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Evaluation The Effect of Some Plant Active Compounds on Bacterial Plasmid

A Thesis

Submitted to the College of Science/ Al- Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology .

By

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الأهداء

غاية الحمد والشكر لولي التوفيق والنعم الله عزوجل

إلى مقام النبي الأعظم محمد رسول الله (صلى الله عليه وآله و سلم) .....

إلى سندي في الحياة (أبي و أمي) .

إلى الشموع التي تنير لي دربي أخواني وأخواتي....

(علي،رسول،رغد،اكرام)

إلى كل من علمني حرف وأوصلني إلى هنا

(أساتذتي الأفاضل)

إلى هؤلاء أقدم جهدي الذي يشكل مستقبل حياتي.

هبة

Aqueous and methanolic extracts of dried aerial parts of *Hibiscus sabdariffa* and *Harpagophytum procumbens* were tested for their antimicrobial and curing activity against two microorganisms *Escherichia coli* HB 101 and *Pseudomonas aeruginosa* RB19 .

Chemical investigation on these extracts showed that *Hibiscus sabdariffa* methanolic extracts contain flavonoids and water extract contain glycosides and Saponins while the *Harpagophytum procumbens* methanolic extracts contain flavonoids and water extract contain glycosides.

The *in vitro* Results showed that water and methanolic extracts of *Hibiscus sabdariffa* and methanolic extract of *Harpagophytum procumbens* have antimicrobial effect against *E.coli* HB 101 and *P. aeruginosa* RB19, while aqueous extract of *Harpagophytum procumbens* have an antimicrobial effect against *P. aeruginosa* RB19 but do not have any effect against *E. coli* HB 101.

The sensitivity of *P. aeruginosa* RB 19 and *E.coli* HB 101 against different antibiotics was examined, and it was found that *E.coli* HB 101 was able to resist ampicillin and tetracycline, while it was sensitive to erythromycin, trimethoprim, chloromphenicol, streptomycin, fusidic acid, vancomycin, imipenem, cephotaxime, cephalothin, cephalexin, gentamicin, amoxycillin, penicillin G, ticarpen and clavulanic acid. While *P. aeruginosa* RB19 was resistant to ampicillin, amoxycillin, chloromphenicol, penicillin G, cephalothin, cephotaxime, tetracycline, and clavulanic acid, while it was sensitive to erythromycin, trimethoprim, gentamicin, streptomycin, Fusidic acid,

ticarpen, cephalexin, vancomycin, and imipenem.

Plasmid profile of *P. aeruginosa* RB19 and *E. coli* HB 101 was studied by extraction the total DNA according to alkaline lysis method, then electrophoresis on agarose gel. Results showed that *E.coli* HB 101 strain was harburing a single small plasmid, while in *P.aeruginosa* RB 19 isolate, there are two plasmids(large and small).

Curing of plasmid DNA was achieved using Ethidium bromide to know the role of this plasmid in the virulence and its ability to antibiotic resistance. Results showed that there were a number of *P. aeruginosa* RB 19 colonies had lost their resistance to tetracycline, ampicillin and cephalothin. While those of *E. coli* HB 101 lost their resistance to ampicillin and tetracycline.

Curing of plasmid DNA was achieved by treating *P. aeruginosa* RB 19 and *E.coli* HB 101 with *Hibiscus sabdariffa* and *Harpagophytum procumbens* extracts showed that many of these colonies were lost their resistance to antibiotics. These results indicated that extracts of those plants were have a curing effect on the bacterial plasmids. *Hibiscus sabdariffa* methanolic extract was the best extract that caused *E.coli* HB 101 to loss resistance to ampicillin and tetracycline and *Harpagophytum procumbens* methanolic extract caused *P. aeruginosa* RB 19 to loss resistance to ampicillin, tetracycline and cephalothin.

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List of Abbreviation

Abbreviation	Meaning
EDTA	Ethylene Diamine tetra-acetic acid
SDS	Sodium Dodecyl Sulphat
TBE	Tris- borate- EDTA
TE	Tris-EDTA
E.coli	*Escherichia coli*
P.aeruginosa	*Pseudomonas aeruginosa*
TLC	Thin layer chromatography
Rf	Rate of flow
W/V	Weight per volume
g/L	Gram /liter
U.V.	Ultraviolet
%	Percent
ºC	Celsius Degree

1. Introduction and Literature Review

1.1 Introduction

Herbal medicine, sometimes referred to as Herbalism, Botanical medicine or Herbology, is the use of plants, in a wide variety of forms for their therapeutic value. Herb plants produce and contain a variety of chemical compounds that acts upon the body and are used to prevent or treat disease (Grabely and Thierickly, 2000).

Today Environmental Studies is being promoted world wide. Herbal treatment and alternative medicinal system is largely being promoted as a consequence of this consciousness. Allopathic treatment gained popularity due to its instant remedial mechanism Particularly from this point, modern medicinal practices have started reviving the traditional herbal mechanisms of treatment(Somova *et al*., 2003).

Medicinal plants have been curing various disorders in humans from the time immemorial and are considered and intermittently associated and integral part of the Indian traditional medicinal system, better known as the Ayurvedic system of medicine (Basu, 2002). It is estimated that about 80,000 species of plants are utilized by the different system of Indian medicine traditional healers and medicine man in India practice and apply few medicinal plants for curing this ailment and are cheap as compared to pharmaceutical drugs (Singh, 2006).

Research on the medicinal plants had increased and different plants have been screened for their antimicrobial activity.In Iraq, many studies were conducted to evaluate the probable use of some plant materials (seeds ,flower, etc) as antimicrobials agents(Azhar, 1998).

In folk medicine, the herbs *Hibiscus sabdariffa* and *Harpagophytum procumbens* were investigated for their photochemical and antimicrobial activity (Auddy *et al*., 2003) *Hibiscus sabdariffa* was used in the treatment of diseases like abscesses, bilious conditions, and it was suggested for use as antibacterial and anticancer agents (Auddy *et al*., 2003). While *Harpagophytum procumbens* (also mentioned as devils claw) was used in the treatment of a wide range of conditions including fever, malaria and indigestion (Gagnier *et al*., 2007). Currently, the major uses of devil's claw are as an anti -inflammatory and pain reliever for joint diseases, back pain, and headache. There is currently wide spread use of standardized devil's claw for mild joint pain in Europe (Loots, 2005).

