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Investigation of Some Chemical Compounds in *Salix Acmophylla* and Evaluate their Activity against Bacterial Biofilms

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Abstract: A major problem for the community is the increase in resistant illnesses, which are mostly caused by the negligent use of antibiotics. The purpose of this study was to investigate the antibacterial and antibiofilm properties for ethanolic extract of *Salix acmophylla* against isolates of *Staphylococcus aureus* (MRSA). By Using a Soxhlet device, leaves of *S. acmophylla* were extracted. Certain reagents were used to identify the active chemical compounds, which were then screened using gas chromatographic–mass spectral (GC-Mass) analysis. The antibiotic susceptibility test was performed to examine the sensitivity pattern of 15 clinical *Staphylococcus aureus* (MRSA) isolates that were found to form biofilms from a variety of clinical samples. *S. acmophylla* extract and sensitive antibiotics were studied for their minimal inhibitory concentrations (MIC and Sub MIC). The findings demonstrated that, in comparison to other components, this plant contains a higher number of glycosides and polyphenolic compounds. 45 chemicals were detected by GC/MS analysis. Eicosane and its isomers made up (21.27%) of the total contents, with 2-Cyclohexen-1-one coming in second with(11.04%) percent. However, in a concentration-dependent way, the ethanolic extract of *S. acmophylla* has a strong antibacterial action against MRSA isolates. The study also showed that, when compared to the most sensitive antibiotic (ciprofloxacin), *S. acmophylla* was quite effective against biofilms. In conclusion, the plant under study can be utilized as a natural and alternative defense against bacterial biofilm-induced chronic illnesses.

Keywords: Biofilms, Anti-Bacterial Agents, Ciprofloxacin.,Salicaceae.

INTRODUCTION

Since ancient times, traditional medical techniques have utilized therapeutic plants, often known as medicinal herbs, for a variety of purposes, including defense and protection against insects, fungi, and bacteria[1].Traditionally, all medical medicines were made from plants, either as raw extracts, mixes or as plant parts (leaf, roots, stem, bark, or flowers) and the alkaloids, terpenoids, polyphenols) and others are just a few of the bioactive substances found in plant extracts. These compounds have a wide range of properties, including antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory and anti-obesity properties[2].They have been applied to several conditions, including skin conditions, rheumatoid arthritis, osteoporosis, and cardiovascular ailments[3]. The active substances extracted from plants are medically and economically important compounds and a safer alternative to many antibiotics[4] The antibacterial properties of certain compounds found in plants, such as flavonoids, phenols, tannins, alkaloids, and saponins, account for their efficiency. The ability of alkaloids to enter bacterial cells and tamper with DNA is what defines them, while phenols are known for their capacity to form complexes with extracellular proteins and the cell wall that cause the bacterial cell membrane to rupture, tannins also function to inhibit transporter enzymes that are present in the cell membrane[5]. *Salix acmophylla* is a kind of flowering plant belongs to the Salicaceae family, which is a unique plant community found in Iraq along the Tigris and Euphrates rivers[6]. It is found throughout Iraq's moist regions and

mountains in the northeast still compared to neighboring nations like Iran and Turkey[7]. It has been discovered that this plant has a variety of medicinal uses for its various components. *S. acmophylla* is well-known for its ability to reduce inflammation and heal conditions including osteoarthritis and rheumatoid arthritis[8]. In addition to other uses in: medical field, in the case of cancer, heart illness, eye disease, dandruff, wound healing antibacterial, antiviral, antifungal, and antioxidant[9].The goal of this study was to examine the antibiofilm and antibacterial effect of *S. acmophylla* extract against the resistance to *Staphylococcus aureus* (MRSA) isolates.

METHODOLOGY

Plant extract preparation

Salix acmophylla leaves were gathered, washed with tap water and then spread out in an electrical oven with adequate air to ensure the plants were quite dry. By Using an electrical blender, dried leaves were ground up and extracted by a Soxhlet equipment with (500 ml) of ethanolic alcohol for eight hours[10].The extract was concentrated in a rotary evaporator and was kept in sterilized containers until they were needed.

