Materials and Methods

2.1 Materials

2.1.1 Equipments

The following equipments and apparatus were used in this study:

Equipment	Company (Origin)
Autoclave	GallenKamp (England)
Balance	Ohans (France)
Compound Light Microscope	Olympus (Japan)
Distillator	GallenKamp
Electrical oven	Memmert (Germany)
Hot plate with magnetic stirrer	GallenKamp
Incubator	GallenKamp
Micropipette	Witeg (Germany)
Millipore filters paper unit	Millipore and Whatman (England)
Minimal Electrophoresis apparatus	BioRad (Italy)
pH-Meter	Metter-GmpH Tdedo (U.K.)
Portable Centrifuge	Hermle labortechnik (Germany)
Rotary Evaporator	Buchi (Switzerland)
Sensitive balance	Delta Range (Switzerland)
Shaker Incubator	GFL (Germany)
Soxhlet	Electrothermal (England)
UV-Transillminator	Vilber Lourmat (France)
Vortex	Buchi (Switzerland)
Water bath	GFL (England)

2.1.2 Chemicals and Enzymes

The following chemicals and enzymes were used in this study:

-Analar (England)

Sucrose, Boric acid.

-BDH (England)

Ethylene diamine tetra-acetic acid (EDTA), Ethidium bromide, Bromophenol blue, Chloroform, Glycerol, $MgCl_2$, K_2SO_4, Ammonia, Sulphuric acid, Acetic anhydride, ferric chloride.

-Biolife (Italy)

Nutrient broth, Agar,

-Difco (USA)

MacConkey agar base

-Fluka (Switzerland)

Sodium dodecyl sulphate (SDS), Tris (hydroxymethyl) aminomethan base (Tris-base), Nutrient agar, Brain Heart Infusion Agar, Tris-hydrochloride (Tris-HCl).

-Merck (Germany)

NaCl

-Enzymes

Lysozyme (BDH-England).

-Sigma (USA)

Agarose.

-Oxoid (England)

Peptone

-Riedel-DeHaeny (Germany)

$MgSO_4.7H_2O$, Cetrimide, Methanol, Hexane.

2.1.3 Culture Media

2.1.3.1 Ready to Use Media:

Medium	Company (Origin)
Brain heart infusion agar	Fluka (Switzerland)
MacConkey agar	Difco (U.S.A)
Nutrient agar	Fluka (Switzerland)
Nutrient broth	Biolife (Italy)

They were prepared as recommended by the manufacturing company and sterilized by autoclaving at 121°C for 15 min.

2.1.3.2 Laboratory Prepared Media

-Citrmide Agar (Stolp and Gadkari, 1984)

It is composed of:

Peptone	20g
$MgCl_2$	4.5g
K_2SO4	10g
Cetrimide	0.3g
agar	15g
Distilled water	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving.

-King A Medium (Starr *et al*., 1981)

It is composed of

Peptone	20 g
Glycerol	10 ml
K_2SO_4	10 g
$MgCl_2$	1.4 g
agar	15 g
Distilled water	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving.

-King B Medium (Starr *et al*., 1981)

It is composed of:

Peptone	20g
Glycerol	10g
$MgSO_4.7H_2O$	3.5g
K_2SO_4	1.5g
agar	15g
Distilled Water	1000 ml

pH adjusted to 7.2 and sterilized by autoclaving.

2.1.4 Buffers and Solutions

2.1.4.1 Plasmid Extraction Buffers and Solutions

- **SET Buffer (Maniatis *et al*., 1982)**

  75mM NaCL

  25mM EDTA

  20mM Tris-Cl

pH was adjusted to 8 and sterilized by autoclave

- **Lysozyme Solution (50 mg/ml) (Kieser, 1995)**

This was prepared by dissolving 50 mg lysozyme enzyme in 1 ml of sterile distilled water, this solution prepared freshly.

- **Sodium Dodecyl Sulphate Solution (SDS) (100 mg/ml) (Kieser, 1995)**

It was freshly prepared by dissolving 10g SDS in 100 ml sterilized distilled water.

- **NaCl Solution** (5M) **(Kieser, 1995)**

- **TE Buffer (Maniatis *et al*., 1982)**

EDTA 1 mM

Tris-Cl 10 mM

pH was adjusted to 8 and sterilized by autoclave.

2.1.4.2 Gel Electrophoresis Buffer (Maniatis *et al*., 1982)

- **5X Tris-Borate-EDTA (TBE) (pH 8.0).**

Tris-base 54 g

Boric-acid 27.5 g

EDTA (0.5 M) 20 ml

Distilled water to 1000 ml

- **Loading Buffer**

Bromophenol blue 0.25 % (w/v)

Sucrose solution 40 % (w/v)

2.1.4.3 Curing Solution

- **SDS Solution**

It was prepared as stock solution of SDS (10 % w/v) in distilled water.

- **Ethidium Bromide Solution 10 mg / ml (Bounchaud *et al*., 1969)**

This solution was prepared by dissolving 0.2 g of ethidium bromide in 20 ml distilled water and stirred on magnetic stirrer for few hours to ensure

that the ethidium bromide has dissolved then it was sterilized by filtration, and stored in a dark bottle at 4ºC.

2.1.4.4 Antibiotic Solutions (Maniatis *et al*., 1982)

They were prepared as follows:

1- Ampicillin, neomycin, trimethoprin, chloromphenicol, gentamicin, aztreonam and streptomycin were prepared as stock solution of 10 mg/ml of antibiotic powders in D.W., sterilized by filtration and stored at -20ºC.

2- Tetracycline was prepared as stock solution of 10 mg/ml of tetracycline hydrochloride in ethanol/water (50% v/v), sterilized by filtration and stored in a dark bottle at -20ºC.