Recently, extracts of medicinal plants were used in enhancement of plasmid curing (Rose *et al*., 2008).

Amis of the study:

1- Detection the presence of active compounds in water and Methanolic extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens* .

2- Studying the biological activity of water and Methanolic extracts as a plasmid curing agents of bacterial plasmids.

1.2 Literature Review

1.2.1 Antimicrobials From Medicinal Plants

Herbal medicines are dietary supplements that contain herbs, either singly or in mixture. A herb is a plant or plant part used for its scent, flavor, and/or therapeutic properties. Products made from botanicals that are used to maintain or improve health have been called herbal supplements, botanicals (Al-Zubaidi *et al*., 2005).

Medicinal plants play an important role in health care, the demand for medicinal plants in health care is about 70-80% medicinal plants are the local heritage with global importance. The traditional use of low profile and less known medicinal plants should be documented to disseminate the therapeutic efficacy to pave the way for preparation of acceptable medicine and to reduce the pressure on over exploited species (Prajapati *et al*., 2006).

The use of plant extracts with known antimicrobial properties, can be a great significance in treatments.A number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial character, due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances; for example, flavonoids, or glycosides compounds (Scalbert, 1994). These compounds can be detected on the plants by a chemicaly and a physicaly ways such as Thin Layer chromatography (TLC) provides a quick, economical and reliable method for rapid screening of pharmaceuticals (Kenyon and Layloff, 2000). The TLC is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer material, silica gel. This layer of adsorbent is known as the stationary phase

(Jonathan *et al*., 2007). After a sample application on the activated plate, the plate is placed in a closed system containing a solvent or solvent mixture (known as the mobile phase). Because different analyses ascend the TLC plate at different rate, separation of compounds is achieved (Fair and Kormos, 2008).

Biological evaluation of extracts is vital to ensure safety and efficacy, these factors are importance if plant extracts to be accepted as valid medicinal agents by the healthcare community and to have a future as therapeutic agents (Simon, 2003).

Resistance to commonly antimicrobial agents, which may be coded by chromosomal or plasmids genes, is an increasing problem in many developing countries due to the over use and misuse of antibiotics which result in their ineffectiveness against multidrug resistant bacteria responsible for many life- threatening diseases. Many of plasmids carrying antibiotic resistance genes can be transferred naturally from one bacterial cell into another by conjugation and transformation (Satta *et al*., 1987).

In folk medicine can be elimination of plasmids that carry gene responsible to antimicrobial agents by using medicinal plants that curing common diseases. Use of plant extracts as curing agents could be sufficient to remove those genes necessary for virulence and resistance because both types are frequently carried by plasmid (Singh, 2006).

Antimicrobial activities of plants against bacterial growth were known and recently there were attempts carried out to study the effect of plants active compounds on the bacterial plasmids. Some of these studies referred that the active compounds of some plants have no effect on bacterial plasmids (Shakibaiee *et al*., 2000). While other studies referred to the effect of some plant active

compounds on bacterial plasmids (Beg and Ahmed, 2000).

Studies by Strohbach (1995) and Marshall (1998) revealed that methanolic extract of *Harpagophytum procumbens* exhibited antibacterial activity against *Escherichia coli* HB 101 and *Pseudomonas aeruginosa* RB19 and used for a variety of medicinal purposes. And Vaidya (2000) revealed that aqueous – methanolic extracts of *Hibiscus sabdariffa* was exhibit antibacterial activity and effect on bacterial plasmid.

1.3 Herbal Plants under the study:

1.3.1 *Harpagophytum procumbens*

Devil's claw is the common name for the *Harpagophytum procumbens*. other names include Devil's claw secondary root, grapple plant and wood spider. The medicinal parts of the *Harpagophytum procumbens* are the dried secondary tuber roots cut into slices or pieces or pulverized, and the macerated thick lateral tubers before they are dry. These are very difficult to cut or pulverize when dry (Hachfeld, 2003).

1.3.1.2 Plant Description and Distribution

Harpagophytum procumbens is widespread in South Africa, The flowers are solitary, large and foxglove- like on short pedicles in the leaf axils they are 7 to 20 cm long, 6 cm in diameter, very woody with long striped rind they have a double row of elastic, arm- like, branched appendages with an anchor-

Figure (1.1) secondary tuber roots of *Harpagophytum procumbens* (Hachfeld, 2004)

like hook, dark seeds with a rough surface (Fig 1.1). The plant lies flat on the ground (Powell, 2001). The plant is perennial, leafy, and has a branched root system and branched prostrate shoot 1 to 1.5m long. The leaves are petiolate and lobed and may be opposite or alternate. The plants flower mainly from about November to April (summer) and have fruits from about January (Hachfeld, 2004).

1.3.1.3 Taxonomy of *Harpagophytum procumbens* (Sekhwela, 1994)

***Domain:** Eukaryota

***Kingdom:** Plantae

***Division:** Magnoliophyta

***Class:** Rosopsida

***Subclass:** Asteridae

***Order:** Asterales

***Family:** Pedaliaceae

***Genus:** *Harpagophytum*

***Species:** *procumbens*

Figure (1.2) The Plant of *Harpagophytum procumbens* (Hachfeld, 2004)

1.3.1.4 Active compounds

The *Harpagophytum procumbens* contain several active compound including: monoterpenes, phenylethanol, acteoside, isoacteoside, Oligosaccharides,and other flavonoids, glycolic acid, polyphenol, antioxidants. In addition to these chemicals leaf is rich in minerals, chlorophyll, amino acids, sterols and vitamins (Chrubasiks *et al*., 2003).

1.3.1.5 Medicinal Uses

The most important components in *Harpagophytum procumbens* are iridoid glycosides and flavonoids. Some of its properties are listed: analgesic, anti-arrhythmic, antibacterial, anti-inflammatory, antirheumatic, diuretic, hypertensive, laxative, purgative sedative, In western medicine it is mainly used for arthritis and rheumatism (Loots, 2005).

It apparently works like cortisone but without the bad side effects of that drug. In its local areas, and increasingly elsewhere, it

has also been used for fever, blood diseases, blood purification, lower back pain and pain in pregnant women, coughs, diarrhea, diabetes, bleeding gums, syphilis, gonorrhea, gout and lumbago (Chrubasiks *et al*., 2004).

