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Antibacterial, Wound healing Activities, and GC-MS analysis of *Myrtus communis*

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KEYWORDS

MDR bacteria, Myrtus communis, Phytochemical analysis, Wound healing.

ABSTRACT:

Infectious diseases represent a high-risk factor for mortality among the general population, especially among immunocompromised patients, especially with the increasing rate of infections caused by multi-drug resistant (MDR) bacteria. On the other side, natural products are preferred over synthetic drugs for their availability, and cheapness, and possessing fewer side effects compared to synthetic drugs. Therefore, researchers have been motivated to develop new antibacterial drugs, especially due to the constant emergence of resistant bacteria to the conventionally used antibacterial agents. This study aimed to satisfy 4-objectives (1) assess the in vitro antibacterial potential of the Libyan Myrtus communis leaves extract against MRSA, Pseudomonas aeruginosa, and Acinetobacter baumannii clinical isolates and (2) assess the in-vivo potential of the extract to treat wounds infected with MDR Pseudomonas aeruginosa, and to (3) phytochemically analyze the extract and (4) Study the effect of long storage period of the extract on its performance against tested bacterial isolates. Standard Disc agar diffusion method and animal model were used for the in-vitro and in-vivo screening, respectively. Traditional phytochemical and GC-MS analyses were used for phytochemical screening. Different antibiotic classes were used for comparison. The results proved that the tested extract has good in-vitro and in-vivo antibacterial activity against MDR bacteria. Flavonoids, Saponins, Tannins, Quinic acid Myrtenol, and fatty acids were found within the extract constituents and suggested as responsible for the good shown activity. The results conclude and introduce Myrtus communis as a resource for promising antibacterial agents having good potential against MDR bacteria.

Introduction:

The world is aware of the danger of the spread of bacterial resistance to antibiotics and the resulting suffering and difficulty in treating infectious diseases caused by these bacteria. This has become a threatening factor for increasing the rate of disease and mortality worldwide. Microbes in general and bacteria in specific may be either harmless or extremely harmful causing infections that are difficult to treat especially those

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caused by multi-drug resistant (MDR) bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* are the most worldwide problematic pathogenic bacteria that exhaust treatment efforts with their rapid emergence of resistance to the commonly pre and newly discovered used antibiotics (1). This rapidly increasing rate of bacterial resistance to antibiotics causes an increasing rate of infectious disease and consequently an increasing rate of morbidity and mortality. These bacterial types are already addressed as nosocomial pathogens that threaten human health and they are responsible for many infectious diseases that if not controlled lead to death, especially in immunocompromised patients, especially with their pronounced ability to form biofilms (2-5). This situation motivates researchers, and pharmaceutical companies to develop new antibacterial drugs, and because of the high costs and undesirable side effects of synthetic drugs, natural remedy resources become preferable and traditionally widely used for their availability and cheapness, and also for their fewer side effects (6)

Natural remedies with herbs are one of the oldest uses known to mankind related to health and well-being and are considered an important source of novel therapeutic agents of good biological activities such as antioxidants, chemotherapeutic agents, anti-hypertensive, antifungal, antiviral, and antibacterial agents (7, 8). It has been documented that many of the plant's secondary metabolites such as alkaloids, flavonoids, tannins, terpenes, quinines, and resins are with less adverse effects compared with used synthetic drugs, and have the potential for many therapeutic activities including antibacterial activity against both human pathogenic Gram-negative and Gram-positive bacteria (9, 10) *Myrtus communis* is one of these plants, aromatic in character, belonging to a big family named Myrtaceae, and comprises more than 5500 species, of which about 16 species are native to the Middle East, and North Africa, where used traditionally as antiseptic, hypoglycaemic, and hypotensive agent, and also in the treatment of some infections (11, 12). **In a step done** by the corresponding author for this article in 2018 within steps in a thesis submitted to fulfill the requirements of the degree of philosophy of doctorate, *Myrtus communis* was tested for its potential to control *MRSA*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* clinical isolates isolated from Libyan patients admitted to a local hospital. The results showed pronounced growth inhibition activity against the three tested bacteria. **As a continuation of the tests conducted in 2018, this study aimed to satisfy three objectives**: (1) to evaluate the *in-vitro* antibacterial activity of this plant collected in 2023 and compare it with that showed from the same plant collected and tested in 2018 against the same tested bacteria, (2) to test if the extract that used in 2018 and stored for about 4-years is still retaining the pronounced activity shown in 2018, and (3) to study the plant *in-vivo* potential (topical) in treating wounds infected with *Pseudomonas aeruginosa* pathogen.

Materials and Methods:

Plant Material: Leaves of the *Myrtus communis* plant were collected twice, first in August 2018, and second in August 2023 from around Al-Bayda city located in the Al Jabal Al Akhdar region, northeast of Libya. The plant was identified and classified by the specialists at the Botany Department, Faculty of Science, Omar Al-Mukhtar University, Libya. Plant leaves were cleaned, air-dried at room temperature, powdered, and kept in well-closed glass brown bottles.

Clinical bacterial isolates: Three different clinical isolates; *MRSA*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* previously proved as MDR species were included in this study. These three isolates are the same that had been tested in 2018 for their sensitivity to the same targeted plant; *Myrtus communis* leaves. Three isolates were stored in 80% glycerol at -20°C.

