GC-MS Analysis of Chloroform Extract of *Myrtus communis* and its Performance against Methicillin-resistant *Staphylococcus aureus*

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Abstract:

Aim: To participate in finding a solution for treating infections caused by Beta-lactam resistant Methicillin-resistant *Staphylococcus aureus* (BLR-MRSA), this study aimed to assess the effectiveness of the extract of chloroform solvent of *Myrtus communis* leaves on growth of clinical BLR-MRSA isolate**. Methods:** Soxhlet and Rotary evaporator apparatus were used for extraction and evaporation and chloroform for extraction. Disc diffusion and Agar dilution assays were used to determine the growth inhibition capacity and the MIC, respectively. Gas-chromatography Mass-Spectra to analyze the extract bioactive constituents. **Result:** The extract showed very promising growth inhibition activity against MRSA with a mean inhibition zone of 67mm and with MIC of 3.125mg/ml. The GC-MS analysis revealed many compounds, the abundant were Cyclohexene, 1,5,5-trimethyl-6-acetyl methyl; Myrtenyl acetate; Pyrrolidine, 1-(1-oxobutyl); Oxalic acid, heptadecyl 1-menthyl ester; Gamma. -Sitosterol; Vit. E; Phytol; Myrtenol; L-. alpha. -Terpineol; Myrtocomulones, and α-Terpineol with percentages of 25.98%, 16.65%, 4.88%, 4.48%, 4.30%, 3.41%, 1.16; 1.82%; 0.50%, and 0.28%, respectively. **Conclusion:** *Myrtus communis* have a valuable bioactive compound proved in this investigation to have a valuable therapeutic impact for the treatment of infections caused by MRSA. **Recommendation:** Further work should be carried out to purify the activity responsible compound/s and formulate a new pharmaceutical dosage-form.

Key Words: Beta-lactam resistant MRSA, *Myrtus communis*, Chloroform**,** GC-MS.

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Introduction:

Methicillin resistant *Staphylococcus aureus* (MRSA) bacterium has the ability to asymptomatically colonize approximately third of the population, the point which caused a wide range of human and animal infections. Many outbreaks of food poisoning have been recorded where MRSA was responsible. Infections caused by the pathogen MRSA are varied from mild infection; skin infection to more severe infection such as osteomyelitis and pneumonia and extend to life-threatening infections such as sepsis, cerebral abscesses, infective endocarditis, and toxic

shock syndrome. Indwelling catheter infections, prosthetic devices, and implant-associated infections are often caused by MRSA. (McCarthy, *et al*., 2015, Peacock and Paterson, 2015, Silva *et al*., 2021). MRSA is considered as a major cause of health care-associated and hospital-acquired bacteremia. Immunocompromised persons, diabetics, and patients on hemodialysis are at higher risk of infection with this pathogen.

What further complicates the situation is the ability of MRSA to form biofilms on biotic and abiotic surfaces; the primary mechanism by which MRSA resists antibiotics. More than 80%0f bacterial infections are mediated by biofilms (Cascioferro *et al*., 2021). By formation of biofilm, MRSA is often responsible for prosthetic devices, implant-associated and indwelling catheter infections (Hassoun *et al*., 2017, Lee *et al*., 2018). Production of biofilm is either high or low depending on the strain of MRSA and the gene type it carries. Genes named, ica genes were documented to have a considerable role in biofilm formation and were mostly found among all MRSA isolates.

A secondary mechanism this pathogen adapts to resist the antibiotics, specifically Beta-lactam antibiotics is via their mecA gene, which enables MRSA to grow in the presence of Penicillin-like antibiotics. In *Staphylococcus aureus* isolates not resistant to methicillin, this mecA gene encodes for the formation of four types of Penicillin-binding proteins (PBP); PBP1, PBP2, PBP3, and PBP4 by which the Beta-lactam antibiotic attach and linked and interfere with the bacterial growth. In MRSA, the mecA gene encodes for another gene; 2a (PBP2a) whose structural features resemble that of the four mentioned types but are not inhibited by β-lactam antibiotics. As recently researchers handle the issue that MRSA documented as a major cause of persistent infection, it is reported by the World Health Organization (WHO) as a high priority pathogen and becomes a major concern globally, the reason for which this experimental study targeted this pathogen in order to find an effective suppressing agent from natural product origin. Specifically, it is becoming difficult to eradicate the infections by specific MRSA strains due to their multi-drug resistance properties and the decrease in the effectiveness of commonly used antibacterial agents as well. In addition, many of the synthetic antibacterial agents have undesirable side effects, the issue which directed this study to find a solution from the reality of medicinal plants.