It also helps with diseases of the liver, gall bladder, kidneys, pancreas, digestive system (peptic ulcers, constipation and lack of appetite) and small joints, as well as hypertension, high cholesterol and tuberculosis. Externally it helps heal ulcers, boils, skin lesions and wounds (Neuwinger, 2000; Powell, 2001).

Devil's claw can be allergenic and is contra-indicated for diabetics and people with duodenal and gastric ulcers. Very high doses may act against blood pressure and cardiac therapy. Raimondo *et al* .,(2005) reported that many local people consider that Europeans use this herb too strongly and damage may result. He noted that if the herb is drink too strong and too often, cancer, kidney damage or arthritis might be caused. High doses may also cause abortion (Grant *et al*., 2007).

1.3.2 *Hibiscus sabdariffa*

This plant have other names: Red Sorrel, Roselle, Jamaica Sorrel, Karkade and Red tea (Leung, 1996). The medicinal parts of the plant are the flowers (Watt and Breyer-Bradndwijk, 1962).

1.3.2.1 Plant Description and Distribution

Hibiscus sabdariffa is widespread in India, China and Thailand It grows world wide in the tropics and is cultivated in Europe and is used in Ayurveda and traditional medicine (Dahanukar *et al*., 2000).

The flowers are solitary, axillary. The calyx is red(Fig 1.3) , the corolla yellow, and the anthers blood red. The fruit is a 2 cm long,

ovoid, many seeded capsule leaves, stem and root, Hibiscus is a 0.15 to 1m bushy annual that is branched from the base. The stems are reddish (Salah *et al*., 2002).

Figure (1.3) The Flower of *Hibiscus sabdariffa* (Salah *et al*., 2002)

1.3.2.3 Taxonomy of *Hibiscus sabdariffa* (Vaidya, 2000)

***Domain:** *Eukaryota*

***Kingdom:** *Plantae*

***Sub kingdom:** *Tracheobionta*

***Super division :** *Spermatophyta*

***Division:** *Magnoliophyta*

***Class:** *Magnoliopsida*

***Sub class:** *Dilleniidae*

***Order:** *Malvales*

***Family:** *Malvaceae*

***Genus:** *Hibiscus*

***Species:** *Hibiscus sabdariffa*

1.3.2.4 Active compounds

The *Hibiscus sabdariffa* contain several active compounds including: fruit acids (15-30%) in particular hibiscus, anthocyans (intensive red), flavonoids, glycosides, saponins and other polyphenol ascorbic acid, glycolic acid, antioxidants (Seca *et al*, 2001).

1.3.2.5 Medicinal Uses

Roselle is an aromatic, a stringent, cooling herb that is much used in the tropics. It is said to have diuretic effects, to help lower fevers (Tsai *et al*, 2002). The leaves are emollient, diuretic, refrigerant and sedative, and very mucilaginous and used as an emollient and as a soothing cough remedy. They are used externally as a poultice on abscesses (Odigie, 2003). .

The flowers contain anthocyanins, and the glycoside hibiscus. These may have diuretic and choleretic effects, decreasing the viscosity of the blood, reducing blood pressure and stimulating intestinal peristalsis. The leaves and flowers are used internally as a tonic tea for digestive and kidney functions (Amin and Hamza, 2006).

The ripe calyces are diuretic. The succulent calyx, boiled in water, is used as a drink in the treatment of bilious attacks. The seeds are diuretic, laxative and tonic. They are used in the treatment of debility (Essa *et al*, 2006).

The bitter root is aperitif and tonic. The plant is also reported to be antiseptic, astringent, digestive, purgative and resolving. It is used as a folk remedy in the treatment of debility, dyspepsia, fever, hangover, heart ailments, hypertension and neurosis (Yang *et al*., 2000).

1.4 Secondary Metabolites

Plants produce large, diverse array of organic compounds that appear to have no direct function in plant growth and development. These substances are known as secondary metabolites, secondary products or natural products. Primary metabolites (protein, carbohydrate and fats) are important in plant physiological process such as growth and development (Hartmann, 1996).

The scientist classified it according to the chemical structure of these compounds, and other classified according to the source of metabolism process (Hartmann, 1996). Secondary metabolites, generally present at 1-3% of dry plant weight, are synthesized in specialized cells at distinct developmental stages and have highly complex structures, making their extraction and purification difficult (Dave, 2003).

1.4.1 Flavonoids

Flavonoids or bioflavonoid are a class of plant secondary metabolites. Flavonoids are most commonly known for their antioxidant activity. However, it is now known that the health benefits, and it is provided that it is acting against cancer and heart disease. All so the result of other mechanisms referred to flavonoids as a bioflavonoid. These terms are largely equivalent and interchangeable, for most flavonoids are biological in origin (Ververidis *et al*., 2007).

Flavonoids are widely distributed in plants and they have many functions including producing yellow or red /blue pigmentation in flowers and protection from attack by microbes and insects. The wide spread of flavonoids, variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, significant quantities in

their diet (Cushnie and Lamb, 2005).

Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. They show anti-allergic, anti- inflammatory, anti- microbial and anti- cancer activity (Felicien, 2008).

Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancers and cardiovascular disease. The beneficial effects of fruit, vegetables, and tea or even red wine have been attributed to flavonoids compounds rather than to known nutrients and vitamins (Hwang *et al*., 2003).

1.4.2 Glycosides

Glycosides are certain molecules in which a sugar part is bound to some other part. Glycosides play numerous important roles in living organisms. Many plants store important chemicals in form of inactive glycosides; if these chemicals are needed, the glycosides are brought in contact with water and an enzyme, and the sugar part is broken off, and make these compounds available for use. Many such plant glycosides are used as medications (Felicien Breton, 2008).

Glycosides are compounds containing a carbohydrate and a non carbohydrate residue in the same molecule. The carbohydrate residue is attached by an acetal linkage at carbon atom 1 to a non - carbohydrate residue or Aglycone. While the non sugar component is known as the Aglycone. The sugar component is called the glycone (Harborne, 1982).

The glycone and aglycone portions can be chemically separated by hydrolysis in the presence of acid. There are also numerous enzymes that can form and break glycosidic bond. The most important cleavage enzyme are the glycoside hydrolyses mutant enzyme glycosynthases have been developed that can from glycosidic bonds in excellent yield (Tsushida and Suzuki, 1996).