Detection the active compounds in leaves extract of S. acmophylla

The Mondal method was followed to identify alkaloids using Dragendroff's reagent[11],whereas flavonoid detection was carried out in accordance with[12]. Conversely, the Evans method involved the use of lead acetate for tannin detection [13]. Saponins also were detected according to [14]. Benedict reagent was used to identify the glycosides [12].

Gas Chromatography –mass spectrometry (GC-MS) analysis

GC/MSD Chem instrument and AcqMethod QC3. were used to identify the active chemical compounds in this study. The carrier gas was helium with mobile phase flow rate set at (1.21 mL) min -1. The temperature of the instrument's oven was raised from (100 °C to 260 °C') at a rate of (10 °C min -1) and the volume per injection was set at 2 Pl. In GC-MS, an electron ionization energy system was used with 70 eV [15].The start and end times were (10 min and 70 min), respectively.

Bacterial Isolates

Bacterial isolates were acquired from the University of Baghdad- College of Sciences, Department of Biology.

Biofilm formation

Methicillin-resistant *Staph. aureus* (MRSA) bacterial isolates were the focusing of the biofilm formation detection process. Bacterial culture was adjusted to McFarland standard no. (0.5) after each isolate was propagated for (24 h) at 37°C in tryptic soy broth containing 1% glucose. In three sterile 96-well polystyrene microplate wells, (200 µl) of an isolated culture was introduced. For a (24h) period, all plates were incubated aerobically at 37°C with their lids on to prevent evaporation. A negative control consisted of three wells with tryptic soy broth free of microorganisms. The growing medium was taken out of the biofilm plate after incubation and cleaned three times with distilled buffer phosphate salt before being

fixed for (1h) at 60°C. After (15 min) at room temperature, an aliquot (200 µl) of crystal violet was applied to the wells. Following three rounds of washing, the plates were left at room temperature for ten minutes with a layer of (0.1%) buffer phosphate salt. Following that, (200 µl) of glacial acetic acid were applied to each hole at a (33%) concentration. The absorbance was measured at (630nm) using an ELISA reader to determine the isolates' potential to form a biofilm[16].

Antibiotics sensitivity test

The isolates were tested for antibiotic susceptibilities using the disc diffusion method in compliance with the guidelines set out by the Clinical and Laboratory Standards Institute (CLSI) (2023). The following antibiotics were used: Rifampin (5 µg), Ciprofloxacin (5 µg), Clindamycin (2 µg), Gentamicin (10 µg), Tetracycline (30 µg), Cefoxitin (30 µg), and Chloramphenicol (30 µg). Hi-media / India was the source of all the antibiotic discs.

Antibacterial activity S. acmophylla on disk diffusion in agar against Methicillin – resistant Staph. aureus (MRSA)

The plant extract's inhibitory efficacy was determined at doses ranging from (100, 80, 40, and 20) mg.ml for the crude extract made with Dimethyl sulfoxide (DMSO). as a diluent. The Mueller Hinton plate surface was evenly coated with the bacterial inoculum using a sterile cotton swab. Each of the four wells received (100 µl) of dilution after the agar was punctured with well of (5 mm) in diameter. (DMSO) considered as control. The plates were then left to incubate for an entire night at 37°C. Following the conclusion of the incubation period, the inhibition zones were measured using a ruler[17].

Determination of Minimal Inhibitory Concentration (MIC) for most sensitive antibiotics and S. acmophylla