2.1.5 Bacterial Strains

Bacterial strains used in this study are listed in the following table:

Bacterial Strains	Phenotype	Source
E. coli HB101 Harboring pBR322	Amp^r, Tc^r	Department of Biotechnology / Al-Nahrain University
Pseudomonas aeruginosa RB19 harboring pSR 101 and pSR 202 plasmids	Neo^r, Crb^r, Tm^r	Department of Biotechnology / Al-Nahrain University
Klebsiella pneumonia MR1	Amp^r, Tc^r, C^r, Atm^r, Cn^r, S^r	Department of Biotechnology / Al-Nahrain University

Atm=Aztronam; Amp=Ampicillin; Cn =Gentamicin; C =Chloramphenicol; Crb =Carbincillin; Tm =Trimethprim; Tc =Tetracycline; S =Streptomycin; Neo =Neomycin; r =Resistance

2.1.6 Antibiotics Discs

The following antibiotic discs are used in the study:

Antibiotic	Code	Concentration (µg)	Source (Origin)
Ampicillin	AMP	10	Bioanalyse LTD (Turky)
Aztreonam	ATM	30	Bioanalyse LTD
Carbenicillin	CRB	100	Bioanalyse LTD
Cefotaxime	CTX	30	Bioanalyse LTD
Chloramphenicol	C	30	Bioanalyse LTD
Gentamicin	CN	10	Bioanalyse LTD
Imipenem	IPM	10	Bioanalyse LTD
Kanamycin	KAN	30	Bioanalyse LTD
Nalidixic acid	NAL	30	Bioanalyse LTD
Neomycin	NEO	30	Bioanalyse LTD
Rifampicin	RIF	10	Bioanalyse LTD
Trimethoprim	TM	30	Bioanalyse LTD
Tobramycin	TOB	10	Bioanalyse LTD
Streptomycin	S	10	Bioanalyse LTD
Tetracycline	TE	30	Bioanalyse LTD

2.2 Methods

2.2.1 Plants Collection

Salix acomophylla, *Urtica dioica* and *Calendula officinalis* were collected from different places of Baghdad city and identified by the Iraqi National Herbarium (Baghdad, Abu-Graib).

2.2.2 Preparation of Plant Extracts (*Salix acomophylla, Urtica dioica and Calendula officinalis*)

1. Hexane Extract

Fifty grams of each plant powder (*Salix acomophylla, Urtica dioica* (leaves) and *Calendula officinalis* flowers) was extracted with 250 ml hexane by soxhlet apparatus for 6 hrs at 40-60ºC, the cooled solution was evaporated to dryness by rotary evaporator at 40ºC and the crude extract was kept until used (Al-Jeboory, 1994).

2. Methanolic Extract

Fifty grams of each plant powder (*Salix acomophylla, Urtica dioica* (leaves) and *Calendula officinalis* flowers) was extracted with 250 ml of methanol 70% by soxhlet apparatus for 6 hrs at 40-60ºC, then the solvent was evaporated under reduced pressure by the rotary evaporator at 40ºC, the crude solid extract was kept until used (Sababi *et al*., 1987).

2.2.3 Detection of Some Active Compounds:

1. Detection of Terpens

One gram of each plant hexane extract Precipitated in a few drops of chloroform, and then a drop of acetic anhydride and a drop of concentrated sulphuric acid were added. Dark blue color appeared represented the presence of terpens in the extracts (Al-Abid, 1985).

2. Detection of Flavonoids

Methnolic extracts for each plant was partitioned with petroleum ether, the aqueous layer was mixed with the ammonium solution. The appearance of dark color is an evidence for the presence of flavonoids (Harborne, 1973).

3. Detection of Glycosides

Equal amount of the plant methanolic extract was mixed with Fehling reagent in a test tube, then boiled in a water bath for 10 min. Red precipitate indicate the presence of glycosides (Shihata, 1951).

4. Detection of Saponins

Five ml of plant methanolic extract was added to 1-3 ml of ferric chloride solution, the appearance of white precipitate indicate the presence of saponins and other phenolic compounds (Stahl, 1969).

2.2.4 Maintenance of Bacterial Strains

Maintenance of bacterial strains was performed according to Maniatis *et al*., (1982) and as following:

2.2.4.1 Short Term Storage

Colonies of bacteria were maintained for periods of few weeks on the surface of nutrient agar media. The plates were tightly wrapped with parafilm and stored at 4°C.

2.2.4.2 Medium Term Storage

Strains of bacteria were maintained in stab culture for periods of a few months. Such cultures were prepared in small screw cupped bottles containing 2-3 ml of agar medium. The cultures were incubated using a sterile straight platinum wire that is dipped into dense liquid culture of bacteria and then stabbed deep into the agar medium.

After proper incubation the cap was wrapped tightly with parafilm to prevent desiccation of the medium and kept at 4°C.

2.2.4.3 Long Term Storage

Bacteria can be stored for many years in nutrient broth medium containing 15% glycerol at low temperature without significant loss viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacteria in small screw-capped vials with final volume of 10 ml and stored at -20°C.

2.2.5 *Pseudomonas aeruginosa* RB19

A. Morphology of Colonies

Shape, color and viscosity of the colonies were studied on plates of nutrient agar (NA) medium after incubation of the isolates on these plates at 37°C for 24 hr.

Gram Stain (Atlas *et al*., 1995)

The specimen was applied to clean slide, then the specimen was fixed by heat, crystal violet was applied (for 1 minute) and excess stain was washed, then Gram's iodine was applied (for 1 min) and the excess was washed with distilled water. Alcohol decolorizing agent was applied and the excess was washed. After that sufranins was applied (for 30 sec) and the excess was washed and finally the slide was dried for examination under the microscope.

B. Biochemical Tests

The following biochemical tests were performed:

-Growing on King A (Cruickshank *et al*., 1975)

Inoculate the bacteria on the plate by streaking and incubate at 37°C for 24 hr. this test was performed to study the production of the characteristic pigment, pyocyanin.