The glycosides possess useful pharmacological properties which are the same as their respective aglycone, which properties include a drive-enhancing (stimulating) activity, an anti-inflammatory, Cardiac drugs, Laxatives, Analgesics, anti bacterial,anti rheumatic and anticancer agent. A ready and therapeutically revenant example is the cardio-active agent digoxin, from common foxglove (Asl and Hossein, 2008).

1.4.3 Saponins

Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources, with Saponins found in particular abundance in various plant species, they are special glycosides grouped phenomenological by the soap-like foaming they produce when shaken in aqueous solutions, and these compound contain non sugar a glycol part and this part called sapogenin (Skene and Sutton, 2006) most of them are steroid compounds in the plant kingdom and it has been found in different parts of the plant but its quantity affected by the level of the plant growth. If the Saponins injected into the blood stream they disrupt red cells, through their effects on plasma membranes (Francis *et al*, 2002).

1.5 Plasmid curing in bacteria

Plasmids are extrachromosomal pieces of double- stranded circular DNA which have the capability to replicate independently of the host chromosome, yet coexist with it. To date, many species of bacteria isolated from diverse habitats are known to contain plasmid DNA. Some plasmids are stable and can be maintained through successive generations by being portioned to each daughter cell during cell division. This allows each cell to receive at least one plasmid copy (Amabile *et al* ., 2005).

In nature, plasmid can be lost spontaneously from a very few cells, but the probability of this loss is extremely low, ranging from 10^{-5} to 10^{-7} (Molnar, 1988) however, the majorities of plasmid are extremely stable, and required the use of curing agents or other procedures that might increase the plasmid loss, and these from the basis of artificial plasmid elimination (Trevors, 1986, and Molnar, 1988) Elimination of antibiotic resistance at high frequency is interested to assert extra chromosomal location of genetic determinants (Amabile, 2005).

In recent years, plasmids have been observed in a wide variety of bacteria. In part, this is due to the development of new procedures that allow the detection, isolation, and molecular characterization of plasmid DNA. When working with some plasmid-containing bacteria, it is often desirable to obtain a plasmid- cured derivative. This allows a direct comparison to be made between the plasmid-containing and plasmid-cured cells (Buchrieser *et al*., 2000).

Some plasmids undergo spontaneous segregation and deletion. However, the majority are extremely stable, and require the use of curing agents or other procedures (elevated growth temperature, acridin orange , Ethidium bromide and sodium dodecyl sulfate (SDS),

to increase the frequency of spontaneous segregation).The usefulness of curing agents is unpredictable in many bacterial strains, as there are no standard protocols applicable to all plasmids. However, there are some procedures that have provided good results with certain species (Dozois *et al*., 2000). Plasmid elimination *in vitro* provides a method of isolating plasmid free bacteria for biotechnology without any risk of inducing mutations(Sambrook *et al*.,1989).

1.6 Mechanism of plasmid curing in bacteria

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eliminating plasmids (Stehling *et al*., 2003).

However, the frequency and extent of this effect was significantly less than might have been expected based on a complex interaction with plasmid DNA. Bacterial plasmids can be eliminated from bacterial species grown as pure or mixed bacterial cultures in the presence of sub-inhibitory concentrations of non-mutagenic heterocyclic compounds (Dozois *et al*., 2003).

The antiplasmid effect of heterocyclic compounds is expressed differentially in accordance with the structural form of the DNA to which they bind. In this manner "extra chromosomal" plasmid DNA that exists in a superhelical state binds more compound than its linear or open-circular form and least to the chromosomal DNA of the bacterium, that carries the plasmid (Rodriguez- Siek *et al*., 2005).

It can also be noted that these compounds are not mutagenic and their antiplasmid effects correlate with the energy. Plasmid elimination is considered also to take place in ecosystems containing numerous bacterial species.

Inhibition of plasmid replication at various stages, as illustrated in the "rolling circle" model (replication, partition, conjugal transfer) may also be the theoretical basis for the elimination of bacterial virulence in the case of plasmid mediated pathogenicity and antibiotic resistance (Sambrook *et al*., 1989).

2. Materials and Methods

2.1 Materials

2.1.1: The following Equipments and Apparatus were used in this study:

Equipments	Company (Origin)
Autoclave	Gallenkamp (England)
Centrifuge	Gallenkamp
Compound light microscope	Olympus
Cooling centrifuge	Sigma (U.S.A)
Distillator	Gallenkamp
Electrophoresis unit	BioRad (Italy)
Electrical oven	Memmert (Germany)
Incubator	Gallenkamp
Micropipette	Witeg (Germany)
Millipore filters unit	Millipore and Whatman(England)
pH- meter	Metter-Gmph Tdedo(U.K.)
Power supply	LKB(Sweden)
Rotary Evaporator	Buchi (Switzerland)
Refrigerator	Ishtar
Sensitive balance	Delta Range(Switzerland)
Shaker Incubator	GFL (Germany)
Soxhlet	Electro thermal (England)
UV- Transillminator	Vilber Lourmat (France)
Vortex	Buchi

Water bath	GFL (Germany)

2.1.2 Chemicals and Enzymes.

The following chemicals and enzymes were used in this study:

Chemicals	Company/ Country
Absolute ethanol (99%)	Iraq
Agar-Agar	Himedia (India)
Agarose	Sigma (USA)
Boric acid	BDH (England)
Bromophenol blue	BDH
Cetrimide	Riedel-Dehaeny(Germany)
Chloroform	BDH
Ethylene Damien tetra-acetic acid	BDH
Ethidium bromide	BDH
Ferric chloride	BDH
Glucose	BDH
Glycerol	BDH
Gelatin	Difco (USA)
Glacial acetic acid	BDH
K_2SO_4	BDH
Lysozyme	BDH
Hydrochloric acid	BDH

Methanol	Riedel-Dehaeny
Methyl red	BDH
$MgCl_2$	BDH
$MgSO_4.7\ H_2O$	BDH
Potassium acetate	Fluka (Switzerland)
Potassium hydroxide (KOH)	Fluka
Phenol	BDH
Peptone	BDH
Sodium hydroxide (NaOH)	Fluka
Sodium dodecyl sulphate (SDS)	Fluka
Sucrose	Himedia
Tris(hydroxymethtl)aminomethan base (Tris-base)	Fluka
Tris-hydrochloride (Tris-HCL)	Fluka

2.1.3 Culture Media

2.1.3.1 Media that used in the study:

Medium	Company (Origin)
Brain heart infusion agar	Fluka (Switzerland)
Brain heart infusion broth	Fluka
Nutrient agar	Fluka
MR- VP media	Himedia(India)

Simmon citrate Agar	Himedia
Nutrient broth	Biolife (Italy)
MacConkey agar	Difco (U.S.A)
Triple sugar Iron Agar	Difco
Urea agar base	Difco

These media were prepared according to the instructions of manufacturers, and sterilized by autoclaving (Collee *et al* .,1996).