Reference Antibiotics Discs:

Amoxicillin 25 µg, Amoxicillin + Clavulanic acid (30µg), Cefotaxime/Sulbactam (40 µg), Ceftazidime 30 µg, Ceftriaxone 30 µg, Ciprofloxacin 30 µg, Imipenem 10 µg, Meropenem 10 µg, Gentamicin 10 µg and Vancomycin 30 µg (VA) are ten standard antibiotics discs manufactured by Bioanalyse@ YSE TibbiMalzemeler San, and used as references in this study. They were purchased from companies selling medicines and medical equipment in Libya.

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Experimental Animals:

Fifteen experimental Swiss Albino Rats weighing 180-200gm were used in this study. Animals were housed separately, and given standard feeding and tap water.

Traditional Phytochemical Screening:

For the active constituents of *Myrtus communis* leaves; Alkaloids, Anthraquinone, Coumarins, Flavonoids, Saponins, Steroids, Tannins, and Triterpenes, were carried out using the methods used by authors cited in(7).

GC-MS analysis:

Of tested extract was analyzed using a Shimadzu GC-MS (Model QP2010-Ultra, Japan) equipped with an Rtx-5MS capillary column (30 m × 0.25 mm × 0.25µm). An electron ionization system with an ionization energy of 70 eV was used to detect the chemical constituents. Helium was used as a carrier gas at a flow rate of 1.61 ml/min, and the mass transfer line and injector temperature were set at 250 and 300 °C, respectively. The temperature program was started from 60 °C with a rate of 10 °C/min to 300 °C as the final temperature degree with a 10-minute hold time. The sample (1 µl) was injected in the split mode with a split ratio of 120:1, the delay time was 2 min and the total running time was 29 min. 23 Identification of constituents for the sample was achieved by comparing their retention index and mass fragmentation patents with those available in the library, the National Institute of Standards and Technology (NIST).

***In-vitro* antibacterial assay:**

Preparation of plant extract: Forty grams of *Myrtus communis* leaves were powdered and thoroughly extracted with enough quantities (150 – 200 ml) of Methanol with the use of a Soxhlet apparatus, the solvent was then evaporated under reduced pressure with the rotary evaporator, and the extract was air-dried and kept in a clean colored tightly closed bottle at 4°C. For the antibacterial assay, a fresh extract solution of a concentration of 100mg/ml was prepared by dissolving 0.2g of the extract in 2 ml of DMSO 10%.

Preparation of bacterial suspension: An overnight nutrient agar growth of each of the three tested isolates was harvested and dissolved in sterile normal saline 0.9% and calibrated with McFarland 0.5 solution.

Antibacterial screening assay: The disc agar diffusion method was used for the determination of the antibacterial activity for both fresh and old plant extracts. Duplicate sterile Discs, 6 mm in diameter (Wattman paper N°1 - Selecta, Germany), after being soaked with 20 µl of a solution of tested extract were placed on Mueller-Hinton agar plates 5 minutes previously seeded with 100µl of the tested bacterial suspension. The plates were incubated at 37°C for 24 hours. The diameters of the inhibition zone were measured in mm. The disc agar diffusion method was also used for testing the susceptibility of tested isolates to the tested antibiotic discs.

Minimum Inhibitory Concentrations (MICs): The micro-dilution method was used to determine the MIC according to the National Committee for Clinical Laboratory Standards Guidelines, 1999 (13), with modifications. Twofold serial dilution of crude extract was prepared directly in a microtiter plate containing Mueller Hinton broth (MHB) to obtain various concentrations (50 – 0.39 mg/ml). The bacterial inoculum was added to each well. Two controls were used (control A contains media and bacteria, and control B contains only media). The plate was covered with a sterile cover and incubated for 24 h at 37°C. Resazurin (20µl) was added to each well of the microtiter plate and incubated at 37°C for 3-4 hrs. The wells containing the bacterial growth turned pink, whereas the wells without bacterial growth remained blue. The MIC was defined as the lowest sample concentration showing no color change (clear) and exhibiting complete inhibition of growth (14, 15).

***In-vivo* antibacterial assay (Topical):**

Pharmaceutical dosage form preparation: To prepare a 100gm ointment of a concentration of 3% of *Myrtus communis* extract, a mix of emulsifying wax (30gm), white soft paraffin (50gm), and Liquid paraffin (20ml) was put in a Pyrex beaker with magnetic stiror on a heater (Hot plate magnetic stiror) at 60 Cº. After all ingredients melted, were poured into a mortar containing 3gm of the extract powder. The mix was mixed well with a glass rod until it got a homogenized

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mixture. The ointment preparation was kept in a clean dry tight container at 4 ºC.

Preparation of wounded animal model: In this assay, fifteen (15) Swiss albino rats were included. A full-thickness wound of was made in the skin of each rat. The hair of the lower back and right flank of animals was fully shaved. Rats were lightly anesthetized by inhalation using halothane. The animals were held in a standard crouching position, and the mobile skin of the flank was gently stretched and held by fingers. A metal circular object measuring 1 cm in diameter was placed on the stretched skin and an outline of the object was traced on the skin using a fine-tipped pen. The wound was made by excising the skin within the border of the object to the level of loose subcutaneous tissue, using sterile forceps and a scalpel blade. The artificial wounds were circular, with a diameter of 1 cm. The first day of the experiment was regarded as the Zero day.