Myrtus communis L (*M.communis*), named Myrtle, is a member of the Myrtaceae family, which constitutes more than 5500 species belonging to 145 genera. It is native growth in many areas throughout the world including North Africa, and is widespread in the Mediterranean region. Traditionally it is used in food preparation as flavoring agent and was addressed as a hypoglycemic, antiseptic agent (Peacock and Paterson, 2015).

The performance of the different parts of the *Myrtus communis* plant collected from different regions against Gram-positive, Gram-negative bacteria, and some fungi, were screened by many researchers. (Ghnaya *et al*., 2013; Taheri, *et al*., 2013; Alizadeh *et al*., 2016; Besufekad *et al*., 2017). Myrtle is used traditionally in many countries since antiquity in cooking and medicinal purposes. Although of the recently increasing scientific interest in this field, still the biological

profile is not fully completed (Aleksic & Knezevic, 2014). In sense of the above introduction, this study is carried out to assess the inhibition performance of the extract of chloroform solvent of the Libyan *Myrtus communis* leaves against clinical Beta-lactam resistant Methicillin-resistant *Staphylococcus aureus*.

Materials and Methods

Plant Material

Myrtus communis plant was collected from the Green Mountain area, Northeast of Libya. The plant was identified and classified by Dr. Hussein Altajouri, department of botany, faculty of Science, Benghazi University, Libya. Plant leaves were separated and washed with tap water, left to dry at room temperature, and then was size minimized by use of blinder. The powdered leaves were kept in a colored bottle.

Tested Bacteria:

A clinical MRSA isolate already proved to resist all antibiotics belong to the class beta-lactam, was obtained from Al-Akeed diagnostic laboratory, Benghazi city, Libya.

Reference Antibiotics Discs:

Three standard antibiotics discs were used in this study; Ciprofloxacin 30 µg (CIP), Gentamicin 10 µg (CN) and Vancomycin 30 µg (VA), and were from Bioanalyse@ YSE TibbiMalzemeler San.

Preparation of plant extract:

With use of 300 ml of 300ml of Chloroform, a weight of 100gm of the previously prepared plant powder was extracted for 4 hours. Soxhlet apparatus and rotary evaporator were used for extraction and evaporation. The yield was calculated, and kept at 4C^{o}. On the day of the antimicrobial assay, a fresh solution was prepared by dissolving 0.1gm of the extract in 1ml of a mixture of petroleum ether and methanol (1:2) in order to get a stock solution of 100mg/ml concentration.

Antimicrobial screening assay:

The antibacterial activity was determining According to Eltawaty *et al*., (2018), where with by use of the disc diffusion concept, a Mueller-Hinton agar petri dish surface was streaked with 100µl of the tested organism suspension which freshly adjusted to a 10^8 CFU/ml, four replicate sterile Discs, 6 mm in diameter (Wattman paper No. 1) were placed on Mueller-Hinton agar petri dish, then 20 µl of a solution of *Myrtus communis* extract was added on each disc. The Petri dishes were then incubated at 37°C for 20 hours. Antibiotics discs were used as a positive reference.

Evaluation of minimum inhibitory concentration (MIC):

Agar dilution method was adopted in this study (Andrews, 2002)

Gas Chromatography Mass-Spectra Screening:

The sample tested for its antimicrobial activity in this study was analyzed by use of Gas Chromatograph-Mass Spectra (model qp2010, Shimadzu). Helium was used as carriage gas with flow rate of 1.61ml/minutes under starting temperature of 60 °C, gradually increased to final temperature of 300 °C; 10 °C /min rate. 300C and 200°C, were the injection port temperature and the ion source temperature used in this study, respectively, while the interface temperature was 250C. The total run time was 29 minutes. By comparison with the data of the library of the National Institute of Standards and Technology, the he sample constituents had been identified.

Statistical Analysis

SPSS package version 20 was used in this study for the statistical analysis, and P value of ≤0.05 as significant value.