-Growth on King B (Cruickshank *et al*., 1975)

Inoculate the bacteria on the plate by streaking and incubate at 37°C for 24 hr**.** then the plates were exposed to UV. This test was performed to study the production of the characteristic pigment, flourescein.

-Growth on Cetrimide Agar (Greenwood *et al*, 1997)

This media was used as a selective medium for *Pseudomonas* spp. The plates were inoculated with bacteria by streaking and incubated at 37°C for 24 hr.

-Growth on MacConkey Agar (Atlas *et al*., 1995)

MacConkey agar is a differential plating medium for the selection and recovery of enterobacteriaceae related enteric Gram negative rods, Lactose is the sole carbohydrate source. Lactose fermenting bacteria produce colonies that are varying shades of red because of the indicator dye (red below pH 6.8) form the production of mixed acids. Colonies of non lactose-fermenting bacteria appear colorless or transparent on this medium.

-Growth at 4°C and at 42°C (Collins and Lyne, 1987)

One of the tests for the species of the genus *Pseudomonas* is by growing the bacterial isolates (or the suspected *Pseudomonas* isolates) on nutrient agar plates and incubated at 4°C and at 42°C for 24 hr.

2.2.6 Antibiotic Sensitivity Test (Atlas *et al*., 1995)

The disc diffusion method was used to test the antibiotic sensitivity of the selected isolate. A sterile cotton swab was dipped into the inoculum's (freshly culture, 18 hr) and the entire surface of the brain heart infusion agar plates was swabbed three times by rotating the plate approximately 60° between streaking to ensure even distribution. Then the discs of the antibiotics were applied and incubated at 37°C. The zone of inhibition was observed after incubation for 18 hr.

2.2.7 Minimum Inhibitory Concentration (MIC) Test

Cells of selected isolates were grown in 5ml nutrient broth, then 0.1ml of each culture were inoculated in series of 5ml fresh nutrient broth containing various concentrations of plant extracts (0, 5, 10, 20, 30, 40, 50, 75, 100, 200, 300, 400 and 500 mg/ml) for both isolates *Pseudomonas aeruginosa* RB19 and *Klebsiella pneumoniae* MR1, then all tubes incubated in 37°C for 24 hrs. 100 µl from each tube were spread on brain heart infusion agar plates and all plates were incubated in 37°C for 24 hrs. The lowest concentration of the plant extract that inhibited the growth of bacterial isolate considered as the minimum inhibitory concentration (MIC).

Note: For all extracts which were insoluble in water, 1ml of ethanol was added to the residue then the volume completed with sterile distilled water.

2.2.8 Plasmid Extraction

DNA extraction was done by salting out method which described by Kieser (1995), as follow:

-Culture of bacteria grown in nutrient broth, was pelleted form 20 ml by centrifugation at 6000 rpm for 15 min.

-The pellet washed with 3 ml of SET buffer and resuspended the cells with 1.6 ml of SET buffer, then freshly prepared lysozyme (final concentration 1 mg/ml) was added and incubated at 37°C for 30 min.

-One ml of 10 % SDS was added, mixed by inversion, then incubated at room temperature for 15 min.

-Add 2 ml of 5 M NaCl, mix thoroughly by inversion.

-An equal volume of chloroform was added, mixed by inversion for 15 min. Then centrifuged (6000 rpm at 4°C) for 20 min.

-The aqueous phase (upper) was transferred to another sterile tube, and 0.6 volume of isopropanol was added, mixed by inversion, and kept at room temperature for 5 min.

-Centrifuged at 13000 rpm for 15 min at 4°C.

-The isoproparnol was discarded and the precipitated DNA dissolved in 100 µl TE buffer and stored at -20C.

2.2.9 Agarose Gel Electrophoresis (Sambrook *et al*., 1989)

Agarose gel (0.7%) were run horizontally in tris-borate-EDTA (TBE 1X). Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells on the gel. Generally, gels were run for 2-3 hrs at 5 v/cm and the gel buffer added up to the level of horizontal gel surface.

Agarose gel were stained with ethidium bromide by immersing them in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 min. DNA bands were visualized by UV illumination at 302 nm on an UV transilluminator. Gels were destained in distilled water for 30-60 min to get rid of background before photographs were taken.

2.2.10 Plasmid Curing

2.2.10.1 Curing of *P. aeruginosa* RB19 Plasmid DNA with SDS

Curing experiments were performed on the *P. aeruginosa* RB19 isolate SDS was used in these experiments as curing agent according to Trevors (1986) and as follows:

Cells of the selected isolate were grown in 5 ml of nutrient broth to mid log phase ($O.D._{600}$ about 0.4-0.5). 0.1 ml samples of each culture were inoculated in a series of 5 ml nutrient broth tubes containing various concentrations of SDS (0, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%). All tubes were incubated at 37°C for 24-48hrs. The growth density of the different tubes was measured visually and compared with the control to determine the effect of each concentration of curing agent on bacterial growth. The lowest concentration of

curing agent that inhibits the growth of the bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken from tubes containing the highest concentration of SDS that still allows bacterial growth and diluted appropriately. Then 0.1 ml samples from proper dilutions were spread on nutrient agar plates and incubated over night at 37°C to score the survival colonies.

2.2.10.2 Curing of *Klebsiella pneumoniae* MR1 Plasmid DNA with Ethidium Bromide

Curing experiments were performed on the *Klebsiella pneumonae* MR1 using ethidium bromide as curing agent, according to Trevors (1986) and as follows:

Cells of the selected isolate were grown in 5 ml of nutrient broth to mid log phase ($O.D._{600}$ about 0.4 – 0.5). 0.1 ml samples of each culture were inoculated in a series of 5 ml fresh nutrient broth tubes containing various concentrations of ethidium bromide (0, 20, 50, 100, 200, 250, 300, 400, 800, 1600 and 3200 μg / ml). All tubes were incubated at 37ºC for 24-48 hrs.