***In vivo* (Topical antibacterial assay):** In this essay, the 15 included prepared rates were divided into three groups each containing 5 rats named G1, G2, and G3. The wounds of all rats were infected with an inoculum of *Pseudomonas aeruginosa* ATCC27853 strain suspension freshly adjusted with 0.5 McFarland standard (10^8-10^9 C.F.U. /ml). The first group (G1) was designed as a non-treated group, where infected wounds were left without treatment, only cleaned with alcohol 70% twice daily. The second group; G2 was designed as the standard treated group, where wounds were treated topically with Tetracycline ointment 3% (bought from a local private pharmacy), while the third group, G3 rats were treated topically with the prepared dosage form; *Myrtus communis* extract 30% ointment. Both Tetracycline ointment 3%, and extract ointment 3% were applied every 12 hours starting from the first day. Means of diameters and standard deviation of all wounds were measured once daily (every 24 hours) and percentages of healing areas were evaluated.

Statistical Analysis: Data were expressed as mean ± SD. Statistical examination was performed utilizing SPSS version 21, One-way analysis of variance (ANOVA) followed by the LSD Post Hoc test.

Results:

***In-vitro* antibacterial screening:** In this study screening of the ability of *Myrtus communis* extract to control the tested MDR bacteria was done three times: in 2018 (old fresh extract: OFE), in 2023 (new fresh extract: NFE), and also, retesting of the extract that had tested in 2018 after about 4-years storage period (old stored extract" OSE). The results cleared that NFE and OFE exhibited equal inhibition zones of 25 ± 0.12mm and 25 ± 0.15 mm, respectively against MRSA, while OSE revealed 24 ± 0.20 mm inhibition zone. For tested *Pseudomonas aeruginosa*, the results said that NFE, OFE, and OSE revealed an inhibition zone of 20 ± 0.10, 21 ± 0.20, and 20 ± 0.10 mm, respectively against this bacterium. However, for the tested *Acinetobacter baumannii*, the study outputs proved that NFE, OFE, and OSE revealed inhibition zones against this bacterium of 21 ± 0.10, 23 ± 0.16, and 22 ± 0.20 mm, respectively (Table 1 & Figure 1).

Table (1): Screening of antibacterial activity of *Myrtus communis* extract against tested clinical bacteria

Isolated bacterial tested	FE2018 mg/ml	Old stored extract (OSE) mg/ml	FE2023 mg/ml
	Diameter of Inhibition Zone (MDIZ*)		
MRSA	25 ± 0.12	24 ± 0.20	25 ± 0.15
Ps. aeruginosa	21 ± 0.20	20 ± 0.10	20 ± 0.10
A. baumannii	23 ± 0.16	22 ± 0.20	21 ± 0.10

MDIZ* (mm) = Mean diameter of growth inhibition zone in mm. Interpretation of results: MDIZ (mm): <9mm zone was considered as inactive; 9-12mm as partially active; while13-18mm was active and >18mm

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was very active, the concentration used 100 mg/ml. **FE2018** = Fresh Extract 2018, **FE2023** = Fresh Extract 2023, **OSE**= Old Stored Extract.

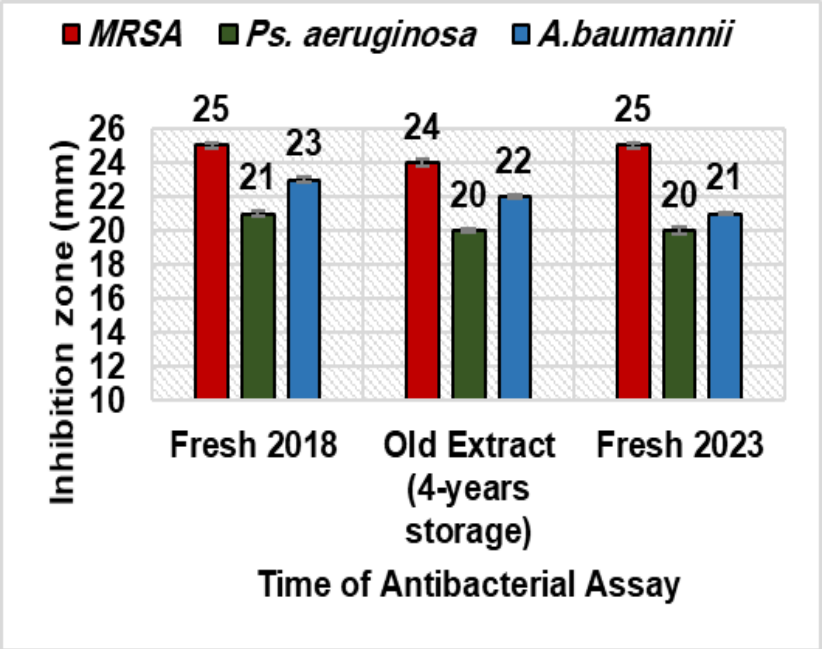

Figure (1): Antibacterial activity of *M. communis* extract against tested bacteria

Minimum inhibitory concentration determination:

When the MICs of the tested extracts were investigated in this study, the results showed equal MICs of 6.25mg/ml for the three extract against *Pseudomonas aeruginosa*, lower equal MICs of ≤ 0.39mg/ml for both OFE and NFE against MRSA, and 0.78mg/ml from OSE against MRSA. For *Acinetobacter baumannii* both OFE and NFE revealed an equal MICs of 6.25mg/l, while OSE, showed a lowest minimum inhibitory concentration of ≤ 0.39 mg/l (Table 2 and Figure 2).