By using dilution procedures, the most sensitive antibiotic's minimum inhibitory concentration (MIC) was ascertained after the inhibitory activity of three different antibiotic types- gentamicin(10µg), ciprofloxacin (5µg), and rifampin (5µg) was observed against the isolated bacterial species. A material that needs to be first dissolved to create a stock solution and then diluted to get the right concentration. Rifampin was diluted using a ratio of (9 ml methanol and 1 ml distilled water (D.W), while (1g) of each antibiotic (gentamicin and ciprofloxacin) was diluted with (10 ml) of D.W. [18]. The obtained extract's inhibitory efficacy was evaluated using the same methodology against bacterial species that had been isolated at a concentration of (20 mg. mL^{-1}). according to[19]. After incubation for (24h) period at 37°C, the turbidity was then measured using a (0.5) McFarland tube after (100 µg) of the bacterial suspension were added to an Eppendorf container along with glucose and heart and brain infusion broth. By using a micropipette, the wells in the plate were filled with three duplicates of each isolate, each with a volume of 200 µl (100 µl of bacterial suspension and 100 µl of plant extract). The most sensitive antibiotics were also added, and the plate was incubated for (24 h) at 37°C.After emptying the plate, it was washed three times with buffer phosphate salt and allowed to dry. Then, by using a micropipette, (20 µl) of the prepared Resazurin sodium salt were added to each hole, and the plate was incubated at 37°C for two hours. Following this, the plate was cleaned three times with solution Salt phosphate and

allowed to dry at room temperature. The Resazurin sodium salt was then prepared (0.015) with (100 D.W.) and mixed with a vortex device.

Antibiofilm activity of S. acmophylla extract and sensitive antibiotic on the biofilm at MIC and sub -MIC concentration

According to method of [20], the MIC and Sub MIC values were obtained for each *S. acmophylla* extract and the most sensitive antibiotic (ciprofloxacin) to assess their impact on the bacterial isolates' ability to form biofilms using the same protocol of the biofilm formation in the method previously mentioned[16].The equation used to calculate the biofilm inhibition rate was Biofilm inhibition (%) = (Control OD- Test OD / Control OD) × 100.

Statistical Analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference groups in study parameters. Least significant difference-LSD was used to significant compare between means. Chi-square test was used to significant compare between percentage 0.05 and 0.01 probability in this study.

RESULTS AND DISCUSSION

Detection the active compounds in Salix acmophylla leaves extract

The components of *S. acmophylla* were compiled in (Table 1). Six groups of mostly active chemicals were found to have produced positive findings in the ethanolic extract of the plant under study. The amount of precipitate or foam that formed during the test indicated a quantitative change in the presence rates. These sums consist of (alkaloids, Flavonoids, Polyphenolic Compounds, Tannins, Saponins, polysaccharide). included a higher number of contained saponins, glycosides and flavonoids in a greater percentage. As for the rest of the compounds, the percentages were medium similar.*S. acmophylla* contains a wide range of flavonoids which are unique to each type, such as flavones, flavanols, flavanones, dihydro flavanols, and isoflavones. It also contains compounds that are used medicinally since antiquity and have been linked to the discovery of acetylsalicylic acid and aspirin. These plants had been traditionally used to treat painful musculoskeletal joint pain conditions, inflammation, fever, antifungal, anticancer, and antioxidant[21].

GC/MS analysis of S. acmophylla leaves extract

GC/MS analysis of *S. acmophylla* leaf extract detected forty-five compounds. The main defined compounds were twenty-one, these compounds with their retention times and percentage of composition were listed in (Table 2) and figure (1). Eicosane and its isomers accounted for (21.27%) of the total constituents, with 2-Cyclohexen-1-one finishing second with (11.04%).Eicosane has antibacterial, anti-inflammatory, and antioxidant properties[22]. (2-cyclohexen-1-one), possess antioxidant qualities, biological action against a range of bacteria, and a cancer-prevention impact [23].

Antibiotics sensitivity test

According to the study's findings, many of the bacterial isolates that were found to be resistant to methicillin were also found to be resistant to cefoxitin (100%), azithromycin

(93.33%), clindamycin (86.67%), chloramphenicol (93.33%), and tetracycline (86.67%). It was responsive, nevertheless, to gentamycin (86.67%), ciprofloxacin (80.0%), and rifampicin (93.33%), figure (2). The results showed that the diversity in antibiotics was significant ($P<0.01$). Methicillin-resistant *Staphylococcus aureus* (MRSA) is always resistant to several antimicrobial drugs, such as cefoxitin, quinolones, macrolides, cephalosporins, tetracycline, penicillin, methicillin, oxacillin, and amoxicillin-clavulanic acid[24]. One of the causes of bacterial isolates' resistance to

beta-lactam antibiotics is the synthesis of lactamases, which are enzymes that break down the beta-lactam ring and so prevent the action of penicillin-group antibiotics[25]. The problem of antibiotic resistance is spreading

throughout the world. Methicillin-resistant *staphylococcus aureus* (MRSA) bacterial infections that have a larger negative impact on health and finances [26].