2.1.3.2 Laboratory prepared media

2.1.3.2.1 Cetrimide Agar (Stolp and Gadkari, 1981) This medium was prepared from the following components:

Component	Concentration(g/L)
Peptone	20
$MgCl_2$	1.5
K_2SO_4	10
Cetrimide	0.3
Agar	15

All components were dissolved in 950 ml distilled water, pH was adjusted to 7.2, and then the volume was completed to 1000 ml with distilled water, and sterilized by autoclaving .

2.1.3.2.2 King A Medium (Starr *et al.*, 1981) This medium was prepared from the following components:

Component	Concentration (g/L)
Peptone	20
K_2SO_4	1.5
$MgCl_2$	1.4
Glycerol	10 ml
Agar	15

All components were dissolved in 950 ml distilled water, pH was adjusted to 7.2, and then the volume was completed to 1000 ml with distilled water, and sterilized by autoclaving.

2.1.3.2.3 King B medium (Starr *et al.*, 1981)

This medium was prepared from the following components:

Component	Concentration (g/L)
Peptone	20
$MgSO_4.7H_2O$	3.5
K_2SO_4	1.5
Glycerol	10 ml
Agar	15

All components were dissolved in 950 ml distilled water, pH was adjusted to 7.2, and then the volume was completed to 1000 ml with distilled water, and sterilized by autoclaving .

2.1.3.2.4 Indol media (Collee *et al*., 1996)

It was prepared by dissolving 1g of peptone in 100ml distilled water and divided into clean and sterilized test tubes (5ml/tube),then sterilized by autoclaving.

2.1.3.2.5 Gelatin medium (Baron and Fine gold, 1994)

This medium was prepared by dissolving 12 gram of gelatin and 3.7 gram of Brain- Heart infusion broth in 90 ml of distilled water, then the volume was completed to 100 ml, pH was adjusted to 6 and sterilized by autoclaving, then the medium was distributed into sterilized test tubes to be contain 5 ml / tube.

2.1.3.2.6 Urease medium (Collee *et al* ., 1996)

This medium was prepared by adding 5ml of 40% urea solution (sterilized by filtration) to 95ml of urea-agar base (sterilized by autoclaving), then the medium was distributed into sterilized test tubes to be contain 5ml/tube.

2.1.3.2.7 Semi –solid agar medium (Collee *et al*., 1996)

This medium was prepared by dissolving 0.5 g of agar, and 0.8g of nutrient broth in 90 ml of distilled water, then the volume was completed to 100 ml with distilled water, pH was adjusted to 7.0 and sterilized by autoclaving. Then the medium was distributed into sterilized test tubes to be contain 5 ml/ tube.

2.1.4 Reagents

Reagents used in this study were prepared according to James and Sherman (1987) as fallows:

2.1.4.1 Indol (Kovac´s reagent)

This reagent was prepared by dissolving 5 g of α- Dimethyl – aminobenzaldehyde in 75 ml of Isoamyl alcohol, then 25 ml of concentrated HCL was added.

2.1.4.2 Voges – Proskaur (VP)

A- VP 1: It was prepared by dissolving 40g of potassium hydroxide in 100 ml of distilled water.

B- VP 2 : It was prepared by dissolving 5 g of α- Naphthol in 100 ml of absolute ethanol.

2.1.4.3 Methyl red reagent

It was prepared by dissolving 0.1 gm of methyl red in 300 ml of ethanol (99%), then 200 ml of distilled water was added.

2.1.4.4 Fehling Reagent (Sarkas *et al* ., 1980)

This reagent consists of two solutions:

Solution A: It was prepared by dissolving 35 g of copper sulfate in 400 ml of distilled water, after shaking the volume was completed to 500 ml with distilled water.

Solution B: prepared by dissolving 7 g of sodium hydroxide and 175 g of sodium-potassium in 400 ml of distilled water, after shaking the

volume was completed to 500 ml with distilled water. Equal volumes of solution A and solution B were mixed well and kept at 4ºC until use.

2.1.4.5 Potassium hydroxide solution (50% W/V) (Sarkas *et al.*, 1980)

This solution was Prepared by dissolving 50 g of potassium hydroxide in 80 ml of distal water, then the volume was completed to 100 ml with distilled water.

2.1.5 Antibiotic Solutions

Antibiotic Solutions were prepared according to Maintains *et al.*, (1982) and as follows.

1- Ampicillin, penicillin G, cephalothin, cephotaxime, amoxycillin and Clavulanic acid were prepared as stock solutions of 10 mg/ml by dissolving 1 g of antibiotic in 90 ml distilled water,then the volume was completed to 100 ml, sterilized by filtration and stored at- 20ºC.

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

2.1.5.1 Antibiotics Discs

The following antibiotic discs were used in the study :

Antibiotic	Code	Concentration (µg/disk)	Source (Origin)
Ampicillin	AMP	10	Oxoid/England
Chlaromphenicol	C	30	Oxoid
Cephotaxime	CE	30	Oxoid
Cephalothin	CK	30	Oxoid
Cephalexin	CL	30	Oxoid
Gentamicin	CN	10	Oxoid
Imipenem	IPM	30	Oxoid
Erythromycin	E	15	Oxoid
Fusidic acid	FA	10	Oxoid
Penicillin G	G	10	Oxoid
Trimethoprim	TM	30	Oxoid
Ticarpen	Tic	75	Oxoid
Tetracycline	TE	30	Oxoid
Vancomycin	VA	30	Oxoid
Streptomycin	S	10	Oxoid
Amoxycillin	AM	20	Oxoid
Clavulanic acid	C	10	Oxoid

2.1.6 Bacterial Strains and isolates

Table (2.1) Bacterial strains and isolates used in the study.