Table (2): Minimum inhibitory concentrations of *M. communis* extract against clinical bacteria

Isolated bacterial tested	**Minimum Inhibitory Concentration (mg/ml)**		
	Fresh 2018	**Old Extract (4-years storage)**	**Fresh 2023**
MRSA	≤ 0.39	0.78	≤ 0.39
Ps. aeruginosa	6.25	6.25	6.25
A. baumannii	6.25	≤ 0.39	6.25

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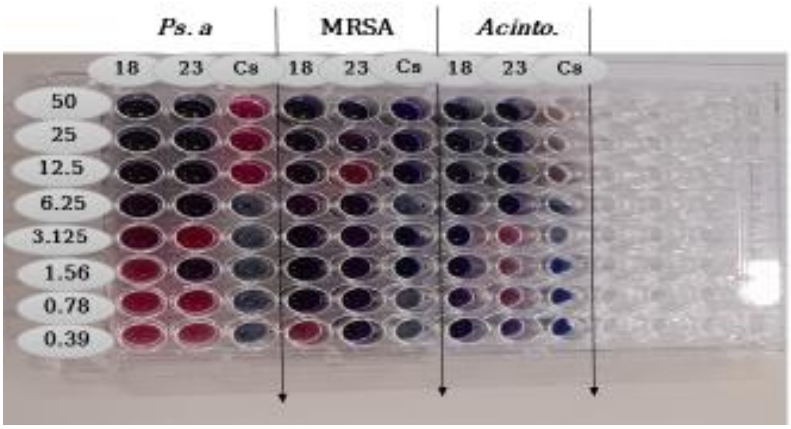

Figure (2): Minimum inhibitory concentration determination with use of Resazurin reagent.

3.3. Antibiogram of clinical isolates: The included clinical isolates were tested for their sensitivity against the ten used antibiotics references, and the results showed that MRSA was resisted all included beta-lactam, and carbapenem antibiotics, and controlled by Vancomycin 10 µg, Gentamicin 10 µg, and Ciprofloxacin 5 µg with inhibition zones of 34± 0.07, 20± 0.7, and 32± 0.1mm, respectively. The results showed that *Pseudomonas aeruginosa* was resisted all tested beta-lactam antibiotic except of Amoxicillin/Clavulanic acid which affected it with an inhibition zone of 11.0 ± 0.0 mm. However, this bacterium was killed by Ciprofloxacin 5µg, Imipenem 10µg, Meropenem 10µg, and Gentamicin 10µg with inhibition zones of 39.2± 0.2, 30.0 ± 0.0, 29.0± 0.0, and 16.9± 0.1mm, respectively. On the other hand, this study results proved that no one of the used antibiotics can affect the tested *Acinetobacter baumannii* since it was resisted the all (Table 3).

Table (3): Antibacterial activity of reference drugs (antibiotic drugs) against isolated bacterial tested:

Antibiotic drugs	**Concentrations**	**MRSA**	***Ps. aeruginosa***	***A. baumannii***
Extract	**100 mg/ml**	**25 ± 0.12**	**21 ± 0.20**	**23 ± 0.16**
Amoxicillin	**25 µg**	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Amoxicillin/Clavulanic acid	**30 µg**	0.00 ± 0.0	11.0 ± 0.0	0.00 ± 0.0
Ceftazidime	**30 µg**	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Ceftriaxone	**30 µg**	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Ciprofloxacin	**5 µg**	32 ± 0.1	39.2± 0.2	0.00 ± 0.0
Imipenem	**10 µg**	0.00 ± 0.0	30.0 ± 0.0	0.00 ± 0.0
Meropenem	**10 µg**	0.00 ± 0.0	**29.0± 0.0**	0.00 ± 0.0
Gentamicin	**10 µg**	20 ± 0.7	16.9± 0.1	0.00 ± 0.0
Vancomycin	**10 µg**	34 ± 0.07	Note Done	Note Done

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3.4. Wound healing assay (antibacterial screening): In wound healing assay, the group (1) rats was completely healed in 11 days. Other groups were healed in 7 days. When the extract ointment 3% applied on rats group with wounds infected by *Pseudomonas aeruginosa* and compared with wounds treated with antibiotics ointment reference, the results showed that with the end of the first day treatment the wounds area percentage was decreased from 100% to 59.169% with tested extract ointment compared with 70.33% with standard reference used (Tetracycline 3% ointment) with no significant difference. Starting from 2nd day treatment the healing rhythm of the wound area was continue decreasing in both standard and sample treated rats groups with highly significant differences, where the probability values were ≤ 0.01 (Table 4).