Results:

Antibacterial Evaluation:

This study was done on a number of 4 replicates of the tested clinical isolates. The four replicates of multi-drug resistant MRSA isolate showed very high sensitivity towards the chloroform extract of *M.communis* leaves and revealed inhibition zones of 60mm ± 1.0, 70mm ± 2.0, 70mm ±2.0, and 68mm ± 1.4, with a mean of growth inhibition zone of 67mm (Photo 1).

The minimum inhibitory concentration shown by the tested extract against tested MRSA was 1.562mg/ml. The extract showed no activity against the other four tested Gram-negative bacteria, whereas, *Ps.aeruginosa*, *A.baumannii*, *K.pneumoniae*, or *E.coli* does not show any sensitivity toward the tested extract. The statistics showed high significance differences (Pv ≤ 0.01) in the performance of the tested extract on both tested Gram-positive bacterium and Gram-negative bacteria. As MRSA was the only affected and inhibited compared with the other tested Gram-negative bacteria, it has been tested for its sensitivity towards Ciprofloxacin 30ug, Gentamicin 10ug, and Vancomycin 30ug and the results showed that the growth of MRSA was inhibited by the three references with inhibition zones of 30.0 ± 3, 18.5±1.1, and 20±1.0, respectively.

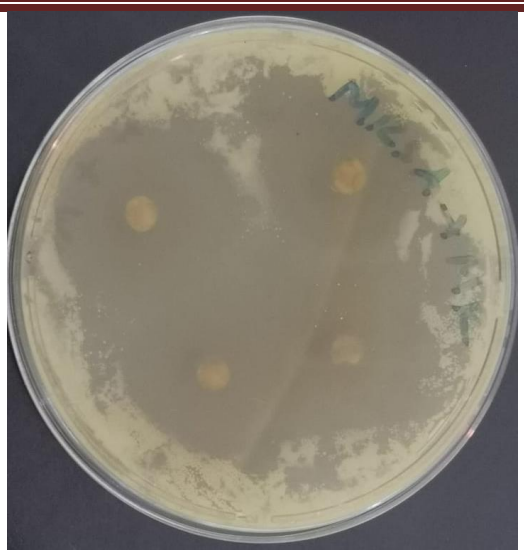

Photo (1): Inhibition zones revealed by tested extract

Gas Chromatography Mass-Spectra Analysis (GC-MS):

The GC-MS analysis in this study revealed out a sixty two (62) compounds as constituents of the chloroform extract of leaves of *M.communis* (Figure 1).

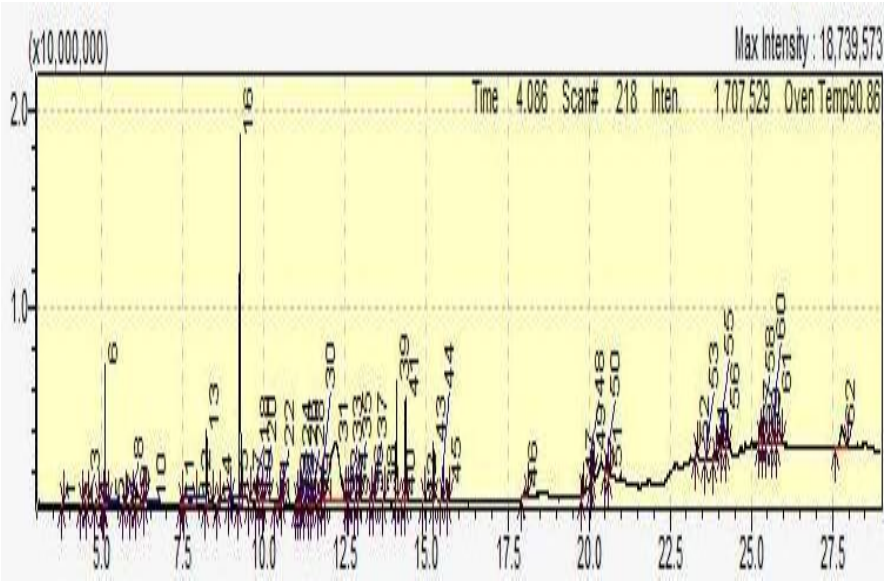

Figure (1) Peaks of GC-MS analysis of tested chloroform extract of leaves of *M.communis*

The highest percentages of related compounds were 25.98%, 16.65%, 4.88%, 4.48%, 4.30%, 3.41%, 1.16; 1.82%; 0.50%, and 0.28% for Cyclohexene, 1,5,5-trimethyl-6-acetylmethyl; Myrtenyl acetate; Pyrrolidine, 1-(1-oxobutyl); Oxalic acid, heptadecyl 1-menthyl ester; Gamma. -Sitosterol; Vit. E; Phytol; Myrtenol; L-. alpha. -Terpineol; Myrtocomulones, and α-Terpineol, respectively (Table, 1) and Figures 2-10, respectively.