Bacterial Strains and isolates	Phenotype	Source
E. coli HB 101 Harboring pBR322	Amp^r, Tc^r	Department of Biotechnology/Al-Nahrain University
P. aeruginosa RB 19	Amp^r, CK^r, CE^r, Amc^r, C^r, P^r, Tc^r	Department of Biotechnology/Al- Nahrain University

CK: Cephalothin; CE: Cephotaxime; Amc: Amoxycillin; Amp: Ampicillin
Tc: Tetracycline; P: Penicillin G; C: Chlaromphenicol; r: Resist

2.1.7 Extraction of plasmid DNA

2.1.7.1 Buffers and solutions

All buffers and solutions for DNA extractions were prepared according to Sambrook *et al* ., (1989) as fallows:

2.1.7.1.1 SET buffer

This solution was prepared from the following components:

Component	concentration (mM)
NaCl	75
EDTA	25
Tris-Cl	20

pH was adjusted to 8 and sterilized by autoclaving.

2.1.7.1.2 Tris– EDTA–Glucose solution (TEG)

This solution was prepared from the following components:

Component	concentration (mM)
Glucose	50
Tris-HCL	25
EDTA	10

pH was adjusted to 8 and sterilized by autoclaving.

2.1.7.1.3 Alkaline sodium dodecyl sulphate Solution

This solution was prepared by dissolving 1g of 1% SDS in 100 ml of freshly prepared 0.2 N NaOH solutions.

2.1.7.1.4 Tris-EDTA (TE) Buffer

This solution was prepared from the following components:

Component	Concentration (mM)
Tris-HCL	10
EDTA	1

pH was adjusted to 8 then sterilized by autoclaving.

2.1.7.1.5 Lysozyme Solution (50 mg/ ml)

This solution was freshly prepared by dissolving 0.5 g of lysozyme in 10 ml of sterile distilled water, and kept at 4 ºC until use.

1.1.7.1.6 Potassium acetate solution

Prepared by mixing 60 ml of Potassium acetate, (5M), 11.8 ml of glacial acetic acid, and 28.5 ml of distilled water. pH was adjusted to 4.8, and kept at 4ºC until use.

2.1.7.1.7 Phenol Solution

Crystalline Phenol was first melted in a water bath at 68ºC, then the soluble phenol was extracted several times with equal volume of Tris-EDTA (0.01M Tris-base and 0.01M Na2EDTA),until pH of the aqueous phase reached to 8, then one drop of 8-hydroxyquinoline was added and kept at 4ºC in a dark bottle.

2.1.7.1.8 Phenol – Chloroform Solution.

This solution was Prepared by mixing two volumes of phenol and one volume of chloroform, stored in a closed dark bottle at 4ºC.

2.1.7.2 Buffer Solutions For Gel Electrophoresis

2.1.7.2.1 5X Tris- Borate- EDTA (TBE)

This solution was prepared by dissolving 54g of Tris base, and 27.5g Boric acid in 900 ml distilled water, then 20 ml of 0.5M EDTA was added, and the volume was completed to 1000 ml with distilled water .

2.1.7.2.2 Loading Buffer

This solution was prepared by dissolving 0.25 of Bromophenol blue and 40 g of sucrose in 90 ml of distilled water then, the volume was completed to 100 ml with distilled water.

2.1.7.3 Curing Solution

2.1.7.3.1 Ethidium Bromide Solution (10 mg/ml)

This solution was prepared by dissolving 1gm of Ethidium bromide in 100 ml of distilled water in a dark bottle and stored at 4ºC until use.

2.2 Methods

2.2.1 Methods of Sterilization

A- Autoclaving

Culture medium, reagent and solutions were sterilized by autoclaving at 121ºC, 15psi for 15min.

B- Filter sterilization

Solutions which are sensitive to heat were sterilized by filtration through Millipore filter unit (0.22 µm).

C- Dry heat sterilization

Glassware were sterilized by dry heat at 180ºC for 3 hours.

2.2.2 Plant Collection.

Harpagophytum procumbens and *Hibiscus sabdariffa* were collected during November 2007 from local markets in Baghdad City and identified kindly by Professor Dr. Ali Al-Musawi (Director of Baghdad University Herbarium) /Department of Biology, College of Science, University of Baghdad. Aerial parts of these plants were cleaned under tap water, and air dried at room temperature for three days, then samples were grounded into powder by electrical grinder, then powdered parts were kept in plastic bags at 4ºC until use (Harborne *et al*., 1975).

2.2.3 Preparation of Plant Extracts.

2.2.3.1 Aqueous Extract

Aqueous extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens* were prepared according to Harborn (1982). Fifty grams of each plant powder was extracted with 250 ml of distilled water at 60ºC in a water bath for 1 hour, after filter the solvent was evaporated under reduced pressure using rotary evaporator at 40ºC, the crude extract was kept at 5ºC until use (Bozkurt, 2006).

2.2.3.2 Methanolic Extract

Methanolic extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens* were prepared according to Harborn(1982). By weighting 50g of each plant powder (*Hibiscus sabdariffa* and *Harpagophytum procumbens*) and extracted with 250 ml of 70 % methanol in Soxhlet apparatus for 6 hrs at 60 ºC, after filter the solvent was evaporated under reduced pressure using rotary evaporator at 40ºC. The crude extract was kept at 5ºC until use.

2.2.4 Detection of Some Active Compounds:

1- Detection of Flavonoids

Flavonoids were detected in both plants (*Hibiscus sabdariffa* and *Harpagophytum procumbens*) according to Harborn (1984) by mixing the complete a mount of solution(A) and (B) prepared as follows:

***Solution (A):** This solution was prepared by adding 5ml of 95% ethanol to 10 g of plant powder, mixed gently at room temperature, then ethanol layer was filtered through wattman filter paper No.1 into sterile test tube.

***Solution (B):** This solution was prepared by adding 10 ml of 50% ethanol to 10ml of 50% aqueous potassium hydroxide solution. The appearance of yellow color evidence of the presence of flavonoids.