Investigation of the susceptibility of isolates to biofilm formation

All the bacterial isolates formed biofilm, although the severity of the biofilm varied. According to the table (3), roughly (86.67%) of the MRSA bacterial isolates developed strong biofilms, while only (13.33%) had moderate biofilms. Methicillin-resistant S*taph. aureus* (MRSA) can develop strains of these pathogens that, in conventional laboratory testing, either become exceedingly resistant to the same antimicrobials or become vulnerable to certain antimicrobials by building the biofilms. This may make treating infectious diseases involving biofilms difficult[27]. The problem of bacterial resistance makes it imperative to investigate the antibacterial properties of new substances. The biofilm or growth method of the bacteria is determined by the cells submerged in their own extracellular matrix. Numerous disorders, including persistent tissue infections like cystic fibrosis and infections of prosthetic joints or catheters, have been connected to bacterial biofilms [28]. Biofilm has a role in the pathophysiology of Methicillin-resistant *Staphylococcus aureus* (MRSA) infections. When bacteria are under stress, their genes express the biofilm gene as a stress response. Because of the slime-like glycocalyx known as biofilm, bacteria can flourish in harsh settings [29]. In addition, they can adhere to and colonies biotic or abiotic surfaces like prosthetic surfaces, which can act as a substrate for microbial adhesion and propagate throughout the body. Methicillin-resistant *Staph. aureus* (MRSA) [30].

Antibacterial activity S. acmophylla on disk diffusion in agar against Methicillin nt Staph. aureus (MRSA)

According to the current investigation, *Salix* leaves extract exhibits a good concentration-dependent antibacterial effectiveness. Against every isolate, the plant extract shown inhibitory qualities. The areas of inhibition were 17± 13, 15 ± 11, 13 ± 0, 11 ± 0 mm at (100, 80, 40, and 20 mg. mL^{-1}), respectively.

Determination of Minimal Inhibitory Concentration (MIC) and (Sub-MIC) for most sensitive antibiotics S. acmophylla and extract:

By using MIC tests, the antibacterial properties of *S. acmophylla* extract and Ciprofloxacin were investigated. The findings**,** Figure (4) demonstrated that the minimum inhibitory concentrations (MIC) for all MRSA isolates were (10 $mg.mL^{-1}$) and (0.25 $\mu g.mL^{-1}$),

respectively, for the plant extract and the most sensitive antibiotic (ciprofloxacin).Conversely, the Sub-MIC value for the plant extract was (5 $mg.mL^{-1}$), whereas the Sub-MIC value for the antibiotic that is most sensitive, ciprofloxacin, was (0.125 $\mu g.mL^{-1}$) for every MRSA isolate.

Antibiofilm activity of S. acmophylla extract and sensitive antibiotic at MIC concentration

According to the findings, most bacterial isolates were more susceptible to the MIC level of *S. acmophylla* ethanolic extract than to the antibiotic Ciprofloxacin. with a percentage of 35%, the Mr1 isolate showed the lowest plant extract antibiofilm activity, whereas the Mr9 isolate showed the highest antibiofilm activity (78%). As indicated in (Table 4), there is a considerable variation in the effectiveness between isolates, with a probability of ($P \leq 0.01$) in the creation of biofilms. The biologically active compounds' selectivity against clinical isolates and the synergistic effects of the crude extract components of biologically active compounds could be the cause of the disparity in the antibacterial efficacy of the extracts[3].