Table (1): Anti-MRSA relative compounds revealed by GC-MS analysis of the chloroform extract of leaves of *Myrtus communis*

No	Name	Retention Time	Area	Area %
1	Cyclohexene 1,5,5-trimethyl-6-acetylmethyl	12.204	57270472	25.98
2	Myrtenyl acetate	9.260	36719760	16.65
3	Pyrrolidine	14.077	10763851	4.88
4	Oxalic acid	14.355	9877128	4.48
5	Gamma.-Sitosterol	27.789	9491210	4.30
6	Vitamin E	25.845	7513291	3.41
7	Phytol	18.027	2552167	1.16
8	Myrtenol (Bicyclo[3.1.1]hept-2-ene-2-methanol, 6,6-dimethyl)	7.583	4010738	1.82
9	L-.alpha.-Terpineol	7.471	1110197	0.50
10	Myrtocomulones (5-Hydroxy-2,2,6,6-tetramethyl-4-cyclohexene-1,3-dione)	8.612	619511	0.28

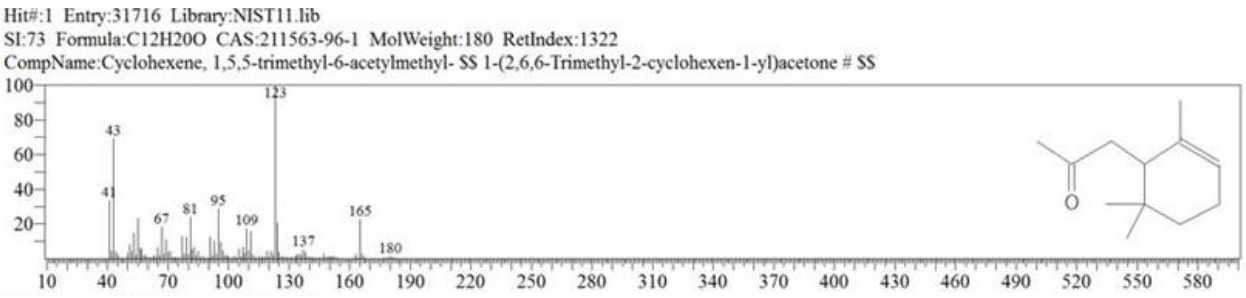

Figure (2): Peak of GC-MS analysis of Cyclohexene, 1,5,5-trimethyl-6-acetylmethyl