2-Detection of Glycosides. (Stahl, 1969)

Equal amounts of aqueous plant extracts prepared in (2.2.3.1) was mixed with Fehling reagent prepared in (2.1.4.4) in a test tube, then the mixture was boiled in a water bath for 10 min with the addition of few drops of diluted H_2SO_4 .Presences of red precipitate indicates a positive result.

3-Detection of Saponins (Stahl, 1969)

Saponins were detected by adding 5ml of aqueous plant extracts prepared in (2.2.3.1) to 1-3 ml of 3% ferric chloride solution. Appearance of white precipitate indicates a positive result .

4- Detection of Flavonoids Compounds by TLC

The procedure of Harborne (1984) was followed, in which a silica gel TLC plate (200×200×0.25mm) was activated in the oven at 110ºC for one hour, and after cooling the plate at room temperature (around 30 minutes), a marginal line was drawn on the upper and lower side of the plate with a distance of 2 centimeters from the margins. On the lower line of the plate, 0.25 ml of concentrated sample (methanol extract) and a Flavonoids standard (rutin) was applied. After around 5-7 minutes, the plate was placed in a jar-closed-system containing a solvent (*n*-BuOH: HOAc: H_2O at a ratio 4:1:5), and the system was left for two hours at room temperature. Then plate was examined under UV light to detect the separated Flavonoids

compounds in methanol extract samples as compared to the Flavonoids standard and the R_F value.

The R_F value is the distance compound moves in the chromatography relative to the solvent front. It is obtained by measuring the distance from the origin to the center of the spot produced by the substance, and this is divided by the distance between the bore line and the solvent front (i.e. the distance the solvent travels). This always appears as a fraction and lies between 0.01 and 0.99. It is convenient to multiply this by 100 and R_F is quoted as R_F (×100) (Harborne, 1984). Such value was used to assess the presence of Flavonoids compounds in the two plant extracts (Table 2-1).

2.2.5 Identification of Bacterial Strains and isolates:

2.2.5.1 Morphological Characteristics (Atlas *et al* ., 1995)

Colonies that were able to grow on the selective media were further identified by studying their morphological characteristics beginning with staining ability, appearance under light microscope, and size, shape, edge, color and transparency of colony on plates.

2.2.5.2 Biochemical Tests:

The following biochemical tests were achieved for the suspected colonies according to Collee *et al*., (1996) and as follows:

1- Motility test

Test tubes containing semisolid agar media, were inoculated with single colony using a straight wire, making a single stab down the center of the tube to about half depth of the medium incubated at 37ºC for 24 hrs. Motile bacteria typically give diffuse, hazy growths that spread, throughout the medium rendering it slightly opaque.

Table (2.2): Properties (R_F values) of common Flavonoids compounds Harborne (1984)

R_F (×100)	
Flavonoid Compounds	Solvent (*n*-BuOH:HOAc:H_2O)
flavonols	
Kaempferol	83
Quercetin	64
Myricetin	43
Azaleatin	48
Flavones	
Apigenin	89
Chrysoeriol	82
Glycosylflavones	
Vitexin	41
Iso-orientin	41

2- Indol production test

This test demonstrates the ability of bacteria to decompose tryptophan to Indol accumulates in the medium. Test tubes containing 1% peptone broth were inoculated with single colony of bacterial strains and incubated at 37ºC for 24-48 hrs, then 0.05 ml of Kovac's reagent prepared in (2.1.4.1) was added. Presence of red ring on the surface of the medium indicates a positive result.

3- Methyl red test

This test was achieved to detect acid production during fermentation of glucose. Test tubes containing MR-VP broth were inoculated with single colony of both strains, and incubated at 37ºC for 48 hrs, then 5 drops of methyl red indicator prepared in (2.1.4.3) was added to each tube, and mixed thoroughly presence of red color indicates a positive result.

4-Voges- Proskaur test

Test tubes containing MR-VP broth was inoculated with single colony of each strains, and incubated at 37ºC for 48 hrs, then 1ml of VP1 and 3 ml of VP2 prepared in (2.1.4.2) were added to 5ml of broth culture and shacked for 30 seconds. Formation of pink to red color indicates a positive result.

5-Citrate utilization test

This test was achieved to detect the ability of bacterial cells to utilize citrate as a sole source for carbon and energy, and ammonium salts as source for nitrogen. Simmon citrate agar medium was streaked with both strains and incubated at 37ºC for 24 hrs, medium color change from green to blue indicates a positive result.

6-Urease Test

This test was achieved to examine the ability of bacterial strains in urease production, by inoculating urea agar slants with both bacterial strains, and incubated at 37ºC for 24 hrs. After incubation, appearance of pink color indicates a positive result.

7- Triple sugar iron agar test

This test was achieved by stabbing and streaking TSI agar slants with single colony of each bacterial strain. After incubation for 24 hrs at 37ºC Result are as following:

Color	pH: slant/Bottom	Result Utilization
Red/Yellow	Alkaline/Acid	Glucose only fermented Peptones utilization
Yellow/Yellow	Acid/Acid	Glucose fermented; lactose and /or sucrose fermented
Red/Red	Alkaline/Alkaline	No fermentation of glucose, lactose or sucrose/ Peptones utilization

While the formation of black precipitate indicated H_2S production, and pushing agar to the top indicate CO_2 formation.

8- Catalase test

This test was achieved to examine the ability of bacterial strains in catalase production by transferring single colony of each strains and smeared into glass slide, then one drop of 3% H_2O_2 was added, appearance of gaseous bubbles indicates a positive result.

9-Gelatinase Test

Gelatin agar was used to detect the ability of bacterial strains to produce gelatinase. This test was achieved by inoculating test tubes containing gelatin agar medium prepared in (2.1.3.2.5) and incubated at 37ºC for 48 hrs, then test tubes were kept in refrigerator at 4ºC for 30

min. positive result was recorded by observing gelatin liquefaction.

10- Growth on King A medium

This test was performed to study the production of pyocyanin pigment. Single colony of each bacterial strain was streaked on king A medium and incubated at 37ºC for 24 hrs. Ability of bacterial strains to grow on this medium indicates a positive result.

11-Growth on King B medium

This test was performed to study the ability of bacterial strains in production of fluorescent pigment. Single colony of each bacterial strain was streaked on King B agar medium and incubated at 37ºC for 24 hrs, then plates were exposed to U.V. light. Presence of fluorescent pigment indicates a positive result.