Table (4): Effect of *M. communis* 3% ointment on an infected wound

Days	Control (*Ps. aeruginosa* infected wound)		*Ps. aeruginosa* infected wound treated with 3% Tetracycline ointment		*Ps. aeruginosa* infected wound treated with 3% *M. communis ointment*)		LSD Post Hoc analysis	
	Mean ± SD	Wound %	Mean ± SD	Wound %	Mean ± SD	Wound %	(P value)	Sig.
0day	3.9a±2.5	100	3.0 a ±0.1	100	2.9 a ±0.8	100	.608	NS
1day	2.9 a ±1.8	-70.459	2.2 a ±0.6	-70.3333	1.8 a ±0.5	-59.169	.295	NS
2day	2.5a±1.0	-83.3069	1.0 b±0.4	-43.2545	1.1 b±0.4	-59.3111	.023	*
3day	2.1 a ±1.1	-81.5	0.7 b±0.2	-69	0.7 b ±0.3	-62.5364	.009	**
4day	1.6 a ±1.0	-74.0905	0.5 b ±0.1	-70.7286	0.4 b ±0.1	-56.4429	.002	**
5day	1.3 a ±0.1	-79.65	0.3 b ±0.1	-59.5	0.2 b ±0.1	-49.6	.002	**
6day	1.0 a ±0.1	-75.6231	0.1 b ±0.1	-33.0333	0.1 b ±0.1	-49.8	.002	**
7day	0.7 a ±0.1	-69	0.0 b ±0.0	0.1	0.0 b ±0.0	0.1	.004	**
8day	0.5±0.4	-70.7286						
9day	0.3±0.2	-59.5						
10day	0.1±0.2	-33.0333						
11day	0.0±0.1	0.1						

3.5. Phytochemical analysis:

The traditional phytochemical analysis of tested methanol extract of leaves of *Myrtus communis* showed presence of high concentration of Flavonoids, and moderate concentration of Saponins and Tannins (Table 5). The GC-MS analysis done in this study reported that the tested extract consists of 32 compounds among them Flavonoid (n=1), Aryl-Aldhyde (n=1), Aldhyde (n=1), Carboxylic acid (n=1), Monoterpenes (n=9), Diterpene (n=2), Monoterpene alcohol (n=1), Terpene (n=1), Ketone (n=2), Fatty acids (n=6), Steroide (n=1), Flavonoide (n=1), Ester (n=1), simple cyclic polyketides (n=1), Aromatic acid (n=1), Heterocyclic (n=1), saturated aliphatic hydrocarbon (n=1) (Table 6). The majority were Quinic acid which constitutes about 28% of the total followed by fatty acids (19%), Aldhyde (7%), Ketones (7%), Diterpenes (6%), and about (3%) for each of other compounds (Figure 3). As shown in table (6) bout 17 compounds (53%) of a total of 32 compounds identified in this study were previously reported as having antimicrobial activity, 13 compounds (40.6%) reported having another biological activity, and two compounds (6.25%) appeared in this study as has not been previously reported for any biological activities; Endo-1,5,6,7-Tetramethylbicyclo[3.2.0]hept-6-en-3-ol, and 2.4-Hexanedione, 5-methyl-3-(2-methyl-1-propenyl).

Table (5): Phytochemical analysis of *Myrtus communis* extract:

Alkaloids	Anthraquinones	Coumarins	Flavonoids	Saponins	Steroids	Tannins	Triterpenes
-	-	-	+++	++	-	++	-

(-): Absent; **(+):** Low concentration; **(++):** Moderate concentration **(+++):** High concentration (16).

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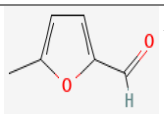
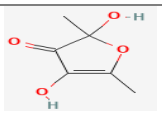
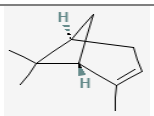
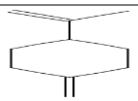
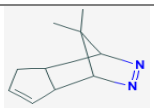
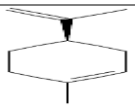
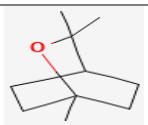
Table (6): Main bioactive compounds distinguished from methanolic extract of *Myrtus communis* leaves:

No.	Number of the compound	R. Time	Area (%)	Formulas	Molecular Weight (g/mol)	Structures	Nature of the compounds	Bioactivity of the compounds (References)
1.	2-Furancarboxaldehyde, 5-methyl-	4.07	0.80	$C_6H_6O_2$	110		Aldehyde	Antibacterial and anti-cancer activities (17), anti-inflammatory (18), antimicrobial activities (19).
2.	2, 4-Dihydroxy-2, 5 dimethyl-3(2H)-furanone	4.23	0.67	$C_6H_8O_4$	144		Carboxylic acid	Antimicrobial effect, anti-biofilm (20).
3.	Beta-Myrcene	4.33	0.04	$C_{10}H_{16}$	136		Monoterpene	Antibacterial properties (21).
4.	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	4.58	0.33	$C_{10}H_{16}$	136		Monoterpene	Antimicrobial effects (22).
5.	Cyclohexane, 1-methylene-4-(1-methylethenyl)-	4.74	0.05	$C_{10}H_{16}$	136		Monoterpene	Anticancer activity (23).
6.	1,4-Methano-1H-cyclopenta (d) pyridazine,	4.86	0.17	$C_{10}H_{14}N_2$	162		Monoterpene	Antimicrobial Activity (24).
7.	D-Limonene	4.91	0.28	$C_{10}H_{16}$	136		Terpene	Anticancer activity (25).
8.	Eucalyptol (1,8-cineol)	4.96	0.20	$C_{10}H_{18}O$	154		Monoterpene	Anti-inflammatory, antioxidant, mucolytic/secretolytic, bronchodilatory, Antiviral, and antimicrobial effects (26).