The growth density of the different tubes was measured visually and compared with the control to determine the effect of each concentration of curing agent on bacterial growth. The lowest concentration of curing agent that inhibited the growth of the bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken form tubes containing the highest concentration of ethidium bromide that still allows bacterial growth and diluted appropriately. Then 0.1 ml samples from proper dilutions were spread on nutrient agar plates and incubated overnight at 37ºC to score the survived colonies.

2.2.10.3 Curing of Plasmid DNA with Plant Extract

Alcoholic extracts of (*Salix acomophylla*, *Urtica dioica* and *Calendula officinalis*), were used in an attempts to cure plasmid content of *Pseudomonas*

aeruginosa RB19 and the pathogenic *Klebsiella pneumoniae* MR1 isolates depending on Trevors (1986).

Cells of the selected isolates were grown in 5ml nutrient broth to mid log phase ($O.D._{600}$ about 0.4-0.5). 0.1ml samples were inoculated in a series of 5 ml fresh nutrient broth containing various concentrations (0, 10, 20, 30, 40, 50, 75, 100, 200, 300, 400 and 500 mg/ml) of hexane and methanolic extracts of the three plants for *Pseudomonas aeruginosa* RB19, and (0, 10, 20, 30, 40, 50, 75 and 100 mg/ml) for *Klebsiella pneumoniae* MR1, All tubes were incubated at 37°C for 24-48 hrs.

The growth density of different tubes was measured visually and compared with control to determine the effect of each concentration of each plant extract on bacterial growth. The lowest concentration of plant extract that inhibited the growth of bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken from tubes containing the highest concentration of each plant extract that still allows bacterial growth and diluted appropriately. Then 0.1ml samples from proper dilutions were spread on nutrient agar plates and incubated overnight at 37°C to score the survived colonies.

2.2.11 Selection of Cured Cells

After treatment of bacterial isolate with standard curing agent and plant extracts and the isolation of survivors on nutrient agar, survivors were analyzed for the presence or absence of drug resistance as a result of eliminating the plasmid by selecting 100 colonies of bacterial isolate from each treatment. These colonies were replica plated (using toothpick) on nutrient agar plates (master plates) and on nutrient agar plates containing an antibiotic to which the original isolate is resistant (Trevors, 1986).

If a colony was able to grow on the master plate but not on the selective agar containing the appropriate antibiotic, it means that the cells of this colony

are cured cells that lost plasmid responsible on resistance to this antibiotic. The percentage of cured cells was determined.

Materials and Methods

2.1 Materials

2.1.1 Equipments

The following equipments and apparatus were used in this study:

Equipment	Company (Origin)
Autoclave	GallenKamp (England)
Balance	Ohans (France)
Compound Light Microscope	Olympus (Japan)
Distillator	GallenKamp
Electrical oven	Memmert (Germany)
Hot plate with magnetic stirrer	GallenKamp
Incubator	GallenKamp
Micropipette	Witeg (Germany)
Millipore filters paper unit	Millipore and Whatman (England)
Minimal Electrophoresis apparatus	BioRad (Italy)
pH-Meter	Metter-GmpH Tdedo (U.K.)
Portable Centrifuge	Hermle labortechnik (Germany)
Rotary Evaporator	Buchi (Switzerland)
Sensitive balance	Delta Range (Switzerland)
Shaker Incubator	GFL (Germany)
Soxhlet	Electrothermal (England)
UV-Transillminator	Vilber Lourmat (France)
Vortex	Buchi (Switzerland)
Water bath	GFL (England)

2.1.2 Chemicals and Enzymes

The following chemicals and enzymes were used in this study:

-Analar (England)

Sucrose, Boric acid.

-BDH (England)

Ethylene diamine tetra-acetic acid (EDTA), Ethidium bromide, Bromophenol blue, Chloroform, Glycerol, $MgCl_2$, K_2SO_4, Ammonia, Sulphuric acid, Acetic anhydride, ferric chloride.

-Biolife (Italy)

Nutrient broth, Agar,

-Difco (USA)

MacConkey agar base

-Fluka (Switzerland)

Sodium dodecyl sulphate (SDS), Tris (hydroxymethyl) aminomethan base (Tris-base), Nutrient agar, Brain Heart Infusion Agar, Tris-hydrochloride (Tris-HCl).

-Merck (Germany)

NaCl

-Enzymes

Lysozyme (BDH-England).

-Sigma (USA)

Agarose.

-Oxoid (England)

Peptone

-Riedel-DeHaeny (Germany)

$MgSO_4.7H_2O$, Cetrimide, Methanol, Hexane.

2.1.3 Culture Media

2.1.3.1 Ready to Use Media:

Medium	Company (Origin)
Brain heart infusion agar	Fluka (Switzerland)
MacConkey agar	Difco (U.S.A)
Nutrient agar	Fluka (Switzerland)
Nutrient broth	Biolife (Italy)

They were prepared as recommended by the manufacturing company and sterilized by autoclaving at 121°C for 15 min.

2.1.3.2 Laboratory Prepared Media

-Citrmide Agar (Stolp and Gadkari, 1984)

It is composed of:

Peptone	20g
$MgCl_2$	4.5g
K_2SO4	10g
Cetrimide	0.3g
agar	15g
Distilled water	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving.

-King A Medium (Starr *et al*., 1981)

It is composed of

Peptone	20 g
Glycerol	10 ml
K_2SO_4	10 g
$MgCl_2$	1.4 g
agar	15 g
Distilled water	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving.

-King B Medium (Starr *et al*., 1981)

It is composed of:

Peptone	20g
Glycerol	10g
$MgSO_4.7H_2O$	3.5g
K_2SO_4	1.5g
agar	15g
Distilled Water	1000 ml

pH adjusted to 7.2 and sterilized by autoclaving.

