _s	10	20
	<i>Escherichia coli</i> 15 _s	5	20
	<i>Escherichia coli</i> 16 _s	10	20
<i>Acenitobacter baumannii</i> 7 _s	2.5	2.5	

At 20µl/ml. *R. Officinalis* E.Os were able to inhibit the growth of gram negative bacteria *K.pneumoniae* 17_s and 18_s, *E. coli* 16_s and *A. baumannii*. *S. aureus* 1_s has been the only strain to be inhibited at 40µl/ml. The results obtained in the present study confirm that *T. ritarum* and *R. officinalis* E.Os possess a significant antibacterial activity against 17 bacterial pathogens representing the most common pathogenic microorganisms. *A. baumannii* and *staphylococcus* strains have been the most sensitive strains to *T. ritarum* oils, on the other hand, the most sensitive strains towards *R. officinalis* have been Gram(+) *streptococci* and *E. coli* for Gram (-) bacteria.

The obtained antibacterial resistance profile is not in accordance with the antibiotic sensitivity testing, which is a clear indicator that the bacterial sensitivity to E.Os is not correlated with its antibiotic resistance profile. For instance, Gram (-) strain *P. aeruginosa* showed a multiresistance towards antibiotics from the families of Quinolones (Ciprofloxacin, Lévofoxacin), Cephalosporins of 1st and 3rd generations (Clindamycin (CL), Céfotaxime (CTX), Ceftriaxone (CRO) Ceftazidime (CTZ) and Céfépime (CEP), Triméthoprime-Sulfaméthoxazole (SXT) and an aminoglycoside (Tobramycin), while it has represented a sensitivity to *R. officinalis* oils with an MIC of 10µl/ml. In turn it appears that all of the tested *K. pneumoniae* strains are resistant to antibiotics belonging to the beta lactamase family whereas most of them have revealed a sensitivity when exposed to sublethal doses of the tested oils (2,5µl/ml and 20µl/ml of Rosmarinus oil for *K. pneumoniae* 12_s and *K. pneumoniae* 17_s; 20µl/ml and 10µl/ml of Thymus oil as MIC for the same strains respectively).

The same conclusion has been observed by other authors [28,29]. *A. baumannii* is known to be one of the main causes behind nosocomial infection, it is also considered to be an opportunistic pathogen mostly occurring in patients with a compromised immunity, it has also been revealed that this strain is capable of developing a resistance to antibiotics at an outstanding rate [30,31].

Table 3. Minimal inhibitory and minimal bactericidal concentrations of *Rosmarinus officinalis* in ($\mu\text{l/ml}$).

Strains		<i>Rosmarinus officinalis</i>	
		MIC	MBC
Gram (+) Cocci	<i>Staphylococcus aureus</i> 1 _s	40	>40
	<i>Staphylococcus aureus</i> 2 _s	10	>40
	<i>Staphylococcus aureus</i> 3 _s	10	>40
	<i>Streptococcus</i> D 10 _s	2.5	>40
	<i>Streptococcus</i> D 11 _s	5	>40
	<i>Enterococcus faecalis</i> 4 _s	>40	>40
Gram (-)Bacilli	<i>Pseudomonas aeruginosa</i> 5 _s	10	>40
	<i>Klebsiella pneumoniae</i> 6 _s	>40	>40
	<i>Klebsiella pneumoniae</i> 12 _s	2.5	>40
	<i>Klebsiella pneumoniae</i> 17 _s	20	>40
	<i>Klebsiella pneumoniae</i> 8 _s	20	20
	<i>Escherichia coli</i> 9 _s	5	>40
	<i>Escherichia coli</i> 13 _s	5	>40
	<i>Escherichia coli</i> 14 _s	>40	>40
	<i>Escherichia coli</i> 15 _s	5	>40
	<i>Escherichia coli</i> 16 _s	20	>40
	<i>Acenitobacter baumannii</i> 7 _s	20	>40

A. baumannii showed the highest antibiotic resistance profile (CIP/CTX/SXT/CAZ/CEM/LEV/and TOB) however, it was the most sensitive against *T. riatarum* oils (2,5 $\mu\text{l/ml}$ for both MIC and MBC), this extreme susceptibility may be attributed to the difference in the strain's membrane structure [32].

E. coli strains (specifically *E. coli* 13_s and *E. coli* 14_s) have presented an equal sensitivity to both oils even though they acquired a resistance to at least one antibiotic from the beta-lactamin family. As for Gram (+) cocci, while all of the tested staphylococci have been resistant to penicillin, kanamycin and Erythromycin, they nonetheless showed a significant sensitivity to both E.Os with MIC and MBC values ranging from 5 $\mu\text{l/ml}$ to 20 $\mu\text{l/ml}$ for Thymus and 10 $\mu\text{l/ml}$ to 40 $\mu\text{l/ml}$ for *Rosmarinus*. Our study is in accordance with Aouan et al. [33] which exhibited the antibacterial effects of *T. riatarum* essential oils against *E. coli*, *K. pneumonia*, *S. aureus* and *A. baumannii*. It has been argued that the main cause for the high antibacterial effect *T. riatarum* E.Os have on microorganisms could be attributed to the presence of borneol as a major component, in fact the percentage of borneol in *T. riatarum* E.Os could reach up to 30 or 40% [34,35]. In addition. Other components such as linalool might also contribute to the E.Os antibacterial effects. The use of linalool can result in permeabilization and disruption of the bacterial membrane therefore enhancing antibiotic penetration [36]. Our results demonstrated a higher antibacterial effect for *R. officinalis* E.Os then that reported by Mekonnen et al. [37] for *S. aureus* (25mm \pm 1,23) and *E. coli* (6 mm \pm 0,21), the contrast observed between both studies could be related to the qualitative and quantitative difference in the chemical composition of plants used in both studies. Several factors interfere in the occurring of different chemotypes of the same species such as endogenous factors (the selection of plant part for E.Os extraction, plant tissue and development stage), and exogenous factors (light, soil, growing site, precipitation and seasonal variations) [38]. This observation has been previously demonstrated by Celiktas et al. [39] who stated that *R. officinalis* E.Os effect on bacterial strains differed in relation to the location and seasonal variations.

4. Conclusion:

The results obtained in this study displayed that both *T. riatarum* and *R. officinalis* essential oils possess antimicrobial activities. The essential oil from *T. riatarum* showed the lowest MIC value against all the tested strains, the *R. officinalis* E.Os have been mostly active against *Micrococaceae* especially towards *Staphylococcus aureus*. These essential oils can be used for application of new therapeutic protocols for resistant infectious diseases. However, to complete this study, it is necessary to know the chemical components of *T. riatarum* and *R. officinalis* E.Os.

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