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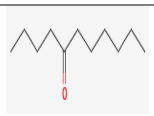
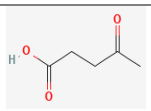
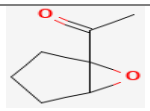
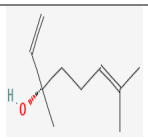
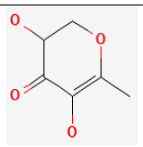
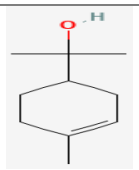
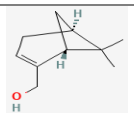
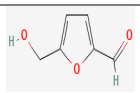
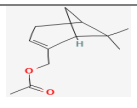
9.	5-Undecanone	5.12	0.72	$C_{11}H_{22}O$	170		Ketone	Anti-inflammatory and analgesic activity (27).
10.	Pentanoic acid, 4-oxo-	5.22	0.36	$C_5H_8O_3$	116		Fatty acid	Hepatoprotective, Flavoring Agent (28)
11.	Cyclopentane, 1-acetyl-1,2-epoxy-	5.72	0.72	$C_7H_{10}O_2$	126		Steroids	Anti-inflammatory, antiviral and bronchodilatory properties (28).
12.	(S)-3,7-dimethyl-1,6 octadien-3-ol	5.89	0.20	$C_{10}H_{18}O$	154		Monoterpene	Cytotoxic and Antioxidant Properties (29).
13.	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP)	6.67	2.38	$C_6H_8O_4$	144		Flavonoid	Antimicrobial, anti-inflammatory and antioxidant capacity (28).
14.	.alpha.-Terpineol	7.33	0.33	$C_{10}H_{18}O$	154		Monoterpene	antioxidant, anti-inflammatory, antimicrobial, anticancer, analgesic, gastroprotective, cardioprotective, neuroprotective, and antidiarrheal effects (30)
15.	(-)-Myrtenol	7.44	0.18	$C_{10}H_{16}O$	152		Monoterpene	Antioxidant, antibacterial, antifungal, antidiabetic, anxiolytic, and gastroprotective activities (31).
16.	5-Hydroxymethylfurfural (Like Honey bee)	7.96	7.79	$C_6H_6O_3$	126		Aldehyde	Antioxidant and antiproliferative activities (32)
17.	Myrtenyl acetate	9.25	1.40	$C_{12}H_{18}O_2$	194		Ester	Antibioflm, cytotoxic, and anti-acetylcholinesteras

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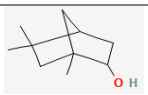
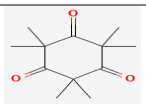
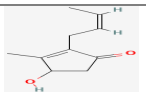
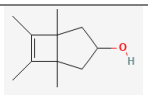
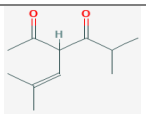
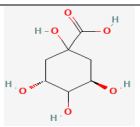
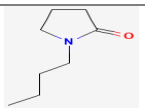
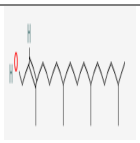
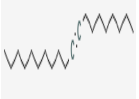
								e activities (33)
18.	Bicyclo(2.2.1)heptan-2-ol, 1,5,5-trimethyl-	9.79	1.91	$C_{10}H_{18}O$	154		Monoterpene	Antibacterial activities (34).
19.	Hexamethylcyclohexane-1,3,5-trione	10.35	0.68	$C_{12}H_{18}O_3$	210		Simple cyclic polyketides	Antimicrobial activity (35).
20.	2-Cyclopenten-1-one, 2-(2-butenyl)-4-hydroxy-3-methyl-, (Z)-	11.94	2.49	$C_{10}H_{14}O_2$	166		Ketone	Antiviral, anti-cancer activities (36).
21.	Endo-1,5,6,7-Tetramethylbicyclo[3.2.0] hept-6-en-3-ol	12.29	11.26	$C_{11}H_{18}O$	166		Other compound	Unknown
22.	2.4-Hexanedione, 5-methyl-3-(2-methyl-1-propenyl)-	12.75	0.41	$C_{11}H_{18}O_2$	182		Ester	Unknown
23.	Quinic acid (Phenolic acid)	13.49	40.02	$C_7H_{12}O_6$	192		Aromatic acid	Antioxidant, antidiabetic, anticancer, antimicrobial, antiviral, aging, protective, anti-nociceptive and analgesic effects (37).
24.	2-Pyrrolidinone, 1-butyl-	14.37	1.87	$C_8H_{15}NO$	141		heterocyclic	Anti-bacterial Anti-fungal (38)
25.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	15.55	1.84	$C_{20}H_{40}O$	296		Diterpene	Anti-inflammatory, antithrombotic, antimicrobial, and antitumor effects.
26.	9-Eicosyne	16.03	0.71	$C_{20}H_{38}$	278		Sat. aliphatic hydrocarbon	Antimicrobial and cytotoxic properties (25).

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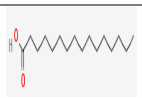
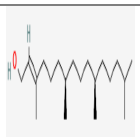
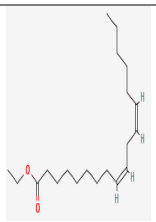
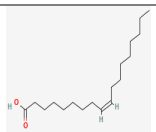
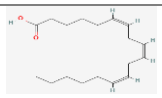
27.	n- Hexadecanoic acid	16.90	3.43	$C_{16}H_{32}O_2$	256		Fatty acid	Antioxidant activity (39).
28.	Phytol	18.43	0.19	$C_{20}H_{40}O$	296		Diterpene	Anti-nociceptive, Antioxidant, anticancer, anti-inflammatory, antimicrobial, diuretic, chemopreventive properties (40).
29.	Linoleic acid ethyl ester	18.65	0.33	$C_{20}H_{36}O_2$	308		Fatty acid	Hypocholesterolemic, nematicide, antiarthritic, hepatoprotective antiandrogenic , hypocholesterolemic, 5-Alpha reductaseinhibitor antihistaminic, anticoronary, insectifuge, antieczemic, antiacne (41).
30.	Oleic acid	18.69	0.16	$C_{18}H_{34}O_2$	282		Fatty acid	Anti-inflammatory, anti-androgenic, anti-cancer, preservative and hypocholesterolemic (42).
31.	Gamolenic acid	18.73	0.50	$C_{18}H_{30}O_2$	278		Fatty acid	Anti-inflammatory (43).
32.	Octadecanoic acid	18.88	0.41	$C_{18}H_{36}O_2$	284		Fatty acid	Antioxidant activity (44).
	Total		**100**					