Antibiofilm activity of S. acmophylla extract and sensitive antibiotic at sub -MIC concentration

According to the findings, most isolates were more susceptible to the Sub-MIC level of *Salix acmophylla* ethanolic extract than to the antibiotic Ciprofloxacin. Table (5) illustrates the variety in the creation of biofilms amongst isolates and their varying levels of efficiency, all of which are statistically significant ($P \leq 0.01$).The bacteria's ability to colonies host cells is enhanced by the biofilm, which also gives them further defense against antibiotics. That is why it is crucial for bacterial survival as a virulence factor [31]. Antibiotic resistance in microorganisms is a known consequence of prolonged and high dosage usage, posing a significant risk to human health. A trend has emerged to employ natural herbal plant extracts as an alternative to antibiotics with the potential to combat antibiotic-resistant bacteria to reduce bacterial pathogenicity, including the production of biofilms[32]. In addition to the bioactive chemicals' selectivity towards clinical isolates. Moreover, the ethanolic extracts' antibiofilm activity is brought about by these bioactive substances. Additionally, the enzyme glycoside hydrolase is present in the herbal extracts. This enzyme aids in the disintegration of glycosidic connections in the polysaccharide chain of the biofilm into smaller subunits or monomers, which inhibits the biofilm [33].

CONCLUSION

The ethanolic extract from *S. acmophylla* leaves has antibacterial and antibiofilm properties and may be a useful source of active chemical components. It can be utilized as a natural and alternative defense against bacterial biofilm-induced chronic illnesses.

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Table (1) Active compounds in the ethanolic extract of *Salix acmophylla* leaves

Test Name	Reagent	Result Ethanolic Extract	Indication
Alkaloids	Dragan Groff	++	Orange, brown precipitate
Flavonoids	7.5%$FeCl_3$ solution	+++	Dark color
Polyphenolic Compounds	ferric chloride 5%solution	++	a-Brown color
	ferric chloride 1%solution	++	b-Dark color
Tannins	Lead Acetate 1%solution	+	creamy precipitate
Saponins	Foam formation	+++	Foam
polysaccharides	a-Anthrone Test	+++	
Carbohydrates and glycosides	b-Benedict reagent	+++	Reddish- brown precipitate

(+++ strong, ++ medium, + weak)

Table (2) Main components of *Salix acmophylla* leaves extract by GC/MS

No	RT (min)	Components	Area%
1	5.36	Cyclohexane	3.55
2	7.33	Toluene	2.43
3	13.72	3-Hexanol	6.86
4	14.13	2-Pentene,3-methyl-, (E)-	3.63
5	17.25	2-Cyclohexen-1-one	11.04
6	17.25	Eucalyptol	1.65
7	25.86	L-. alpha. –Terpineol	2.19
8	32.00	Acetamide, N-methyl-N-(2-propynyl)	2.78
9	39.98	gamma. –Cadinene	1.59
10	51.83	Cyclohexanol,1-ethynyl-2-methyl-, cis-	1.93
11	54.00	Nonadecane	7
12	55.91	1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	1.30
13	59.00	Heneicosane	9.19
14	60.21	Carvacrol, TBDMS derivative	0.68
15	60.42	9-Octadecenoic acid (Z)-	6.54
16	66.70	1H-Imidazole,4,5 dihydro-2- (pheny methyl)-	6.42
17	66..99	2-Methyl-5H-dibenz [b, f] azepin	2.68
18	66.93	1,2 Bis(trimethylsilyl)benzene	1.11
19	67.02	1-methyl-4-phenyl-5-thioxo-1,2,4-t riazolidin-3-one	1.15
20	63.90	Eicosane and its isomers	2.27
21	59.30	ICOSANE	10.97