2.1.4 Buffers and Solutions

2.1.4.1 Plasmid Extraction Buffers and Solutions

- **SET Buffer (Maniatis *et al*., 1982)**

  75mM NaCL

  25mM EDTA

  20mM Tris-Cl

pH was adjusted to 8 and sterilized by autoclave

- **Lysozyme Solution (50 mg/ml) (Kieser, 1995)**

This was prepared by dissolving 50 mg lysozyme enzyme in 1 ml of sterile distilled water, this solution prepared freshly.

- **Sodium Dodecyl Sulphate Solution (SDS) (100 mg/ml) (Kieser, 1995)**

It was freshly prepared by dissolving 10g SDS in 100 ml sterilized distilled water.

- **NaCl Solution** (5M) **(Kieser, 1995)**

- **TE Buffer (Maniatis *et al*., 1982)**

EDTA 1 mM

Tris-Cl 10 mM

pH was adjusted to 8 and sterilized by autoclave.

2.1.4.2 Gel Electrophoresis Buffer (Maniatis *et al*., 1982)

- **5X Tris-Borate-EDTA (TBE) (pH 8.0).**

Tris-base 54 g

Boric-acid 27.5 g

EDTA (0.5 M) 20 ml

Distilled water to 1000 ml

- **Loading Buffer**

Bromophenol blue 0.25 % (w/v)

Sucrose solution 40 % (w/v)

2.1.4.3 Curing Solution

- **SDS Solution**

It was prepared as stock solution of SDS (10 % w/v) in distilled water.

- **Ethidium Bromide Solution 10 mg / ml (Bounchaud *et al*., 1969)**

This solution was prepared by dissolving 0.2 g of ethidium bromide in 20 ml distilled water and stirred on magnetic stirrer for few hours to ensure

that the ethidium bromide has dissolved then it was sterilized by filtration, and stored in a dark bottle at 4ºC.

2.1.4.4 Antibiotic Solutions (Maniatis *et al*., 1982)

They were prepared as follows:

1- Ampicillin, neomycin, trimethoprin, chloromphenicol, gentamicin, aztreonam and streptomycin were prepared as stock solution of 10 mg/ml of antibiotic powders in D.W., sterilized by filtration and stored at -20ºC.

2- Tetracycline was prepared as stock solution of 10 mg/ml of tetracycline hydrochloride in ethanol/water (50% v/v), sterilized by filtration and stored in a dark bottle at -20ºC.

2.1.5 Bacterial Strains

Bacterial strains used in this study are listed in the following table:

Bacterial Strains	Phenotype	Source
E. coli HB101 Harboring pBR322	Amp^r, Tc^r	Department of Biotechnology / Al-Nahrain University
Pseudomonas aeruginosa RB19 harboring pSR 101 and pSR 202 plasmids	Neo^r, Crb^r, Tm^r	Department of Biotechnology / Al-Nahrain University
Klebsiella pneumonia MR1	Amp^r, Tc^r, C^r, Atm^r, Cn^r, S^r	Department of Biotechnology / Al-Nahrain University

Atm=Aztronam; Amp=Ampicillin; Cn =Gentamicin; C =Chloramphenicol; Crb =Carbincillin; Tm =Trimethprim; Tc =Tetracycline; S =Streptomycin; Neo =Neomycin; r =Resistance

2.1.6 Antibiotics Discs

The following antibiotic discs are used in the study:

Antibiotic	Code	Concentration (µg)	Source (Origin)
Ampicillin	AMP	10	Bioanalyse LTD (Turky)
Aztreonam	ATM	30	Bioanalyse LTD
Carbenicillin	CRB	100	Bioanalyse LTD
Cefotaxime	CTX	30	Bioanalyse LTD
Chloramphenicol	C	30	Bioanalyse LTD
Gentamicin	CN	10	Bioanalyse LTD
Imipenem	IPM	10	Bioanalyse LTD
Kanamycin	KAN	30	Bioanalyse LTD
Nalidixic acid	NAL	30	Bioanalyse LTD
Neomycin	NEO	30	Bioanalyse LTD
Rifampicin	RIF	10	Bioanalyse LTD
Trimethoprim	TM	30	Bioanalyse LTD
Tobramycin	TOB	10	Bioanalyse LTD
Streptomycin	S	10	Bioanalyse LTD
Tetracycline	TE	30	Bioanalyse LTD

2.2 Methods

2.2.1 Plants Collection

Salix acomophylla, *Urtica dioica* and *Calendula officinalis* were collected from different places of Baghdad city and identified by the Iraqi National Herbarium (Baghdad, Abu-Graib).

2.2.2 Preparation of Plant Extracts (*Salix acomophylla, Urtica dioica and Calendula officinalis*)

1. Hexane Extract

Fifty grams of each plant powder (*Salix acomophylla, Urtica dioica* (leaves) and *Calendula officinalis* flowers) was extracted with 250 ml hexane by soxhlet apparatus for 6 hrs at 40-60ºC, the cooled solution was evaporated to dryness by rotary evaporator at 40ºC and the crude extract was kept until used (Al-Jeboory, 1994).

2. Methanolic Extract

Fifty grams of each plant powder (*Salix acomophylla, Urtica dioica* (leaves) and *Calendula officinalis* flowers) was extracted with 250 ml of methanol 70% by soxhlet apparatus for 6 hrs at 40-60ºC, then the solvent was evaporated under reduced pressure by the rotary evaporator at 40ºC, the crude solid extract was kept until used (Sababi *et al*., 1987).

2.2.3 Detection of Some Active Compounds:

1. Detection of Terpens

One gram of each plant hexane extract Precipitated in a few drops of chloroform, and then a drop of acetic anhydride and a drop of concentrated sulphuric acid were added. Dark blue color appeared represented the presence of terpens in the extracts (Al-Abid, 1985).