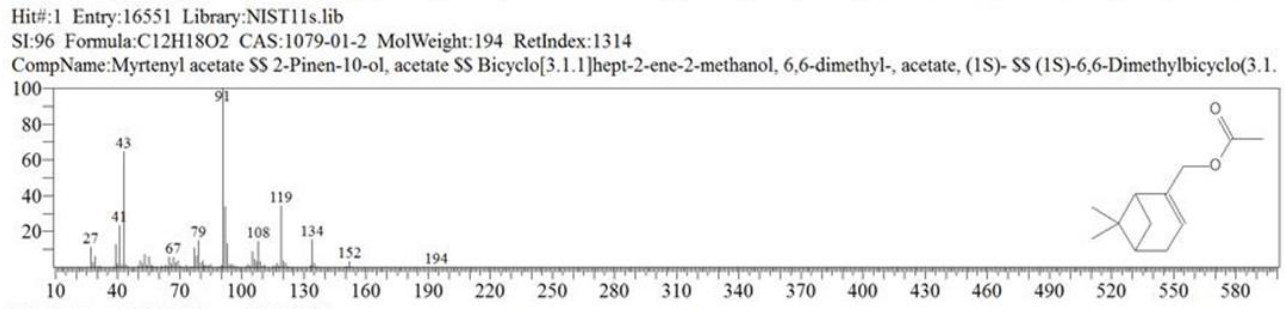

Figure (3): Peak of GC-MS analysis of Myrtenyl acetate

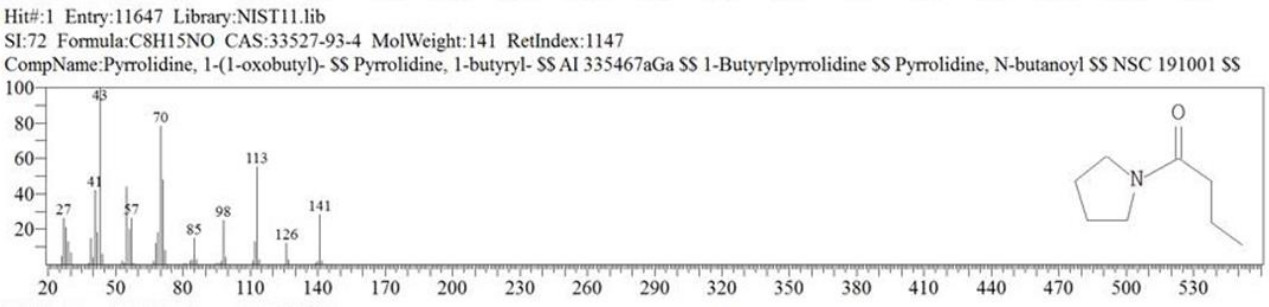

Figure (4): Peak of GC-MS analysis of Pyrolidine

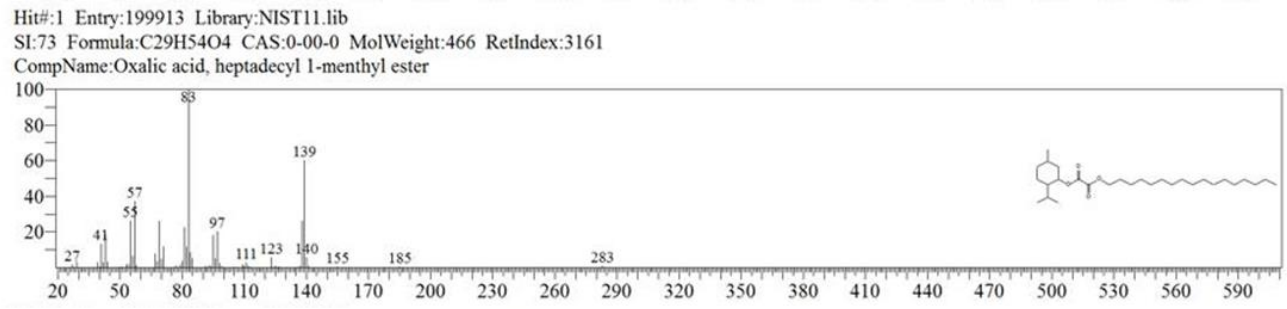

Figure (5): Peak of GC-MS analysis of Oxalic acid

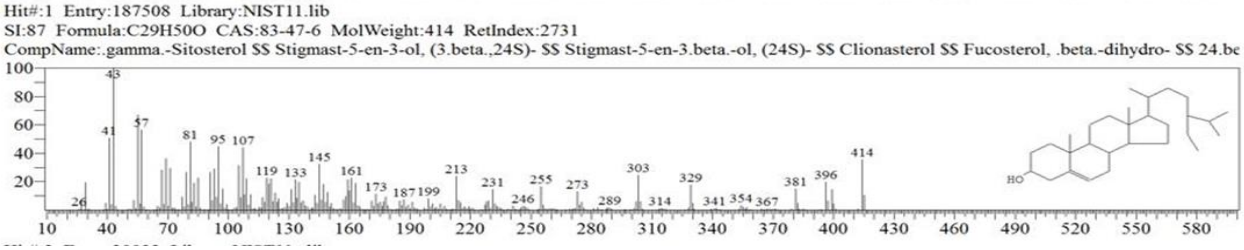

Figure (6): Peak of GC-MS analysis of Gamma-Sitosterol

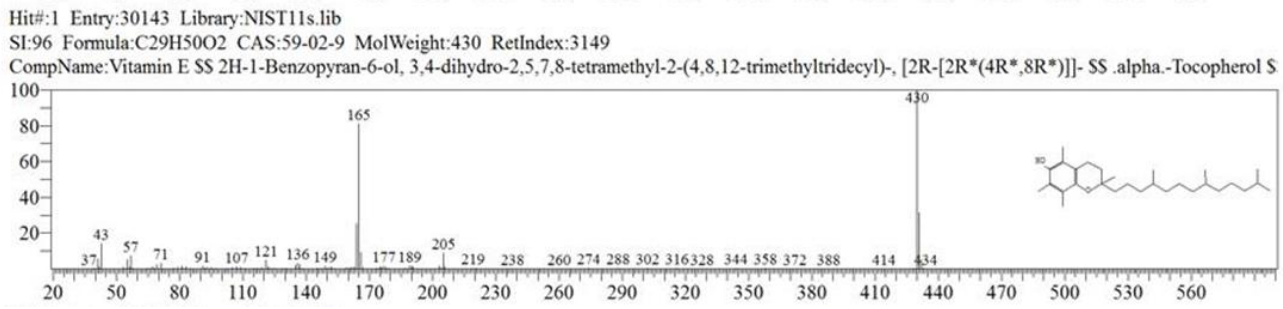

Figure (7): Peak of GC-MS analysis of Vit.E

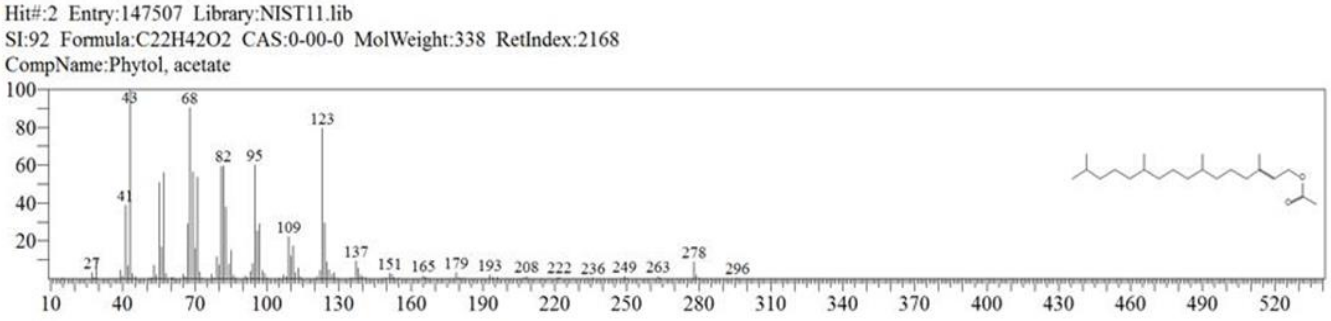

Figure (8): Peak of GC-MS analysis of Phytol

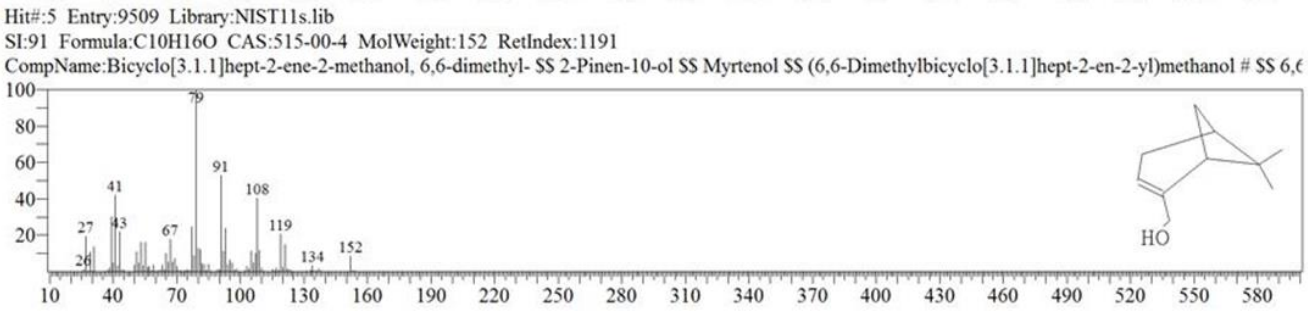

Figure (9): Peak of GC-MS analysis of Myrtenol

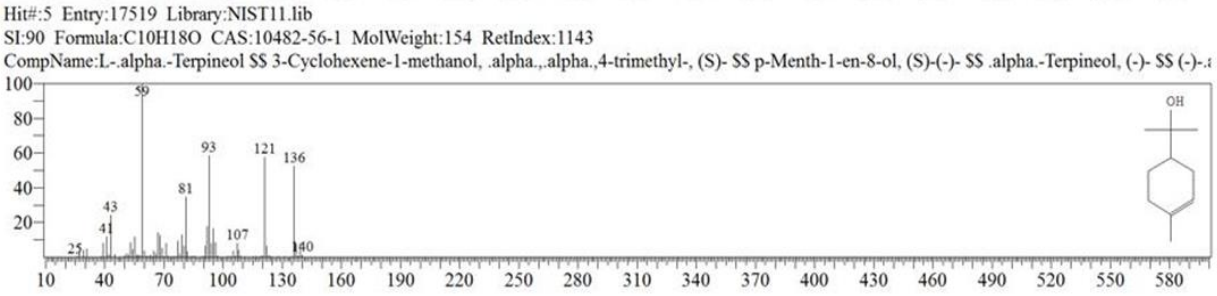

Figure (10): Peak of GC-MS analysis of L-alpha Terpinol

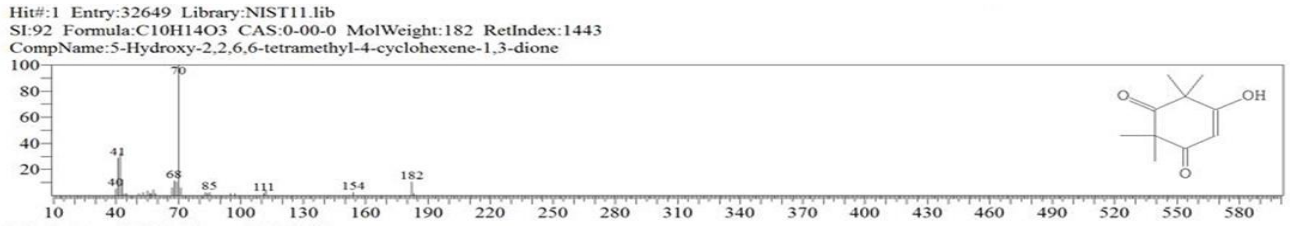

Figure (11): Peak of GC-MS analysis of Myrtocomulones

Discussion:

As shown in photo no. (1), the growth inhibition zones of the four replicates are overlapped each other, which indicates the powerful inhibitory activity, the tested extract has against the clinical MRSA isolate. Many previous studies reported and proved that *Myrtus communis* has good growth inhibition performance on *Staphylococcus aureus*, either standard strains or community and clinical isolates, but most of them were not used chloroform as a solvent and used Methanol instead. Studies in Libya, Morocco, Ethiopia, and Yamen were tested the methanol extract of *Myrtus communis* leaves collected from these different countries with against Standard *Staphylococcus aureus* strains; ATCC 25923 and CECT 976, and clinical MRSA, and the revealed inhibition zones were ranged from 9mm to 39mm, the zones which are less from what revealed in this study with the use of chloroform as solvent (Abouzeed *et al*., 2031; Bouyahya *et al*., 2016; Sisay *et al*., 2019; Abdulqawi and Quadri, 2021).

In addition, the study carried out by Eltawaty research team, 2020 was used methanol to extract the same part of this study plant which was collected from the same area and tested for its effect on both standard strain ATCC23925 and clinical MRSA and found that the extract actively inhibited the bacterial growth with inhibition zones 22mm and 25mm, respectively and showed MIC of 6.25mg/ml against the standard tested strain. Compared to our result and despite the different solvents used in their study and ours, and even though our study showed a larger inhibition zone with smaller MIC, their study is in an agreement with ours in that the leaves of *M. communis* contain bioactive compounds with the same polarities as methanol and chloroform and can inhibit the growth of MRSA.