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4. Discussion:

The consumption of antibiotics is on a high increase worldwide, especially in developing countries. This increase is due to the increasing rate of recurrent and difficult treatable infections, particularly those caused by multidrug-resistant bacteria. Even though the human misuse of antibiotics stands for the rapid and increasing emergence of bacterial resistance phenomenon to antibiotics, but also the use of antibiotics in other purposes rather than human medicine such as animal husbandry and agriculture stands behind this phenomenon too. In 2019 the Centers for Disease Control and Prevention reported that about 35000 and 33000 death cases in the US and European Union, respectively with an economic loss of about 1.5 billion dollars were due to recurrent and difficult treatable infections. That was in 2019, and now we are in the beginning of 2024, and the resistance phenomenon still in struggle, so the disaster surrounding us could be imagined. Aware of this surrounding danger, we decided to move forward with this study to complement what preceded it and continue to reach our desired goal, which is to find a solution that eliminates human pathogenic bacteria that are resistant to antibiotics and find the solution to treat infectious diseases. This study has studied antibacterial activity of the methanol extract of *Myrtus communis* plant leaves three times, and the results proved the pronounce good activity of this extract against three of the most worldwide problematic bacteria; MRSA, *Pseudomonas aeruginosa* and *Acinetobacter baumanii* where all were inhibited by the extract with an active inhibition zone ranged from 20mm to 25mm. The good activity shown against MRSA is agreed with that revealed from a study done by (45), where they reported that *Myrtus communis* leaves extract inhibited the growth of MRSA with an active inhibition zone of 19mm, with the note that they were collected the plant material from a garden in Egypt and tested an aqueous leaves extract, not methanol as this study did. However, a study done by (46), where they were tested the same plant part (leaves) and the same used solvent (methanol), and also, they were collected the plant from the same region, but their result disagreed with this study in that they reported a weak inhibition zone of 9mm against MRSA. This variation may due to the different collection time where Abouzeed team were collected the plant material in the spring season (2010) while in this study it has been collected twice; in the summer seasons of 2018 and 2023 year. In addition, the solvent concentration used was differ; 80% Methanol and absolute Methanol were used by Abouzeed team and this study, respectively. It is well documented that the variation in ecosystem and seasons affect the type and concertation of the plant secondary metabolite, the phenomenon which may stands behind the variation in the antibacterial activity of the same plant part extracted with same condition. Anyway**,** (47), in their study agreed with this and reported a good inhibition zone from methanol leaves extract of *Myrtus communis* against *Staphylococcus aureus* which showed a bit higher sensitivity towards Gentamicin 22mm in-front of 20mm shown in this study against same antibiotic. Also, Mansouri's study proved that this plant extract showed an active inhibition zone against *Pseudomonas aeruginosa* of 15mm, the result which agreed with this study output which proved that the extract revealed a higher inhibition zone of 21mm against this bacterium. The differ between our study and Mansouri's study was the sensitivity the tested *Pseudomonas aeruginosa* bacterium where ours showed higher sensitivity (20mm) than theirs (15mm) towards Gentamicin antibiotic, and this could be happened with the difference between the bacterial different strains.

The resistance of all beta-lactam and most of beta-lactam antibiotics by both MRSA and *Pseudomonas aeruginosa*, respectively indicates to that these two isolates are a beta-lactamase enzyme producer. In addition, MRSA could be addressed as a carbapenemase enzyme producer as it resisted both used carbapenems antibiotics; Imipenem and Meropenem. Although our extract appeared as very active and the more active against MRSA compared with Gentamicin, but it appeared lower in activity compared with Ciprofloxacin and Vancomycin, while it was the only effective one compared with other tested antibiotics against that MRSA. In addition, the tested extract in this study appeared as the only effective agent against *Pseudomonas aeruginosa* compared to the beta-lactam antibiotics, but even though the extract performance is good but it was lower than that shown from Ciprofloxacin, Imipenem, Meropenem, and Gentamicin, but if we talk the side effects of these synthetic antibiotics in our consideration, the *Myrtus communis*