2. Detection of Flavonoids

Methnolic extracts for each plant was partitioned with petroleum ether, the aqueous layer was mixed with the ammonium solution. The appearance of dark color is an evidence for the presence of flavonoids (Harborne, 1973).

3. Detection of Glycosides

Equal amount of the plant methanolic extract was mixed with Fehling reagent in a test tube, then boiled in a water bath for 10 min. Red precipitate indicate the presence of glycosides (Shihata, 1951).

4. Detection of Saponins

Five ml of plant methanolic extract was added to 1-3 ml of ferric chloride solution, the appearance of white precipitate indicate the presence of saponins and other phenolic compounds (Stahl, 1969).

2.2.4 Maintenance of Bacterial Strains

Maintenance of bacterial strains was performed according to Maniatis *et al*., (1982) and as following:

2.2.4.1 Short Term Storage

Colonies of bacteria were maintained for periods of few weeks on the surface of nutrient agar media. The plates were tightly wrapped with parafilm and stored at 4°C.

2.2.4.2 Medium Term Storage

Strains of bacteria were maintained in stab culture for periods of a few months. Such cultures were prepared in small screw cupped bottles containing 2-3 ml of agar medium. The cultures were incubated using a sterile straight platinum wire that is dipped into dense liquid culture of bacteria and then stabbed deep into the agar medium.

After proper incubation the cap was wrapped tightly with parafilm to prevent desiccation of the medium and kept at 4°C.

2.2.4.3 Long Term Storage

Bacteria can be stored for many years in nutrient broth medium containing 15% glycerol at low temperature without significant loss viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacteria in small screw-capped vials with final volume of 10 ml and stored at -20°C.

2.2.5 *Pseudomonas aeruginosa* RB19

A. Morphology of Colonies

Shape, color and viscosity of the colonies were studied on plates of nutrient agar (NA) medium after incubation of the isolates on these plates at 37°C for 24 hr.

Gram Stain (Atlas *et al*., 1995)

The specimen was applied to clean slide, then the specimen was fixed by heat, crystal violet was applied (for 1 minute) and excess stain was washed, then Gram's iodine was applied (for 1 min) and the excess was washed with distilled water. Alcohol decolorizing agent was applied and the excess was washed. After that sufranins was applied (for 30 sec) and the excess was washed and finally the slide was dried for examination under the microscope.

B. Biochemical Tests

The following biochemical tests were performed:

-Growing on King A (Cruickshank *et al*., 1975)

Inoculate the bacteria on the plate by streaking and incubate at 37°C for 24 hr. this test was performed to study the production of the characteristic pigment, pyocyanin.

-Growth on King B (Cruickshank *et al*., 1975)

Inoculate the bacteria on the plate by streaking and incubate at 37°C for 24 hr**.** then the plates were exposed to UV. This test was performed to study the production of the characteristic pigment, flourescein.

-Growth on Cetrimide Agar (Greenwood *et al*, 1997)

This media was used as a selective medium for *Pseudomonas* spp. The plates were inoculated with bacteria by streaking and incubated at 37°C for 24 hr.

-Growth on MacConkey Agar (Atlas *et al*., 1995)

MacConkey agar is a differential plating medium for the selection and recovery of enterobacteriaceae related enteric Gram negative rods, Lactose is the sole carbohydrate source. Lactose fermenting bacteria produce colonies that are varying shades of red because of the indicator dye (red below pH 6.8) form the production of mixed acids. Colonies of non lactose-fermenting bacteria appear colorless or transparent on this medium.

-Growth at 4°C and at 42°C (Collins and Lyne, 1987)

One of the tests for the species of the genus *Pseudomonas* is by growing the bacterial isolates (or the suspected *Pseudomonas* isolates) on nutrient agar plates and incubated at 4°C and at 42°C for 24 hr.

2.2.6 Antibiotic Sensitivity Test (Atlas *et al*., 1995)

The disc diffusion method was used to test the antibiotic sensitivity of the selected isolate. A sterile cotton swab was dipped into the inoculum's (freshly culture, 18 hr) and the entire surface of the brain heart infusion agar plates was swabbed three times by rotating the plate approximately 60° between streaking to ensure even distribution. Then the discs of the antibiotics were applied and incubated at 37°C. The zone of inhibition was observed after incubation for 18 hr.

2.2.7 Minimum Inhibitory Concentration (MIC) Test

Cells of selected isolates were grown in 5ml nutrient broth, then 0.1ml of each culture were inoculated in series of 5ml fresh nutrient broth containing various concentrations of plant extracts (0, 5, 10, 20, 30, 40, 50, 75, 100, 200, 300, 400 and 500 mg/ml) for both isolates *Pseudomonas aeruginosa* RB19 and *Klebsiella pneumoniae* MR1, then all tubes incubated in 37°C for 24 hrs. 100 µl from each tube were spread on brain heart infusion agar plates and all plates were incubated in 37°C for 24 hrs. The lowest concentration of the plant extract that inhibited the growth of bacterial isolate considered as the minimum inhibitory concentration (MIC).

Note: For all extracts which were insoluble in water, 1ml of ethanol was added to the residue then the volume completed with sterile distilled water.

2.2.8 Plasmid Extraction

DNA extraction was done by salting out method which described by Kieser (1995), as follow:

-Culture of bacteria grown in nutrient broth, was pelleted form 20 ml by centrifugation at 6000 rpm for 15 min.

-The pellet washed with 3 ml of SET buffer and resuspended the cells with 1.6 ml of SET buffer, then freshly prepared lysozyme (final concentration 1 mg/ml) was added and incubated at 37°C for 30 min.

-One ml of 10 % SDS was added, mixed by inversion, then incubated at room temperature for 15 min.

-Add 2 ml of 5 M NaCl, mix thoroughly by inversion.

-An equal volume of chloroform was added, mixed by inversion for 15 min. Then centrifuged (6000 rpm at 4°C) for 20 min.