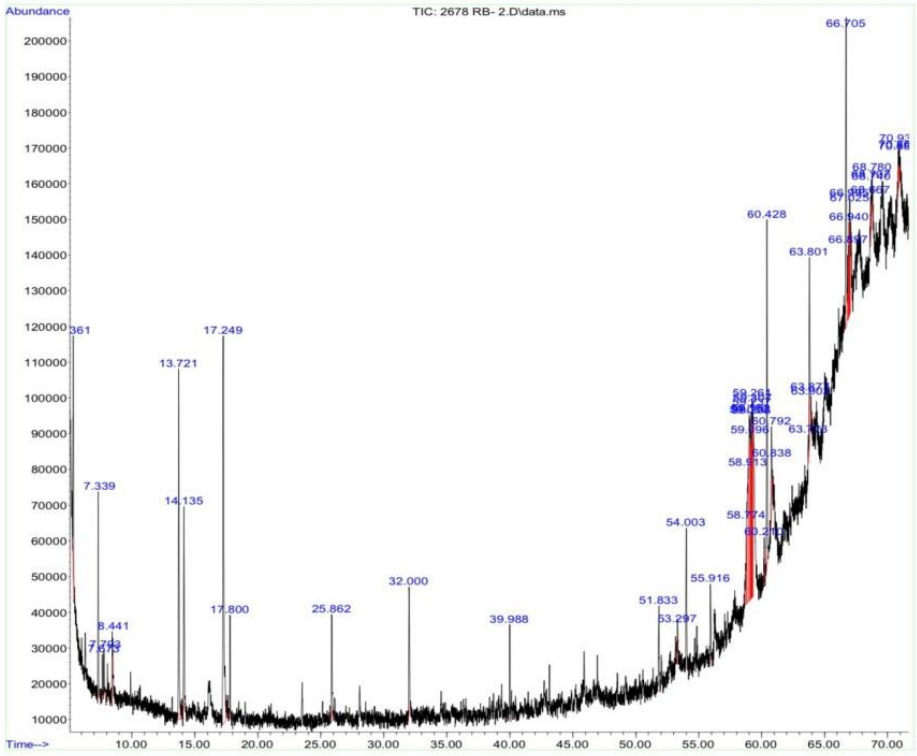

Figure (1) GC/MS analysis of *Salix acmophylla* leaves extract

Table (3) Biofilm formation by Methicillin – resistant *staphylococcus aureus* (MRSA) bacterial isolates.

Biofilm formation	No.	Percentage (%)
Strong	13	86.67
Moderate	2	13.33
Weak	0	0.00
Total	15	100
Chi-Square (χ^2)	---	9.836 **
P-value	---	0.0002

** (P≤0.01).

Table (4): The activity of MIC for *Salix acmophylla* extract and antibiotic on Biofilm

Bacteria	*Salix acmophylla* extract 10 mg. mL^{-1}	Antibiotic Ciprofloxacin 0.25 µg. mL^{-1}	P-value
1	35%	43%	0.0008 **
2	76%	41%	0.0001 **
3	37%	40%	0.0006 **
4	58%	55%	0.0001 **

5	55%	53%	0.802 NS
6	65%	65%	1.00 NS
7	66%	61%	0.0398 *
8	58%	51%	0.0041 **
9	78%	75%	0.159 NS
10	45%	63%	0.0084 **
11	61%	51%	0.072 NS
12	60%	52%	0.0001 **
13	51%	60%	0.0023 **
P-value	0.0001 **	0.0001 **	---

* (P≤0.05), ** (P≤0.01).

Table (5) Effect of Sub MIC of *Salix* extract and antibiotic on Biofilm

Bacteria	***Salix acmophylla* extract 5 mg. mL^{-1}**	**Antibiotic Ciprofloxacin 0.125 µg. mL^{-1}**	**P-value**
1	32%	20%	0.0074 **
2	73%	39%	0.0001 **
3	35%	18%	0.0001 **
4	56%	36%	0.0091 **
5	33%	43%	0.074 NS
6	66%	67%	0.502 NS
7	56%	38%	0.0036 **
8	55%	48%	0.0057 **
9	74%	75%	0.648 NS
10	41%	60%	0.0081 **
11	12%	5%	0.0094 **
12	58%	49%	0.0002 **
13	49%	58%	0.0007 **
P-value	0.0001 **	0.0001 **	---