No study has been found used chloroform as solvent except one Ethiopian study done by Besufekad and his colleagues, 2017, who reported that the chloroform extract of leaves of *M.communis* was inhibit the growth of the standard *Staphylococcus aureus* ATCC 27853 with an inhibition zone of only 2.2mm. Even though the circumstances of this study and ours are the same from the side of the part and solvent used, they were tested the extract against a standard strain while ours tested the performance of our extract a clinical multidrug-resistant MRSA and showed much higher growth inhibition activity with a zone of 68mm. The differences in ecosystem and

environment between Libya and Ethiopia in addition to the difference in the characteristics of both tested standard strain and clinical isolate of *Staphylococcus aureus* might be direct causes for this difference in biological performance.

When the beta-lactam resistant MRSA isolates were investigated for their sensitivity towards the antibiotics references used, the highest inhibition zone was shown with Ciprofloxacin, followed by Vancomycin and then Gentamycin, respectively, but our tested extract appeared as much as more active than the activity revealed by the used antibiotic references and showed higher growth inhibition zone, by the fold compared to Ciprofloxacin and about 3 fold compared with Gentamicin and Vancomycin, the drug of choice commonly used for treating of MRSA infection. As our extract gave a performance exceed what shown by the antibiotic commonly used for the treatment of MRSA infections, this study suggests this result as a pronouncing door is opening for the scoop of the presence of a new pharmaceutical agent/s in *M.communis* can effectively fight multi-drug resistant MRSA.

This study pushed us to a hypothesis that says that *M.communis* leaves have more than one growth inhibition strategy against MRSA, and this hypothesis suggests that one of these strategies may be the same as Ciprofloxacin via inhibition of cell division by interfering with DNA synthesis by inhibiting topoisomerase I enzyme (DNA gyrase) or by interfering with protein synthesis by binding with bacterial ribosomal sub-units; 30S!, as Gentamicin do. In addition, this study hypothesizes that the tested extract may have specific receptors on the tested MRSA wall surface, rather than the penicillin-binding protein receptors where it strongly binds to and penetrates the MRSA cell wall to reach the cytoplasm and disturb DNA and protein synthesis or even disrupt it.

This result directed this study to use GC-MS to analyze and figure out what are biologically active compounds present in the leaves of *M.communis* with a polarity same of chloroform and responsible for this high performance against this worldwide problematic bacteria; MRSA. GC-MS analysis showed that the tested extract contains 62 compounds that comprise previously identified compounds and unidentified compounds. Of the 62 compounds, ten compounds were proved to possess antibacterial activity.

Cyclohexene 1,5,5-trimethyl-6-acetylmethyl was revealed in this study as the highest percentage, it has been reported by Ben Hsouna *et al*., (2014), as has good antibacterial activity against *S.aureus*. Also, previous studies have been documented that the other nine compounds revealed in this study have a good performance against MRSA. Myrtenyl acetate and Myrtenol compounds that appeared in this study are well known to have remarkable antibiofilm activity (Kırmızıbekmez, *et al*., 2009, Hennia et al., 2019; Selvaraj et al., 2019; Cordeiro *et al*., 2020). Furthermore, the occurrence of Myrtocomulone in the results supports the good performance shown by the tested extract against MRSA, since this compound was reported by Vergalito, 2020, as having the ability to interfere and prevent the formation of bacterial biofilms. Upon all this with the previous result recorded by Yahya, 2021, which says that Gamma. -Sitosterol has antimicrobial

activities in general, this study suggests that the remarkable growth inhibition activity of chloroform extract of *M.commuis* leaves shown in this study against MRSA referred to these bioactive constituents.

By these valuable revealed compounds, it becomes sound to us that this extract has an anti-biofilm activity which may be combined with one or all of the three strategy pathways mentioned above can kill the bacteria and this hypothesis needs more study for either confirmation or more illustration

Moreover, this study results informed us that the tested extract has no activity against tested Gram-negative bacteria. Although many studies are in disagreement with our result, and despite they were used ethanol and methanol in the extraction process and not chloroform like ours, we see that more study should be carried out about the effectiveness of the tested extract on Gram-negative bacteria to stand on the exact fact of this result.

Conclusion:

Myrtus communis has been proved by this study having secondary metabolites with polarity same of the chloroform for which bta-lactam resistant Methicillin-resistant *Staphylococcus aureus* showed very high susceptibility, for which this study introduces *Myrtus communis* as a good resource of new pharmaceutical therapeutic agents could help in the management and treatment of infections caused by MRSA.

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