-The aqueous phase (upper) was transferred to another sterile tube, and 0.6 volume of isopropanol was added, mixed by inversion, and kept at room temperature for 5 min.

-Centrifuged at 13000 rpm for 15 min at 4°C.

-The isoproparnol was discarded and the precipitated DNA dissolved in 100 µl TE buffer and stored at -20C.

2.2.9 Agarose Gel Electrophoresis (Sambrook *et al*., 1989)

Agarose gel (0.7%) were run horizontally in tris-borate-EDTA (TBE 1X). Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells on the gel. Generally, gels were run for 2-3 hrs at 5 v/cm and the gel buffer added up to the level of horizontal gel surface.

Agarose gel were stained with ethidium bromide by immersing them in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 min. DNA bands were visualized by UV illumination at 302 nm on an UV transilluminator. Gels were destained in distilled water for 30-60 min to get rid of background before photographs were taken.

2.2.10 Plasmid Curing

2.2.10.1 Curing of *P. aeruginosa* RB19 Plasmid DNA with SDS

Curing experiments were performed on the *P. aeruginosa* RB19 isolate SDS was used in these experiments as curing agent according to Trevors (1986) and as follows:

Cells of the selected isolate were grown in 5 ml of nutrient broth to mid log phase ($O.D._{600}$ about 0.4-0.5). 0.1 ml samples of each culture were inoculated in a series of 5 ml nutrient broth tubes containing various concentrations of SDS (0, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%). All tubes were incubated at 37°C for 24-48hrs. The growth density of the different tubes was measured visually and compared with the control to determine the effect of each concentration of curing agent on bacterial growth. The lowest concentration of

curing agent that inhibits the growth of the bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken from tubes containing the highest concentration of SDS that still allows bacterial growth and diluted appropriately. Then 0.1 ml samples from proper dilutions were spread on nutrient agar plates and incubated over night at 37°C to score the survival colonies.

2.2.10.2 Curing of *Klebsiella pneumoniae* MR1 Plasmid DNA with Ethidium Bromide

Curing experiments were performed on the *Klebsiella pneumonae* MR1 using ethidium bromide as curing agent, according to Trevors (1986) and as follows:

Cells of the selected isolate were grown in 5 ml of nutrient broth to mid log phase ($O.D._{600}$ about 0.4 – 0.5). 0.1 ml samples of each culture were inoculated in a series of 5 ml fresh nutrient broth tubes containing various concentrations of ethidium bromide (0, 20, 50, 100, 200, 250, 300, 400, 800, 1600 and 3200 μg / ml). All tubes were incubated at 37ºC for 24-48 hrs.

The growth density of the different tubes was measured visually and compared with the control to determine the effect of each concentration of curing agent on bacterial growth. The lowest concentration of curing agent that inhibited the growth of the bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken form tubes containing the highest concentration of ethidium bromide that still allows bacterial growth and diluted appropriately. Then 0.1 ml samples from proper dilutions were spread on nutrient agar plates and incubated overnight at 37ºC to score the survived colonies.

2.2.10.3 Curing of Plasmid DNA with Plant Extract

Alcoholic extracts of (*Salix acomophylla*, *Urtica dioica* and *Calendula officinalis*), were used in an attempts to cure plasmid content of *Pseudomonas*

aeruginosa RB19 and the pathogenic *Klebsiella pneumoniae* MR1 isolates depending on Trevors (1986).

Cells of the selected isolates were grown in 5ml nutrient broth to mid log phase ($O.D._{600}$ about 0.4-0.5). 0.1ml samples were inoculated in a series of 5 ml fresh nutrient broth containing various concentrations (0, 10, 20, 30, 40, 50, 75, 100, 200, 300, 400 and 500 mg/ml) of hexane and methanolic extracts of the three plants for *Pseudomonas aeruginosa* RB19, and (0, 10, 20, 30, 40, 50, 75 and 100 mg/ml) for *Klebsiella pneumoniae* MR1, All tubes were incubated at 37°C for 24-48 hrs.

The growth density of different tubes was measured visually and compared with control to determine the effect of each concentration of each plant extract on bacterial growth. The lowest concentration of plant extract that inhibited the growth of bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken from tubes containing the highest concentration of each plant extract that still allows bacterial growth and diluted appropriately. Then 0.1ml samples from proper dilutions were spread on nutrient agar plates and incubated overnight at 37°C to score the survived colonies.

2.2.11 Selection of Cured Cells

After treatment of bacterial isolate with standard curing agent and plant extracts and the isolation of survivors on nutrient agar, survivors were analyzed for the presence or absence of drug resistance as a result of eliminating the plasmid by selecting 100 colonies of bacterial isolate from each treatment. These colonies were replica plated (using toothpick) on nutrient agar plates (master plates) and on nutrient agar plates containing an antibiotic to which the original isolate is resistant (Trevors, 1986).

If a colony was able to grow on the master plate but not on the selective agar containing the appropriate antibiotic, it means that the cells of this colony

are cured cells that lost plasmid responsible on resistance to this antibiotic. The percentage of cured cells was determined.

Chapter Two

Materials and Methods

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Conclusions and Recommendations

4.1Conclusions

1. The presence of some secondary metabolites in *Salix acomophylla*, *Urtica dioica* and *Calendula officinalis* including flavonoids, glycosides, saponins and terpens.

2. Methanolic and hexane extracts of *Salix acomophylla*, *Urtica dioica* and *Calendula officinalis* showed inhibitory effect on *Pseudomonas aeruginosa* RB19 and pathogenic *Klebsiella pneumoniae* MR1 growth.

3. Flavonoids, glycosides, saponins and terpens extracted from *Salix acomophylla*, *Urtica dioica* and *Calendula officinalis* are effective curing agents in elimination of plasmids responsible for antibiotics resistance in *Pseudomonas aeruginosa* RB19 and *Klebsiella pneumoniae* MR1.