** (P≤0.01).

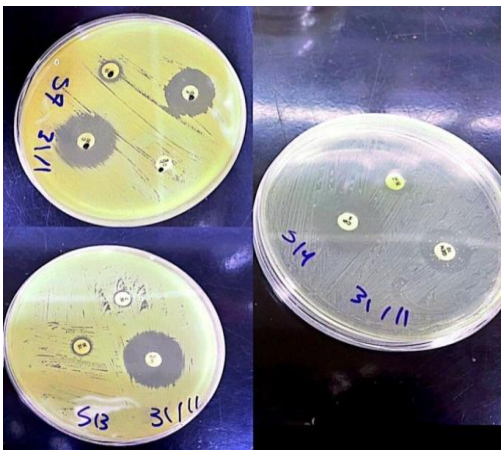

Figure (2) susceptibility test results for three isolates Methicillin – resistant *staphylococcus aureus* (MRSA)

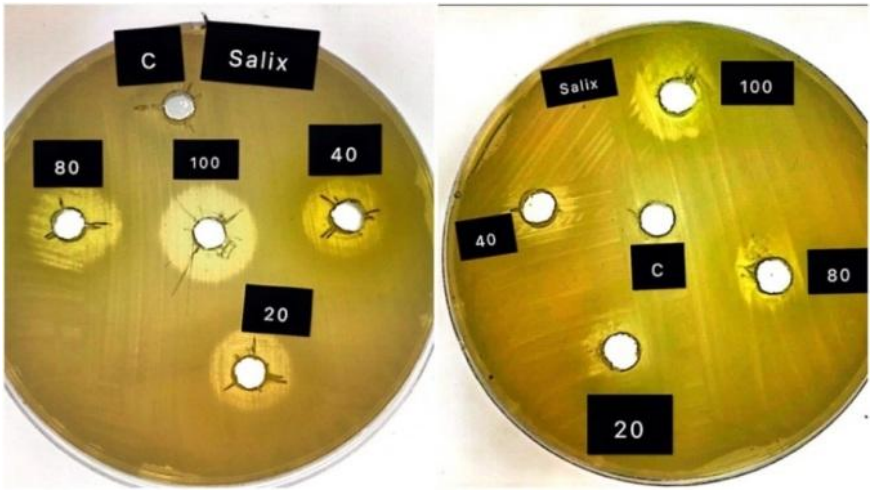

Figure (3) Zones of inhibition of Methicillin – resistant *staphylococcus aureus (MRSA)* bacterial isolates by ethanolic extracts of (*Salix acmophylla*)

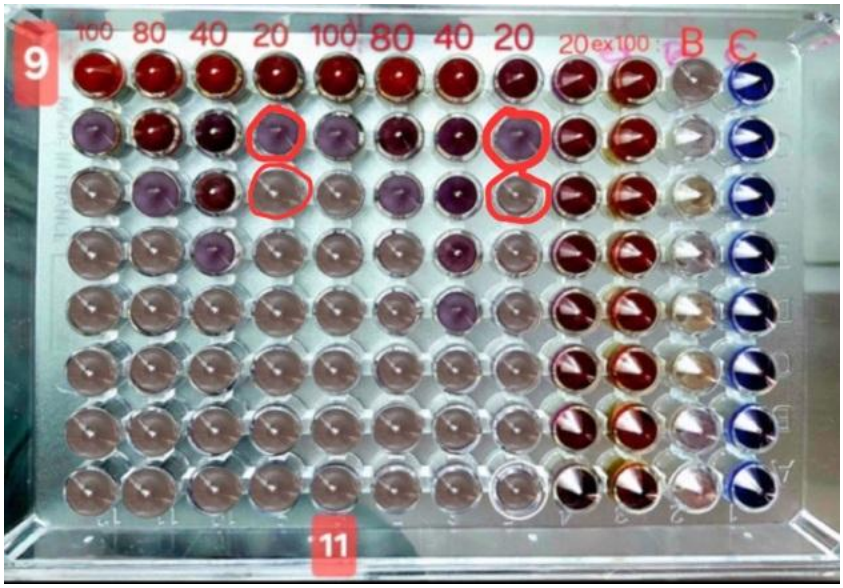

Figure (4) Determination of Minimal Inhibitory Concentration (MIC) and (Sub-MIC) for

(*Salix acmophylla)* extracts against some isolates ofMethicillin – resistant *staphylococcus aureus* (*MRSA*)