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extract could be addressed as the best agent in this study that can control and inhibit the growth of MRSA and *Pseudomonas aeruginosa*. Also, this study results highlighted the threat danger surrounding us from *Acinetobacter baumannii* bacterium specially for immunocompromised patients, where it was resisted all tested antibiotics. This pathogen is responsible for a number of nosocomial infections such as urinary tract infection, skin and soft tissue infection, bacteraemia, meningitis, and pneumonia which is being the most frequently reported infection (48). This study focused on using different antibiotics covering all classes of antibiotics and covering all different antibiotic mechanisms. Since all the antibiotics tested failed to control the tested *Acinetobacter baumannii* bacterium, but our *Myrtus communis* leaves extract effectively controlled and inhibit the growth of this pathogen with high inhibition zone (23 mm), and with a minimum inhibitory concentration of ≥ 0.39mg/ml. This MIC (≥ 0.39mg/ml) is the same MIC by which the tested MRSA was inhibited in this study, in time that lower MIC (6.25mglml) was revealed to diminish and killed *Pseudomonas aeruginosa* by this extract. These MIC values offer a clear indication that *Myrtus communis* constitutes a good and promising source of agents of natural origin that can control these worldwide problematic bacteria. The occurrence of Flavonoids, Saponins, and Tannins support the good revealed antibacterial activities where these compounds are previously well confirmed for their antibacterial activity. This study referred the promising growth inhibition activity shown by *Myrtus communis* plant against the tested bacteria to the high presence (53%) of varied compounds that are known to have antimicrobial. The occurrence of Quinic acid as a major compound constitutes (40%) of total of 32 detected compound, and also the occurrence of the fatty acids stands behind the good revealed antibacterial activity (49, 50). As proved by (51), Quinic acid was inhibited the growth of the *Staphylococcus aureus* ATCC 6538 strain by two mechanisms; inhibit the protein synthesis via disruption of aminoacyl-tRNA synthesis, and by inhibition of both cell division and cell wall synthesis via interfering the synthesis of the synthesis of peptidoglycan and L-lysine. Bai and his team targeted a Methicillin sensitive *Staphylococcus aureus* (*S. aureus* ATCC 6538 strain), while our study targeted a clinical Methicillin resistant *Staphylococcus aureus* (MRSA). This pushed us to recommends more further investigation to isolate and identify the pure compound/s responsible for the revealed activity, and to determine the mechanism/s of action by which it/they controlled these risky bacteria. Also, this study recommends further studies carried out to investigate and explore more biological activities could be offered by this Libyan wild grow plant especially that of the two unclassified compound appeared in the GC-MS analysis done in this study (Endo-1,5,6,7-Tetramethyl bicyclo[3.2.0]hept-6-en-3-ol, and 2.4-Hexanedione, 5-methyl-3-(2-methyl-1-propenyl).

Myrtus communis methanol leaves extract dosage form (3% ointment) was proved in this study for its ability to treat and accelerate healing of wounds infected with *Pseudomonas aeruginosa* bacterium where it showed an activity equal to that offered from the antibiotic dosage form that used as positive control in this study (Tetracycline 3% ointment). Starting from second day treatment a significant difference (p value ≤ 0.05) was observed in the percentage of healing area between the three rats groups; non-treated infected rats, infected rats treated with Tetracycline antibiotic and infected rats treated with *Myrtus communis* extract dosage form. Both tested Tetracycline and extract ointments dosage forms were healed the wounds in an equal time period (7 days), while 11 days lasts for the wounds without treatment (only cleaned daily with an antiseptic) to be completely healed. The equality in healing period of wounds between the control and extract treated groups was observed with a high significant difference between both groups at the completely healed point. This hypothesized that the plant extract can offer the same activity of the control used (antibiotic) but with different mechanism. It is known that *Pseudomonas aeruginosa* and their endotoxins are factors cause prolonged elevation of pro-inflammatory cytokines such as interleukin-1(IL-1) and TNF-α and then prolonged the inflammatory phase, the matter which lead to impairment of wound healing. In-front of that tested extract showed high anti *Pseudomonas aeruginosa* activity equal to the drug control and completely healed the infected wound, this study suggests this plant as a good resource for agent/s can control wound infections caused by this bacterium, and also, this study recommends further deep investigations to determine the mechanism of action by which *Myrtus communis*

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ointment controlled the *Pseudomonas aeruginosa* and completely treated the wounds. Also this study recommend further deep research about presence of any effect/s might be this plant have on the skin layers; epidermis, dermis, and the hypodermis. We are planning to test the same plant extract in a creamy dosage form to test our hypothesis said that the cream dosage form will highly accelerate the wound healing corresponding to the hydrophilicity of the skin stratum corneum through which the active ingredient in a creamy dosage form will penetrate faster than from ointment dosage from. To summarize the *Myrtus communis* plant forms a magic chance for study areas especially that concerned with promoting health wellbeing. The point this study flashed is the ability of the tested extract to retain the antibacterial activity against tested bacterial pathogens since the extract that was stored for a long period (years) showed an antibacterial activity close to that shown by the fresh one. This push to say that this plant contains highly stable compounds that have good antibacterial activity against MDR bacteria, and this offers a good resource for new antibacterial agents with long shelf life.

5. Conclusion: This study aimed to investigate the inhibitory performance of methanol crude extract of the leaves of *Myrtus communis* plant against MDR human pathogenic bacteria that responsible for many serious diseases lead to death if not treated, and the results proved that this extract contains valuable bioactive substances that showed a pronounce activity against tested pathogens. This study introduces this plant as a source of stable valuable agents could be used for synthesis of new antibacterial agents. Furthermore, this study recommends more investigations to be performed to isolate and purify the pure compound/s responsible for the good activity shown by the tested extract, and also to figure out other biological activities this plant might have.

5. Acknowledgments:

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6. Conflict of Interest:

The authors declare no conflict of interest.

7. Authors' Declaration:

The authors declare that all works done in this manuscript are original and they are responsible for any claims relating to the content.

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