4. *Salix acomophylla* methanolic extract was the best extract which gives the highest percentage of *Pseudomonas aeruginosa* RB19 and *Kelebsiella pneumonae* MR1 cured colonies.

4.2 Recommendations

1. Different studies on the activity of *Salix acomophylla*, *Urtica dioica* and *Calendula officinalis* as antimicrobial and curing agent against other microorganisms.

2. Advanced methods for isolation, purification and identification of flavonoids, glycosides, saponins and terpens of *Salix acomophylla*, *Urtica dioica* and *Calendula officinalis* are necessary in future studies.

3. Study the mechanism of plasmid curing carried out by Plant active compounds.

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Summary

Hexane and methanolic extract of dried aerial parts of *Salix acomophylla*, *Urtica dioica* and *Calendula officinalis* were tested for their antimicrobial and curing activity against two microorganisms: *Pseudomonas aeruginosa* RB19 and *Klebsiella pneumoniae* MR1.

Chemical investigation on these extracts showed that *Salix acomophylla*, *Urtica dioica* and *Calendula officinalis* methanolic extracts contain flavonoids, glycosides and saponins while the hexane extracts contain terpens.

The *in vitro* test showed that all extracts of *Salix acomophylla*, *Urtica dioica* and *Calendula officinalis* have antimicrobial activity against *Pseudomonas aeruginosa* RB19 and *Klebsiella pneumoniae* MR1 growth.

Plasmid profile of *Pseudomonas aeruginosa* RB19 isolate was determined and results showed that, the isolate has a small plasmid DNA bands.

To study the plant extracts activity against *Pseudomonas aeruginosa* RB19 and *Klebsiella pneumoniae* MR1 plasmids, these isolates were treated firstly with standard curing agents. Results of *Pseudomonas aeruginosa* RB19 treatment with SDS showed that some colonies of *Pseudomonas aeruginosa* RB19 isolate lost resistance to neomycin and trimethoprime. And obtain a number of colonies of *Kelebsiella pneumonae* MR1 lost their resistance to a number of antibiotics after treated with ethidium bromide. Results showed that there were three types of cured colonies, colonies which lost one plasmid (that lost resistance to aztreonam or that lost chloramphenicol resistance), colonies lost two plasmids (that lost chloramphenicol and aztreonam resistance) and colonies lost three plasmids (that lost resistance to ampicilline, chloramphenicol and aztreonam).

Treatment of *Pseudomonas aeruginosa* RB19 and *Kelebsiella pneumonae* MR1 with *Salix acomophylla, Urtica dioica* and *Calendula officinalis* extracts showed that many of these colonies were lost their resistance to antibiotics; these results indicated that extracts of these plants were have a curing effect on the bacterial plasmids and *Salix acomophylla* methanolic extract was the best extract which give the highest percentage of *Pseudomonas aeruginosa* RB19 and *Kelebsiella pneumonae* MR1 cured colonies.

الخلاصة

أستهدف البحث دراسة فعالية مستخلص الميثانول والهكسان للاجزاء الهوائية لنباتات الصفصاف والقريص والاقحوان ضد نمو الاحياء المجهرية خارج الجسم الحي وقدرتها على تحييد البلازميدات لنوعين من البكتريا: *Pseudomonas aeruginosa* RB19 و *Klebsiella penumoniae* MR1.

بينت نتائج الكشف الكيميائي للمستخلصات النباتية احتواء مستخلص الميثانول لنباتات الصفصاف والقريص والأقحوان على الفلافينويدات والكلايكوسيدات والصابونينات وأحتواء مستخلص الهكسانِ ِعلى التيربينات.

أوضحت الاختبارات خارج الجسم الحي أن جميع المستخلصات لنباتات الصفصاف والقريص والاقحوان لها فعالية ضد النمو البكتيري.

حدد المحتوى الجيني لعزلة *Pseudomonas aeruginosa* RB19 وأظهرت النتائج أحتواء العزلة على حزم بلازميدية صغيرة.

لدراسة فعالية المستخلصات النباتية في تحييد بلازميدات *Pseudomonas aeruginosa* RB19 و *Klebsiella penumoniae* MR1 فقد عوملت أولاً بمواد محييدة قياسية. أظهرت نتائج التحييد باسخدام مادة الـ SDS الحصول على عدد من المستعمرات التي فقدت قابليتها على مقاومة النيومايسين والترايميثوبريم للعزلة *Pseudomonas aeruginosa* RB19، والحصول على عدد من المستعمرات لعزلة *Klebsiella pneumoniae* MR1 فقدت مقاومتها لعدد من المضادات الحيوية بعد معاملتها بمادة الـ ethidium bromide . وتبين وجود ثلاثة أنواع من المستعمرات المحييدة، مستعمرات فقدت بلازميد واحد (التي فقدت مقاومة الـ aztreonam أو تلك التي فقدت مقاومة الـ chloramphenicol، مستعمرات فقدت بلازميدين (التي فقدت مقاومة الـ chloramphenicol و الـ aztronam) و مستعمرات فاقدة لثلاث بلازميدات (التي فقدت مقاومة الـ ampicillin و الـ chloramphenicol و الـaztronam).

أظهرت نتائج معاملة عزلتي *Pseudomonas aeruginosa* RB19 و *Klebsiella pneumoniae* MR1 مع مستخلصات الصفصاف والقريص والاقحوان إن العديد من المستعمرات فقدت قابليتها على مقاومة المضادات الحيوية، مما يشير الى قابلية المستخلصات النباتية على تحييد البلازميدات البكتيرية، وأن المستخلص الميثانولي للصفصاف هو أفضل

المستخلصات في تحييد بلازميدات عزلتي *Pseudomonas aeruginosa* RB19 و*Klebsiella pneumoniae* MR1.