12-Growth on Cetrimide agar

This medium was used as a semi-selective medium for *Pseudomonas* spp., *Pseudomonas* isolate was streaked on Cetrimide agar plates and incubated at 37ºC for 24 hrs. Presence of bacterial colonies indicates a positive result.

13- Growth on MacConkey Agar

MacConkey agar is a differential plating medium for the selection and recovery of enterobacteriaceae related enteric Gram negative rods, Lactose is the sole carbon source. Lactose fermenting bacteria produces colonies that are varying in shade 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.

2.2.6 Maintenance of Bacterial Strains

Maintenance of Bacterial strains was performed according to Johnson *et al*., (1988) as the following:

- **Short- Term Storage**

  Bacterial strains were maintained for few weeks on nutrient agar plates, Plates were wrapped tightly with parafilm, and then stored at 4ºC.

- **Medium- Term Storage**

  Bacterial strains were maintained For few months by stabbing nutrient agar slants in a screw capped tubes containing 5-8 ml of nutrient agar medium and stored at 4ºC.

- **Long Term Storage**

  Single colony was used to inoculated in Braine - heart infusion broth and incubated at 37ºC for 24hrs, then 8.5ml of cell suspension was mixed with 1.5 ml of glycerol, and stored at -20ºC.

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

Disc diffusion method was used to test the antibiotic sensitivity of the selected isolates. A sterile cotton swab was dipped into freshly culture(18 hrs), and the entire surface of the brain- heart infusion agar plates were swabbed three times by rotating the plate approximately 60º between streaking to ensure even distribution, then discs of the antibiotic were applied and incubated at 37ºC. Zones

of inhibition was observed after incubation for 18 hrs.

2.2.8 Minimum Inhibitory Concentration of Plant Extracts (Saxena *et al* ., 1995)

From a fresh culture of each isolates, a portions of 100µl were taken and used to inoculate series of test tubes containing 5ml of nutrient broth with different concentrations of plant extracts (0,50, 100, 200, 300, 400, 500, 750, 1000, 1250, 1500mg/ml), all tubes were incubated in 37ºC for 24 hrs with shaking, then 100µL aliquots of clear cultures were taken and spread on brain-heart infusion agar plates and incubated at 37ºC for 24 hrs. The lowest concentration of the plant extract that inhibits the growth of bacterial isolates considered as the minimum inhibitory concentration (MIC).

2.2.9 Plasmid DNA Extraction

Plasmid DNA extraction from bacterial isolates was achieved to the alkaline lysis method described by Maintains *et al*., (1982) as fallows:

1- Bacterial Cells were harvested from 50 ml of fresh cultures of each isolates by centrifugation at 6000 rpm for 20 min. The supernatant was discarded, while the precipitate of bacterial cells was washed twice with 30 ml of SET buffer and centrifuged under the same conditions.

2- Bacterial precipitate was resuspended in 1ml of an ice –cold solution of TEG buffer containing 5 mg/ml lysozyme and let to stand at room temperature for 5min.

3- Two ml of a freshly prepared solution of 0.2 N NaOH and 1% SDS were added respectively, then tubes were capped and mixed by gentle invertion for several times, then tubes were let to stand in an ice bath for 10 min.

4- Aliquots of 1.5 ml of an ice- cold solution of a 5M Potassium acetate (pH= 4.8) were added, then tubes were caped and mixed gently by invertion, let to stand in an ice bath for 10 min, then Centrifuged at 6000 rpm for 20 min at 4ºC.

5- Supernatant were transferred into another sterile eppendorf tubes, an equal volume of Phenol- chloroform was added, mixed by inversion for several times, then centrifuged at 10000 rpm for 20 min at 4ºC.

6- The aqueous phase (upper) was transferred to another sterile eppendorf tubes and two volume of a cold absolute ethanol was added to the aqueous phase and incubated at -20 ºC for 1 hour.

7- Tubes were Centrifuged at 13000 rpm for 15 min at 4ºC, then ethanol layer was removed gently and the precipitated DNA was dissolved in 250µL of TE buffer and stored at -20ºC.

2.2.10 Gel Electrophoreses (Maniatis *et al*., 1982).

Agarose gel was prepared by dissolving 0.7g of agarose in 100 ml of TBE buffer (1X), and heated in boiling water bath until complete dissolvance, then the temperature of the solution was dropped down to 50ºC and one drop of Ethidium bromide was added to a

final concentration of 0.5 µg/ml, then warm agarose solution was poured gently into the apparatus tray and let to stand at 25ºC until complete solidification then it was transferred to the apparatus tank and immersed by TBE buffer solution (1x).

DNA samples were mixed with 1/10 volume of loading buffer and were loaded into the wells of the submerged gel, and were being run in a current field (5volt/cm) for 2-3 hrs, then DNA bands were virtually detected using U.V. Transillminator (illumination at 302 nm), the gels were then destined in distilled water for 30-60 min to get ride of background before photographs were taken.

2.2.11 Plasmid curing.

2.2.11.1 Curing of Bacterial Plasmids:

To examine the ability of *Harpagophytum procumbens* and *Hibiscus sabdariffa* extracts as curing agents for bacterial plasmids, two bacterial strains were used for this purpose, standard strain of *E.coli* HB 101 harboring pBR322 and local isolate of *P.aeruginosa* harboring RB19 plasmid detected in previous study .The Plant extracts were used as a curing agent in addition to Ethidium bromide as a standard curing agent for bacterial plasmids (Trevors, 1986).

2.2.11.1.1 Curing with Ethidium bromide

Curing experiments were performed on *P. aeruginosa* RB19 and *E.coli* HB101 by using Ethidium bromide as standard curing agent, according to Salzono *et al*., (1992). by inoculating 5 ml of nutrient broth with 0.1 ml of each cultures of *E. coli* HB 101 and *P. aeruginosa* RB19 to mid log phase (O.D.600 about 0.4- 0.5). Then 0.1 ml of each culture was used to inoculate series of test tubes containing 5 ml

nutrient broth with different concentrations of Ethidium bromide (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5 mg/ ml). All tubes were incubated at 37ºC for 24- 48 hrs with shaking.

The growth density in different test tubes was noticed visually and compared with the control treatment to determine the effect of each concentration of curing agent on bacterial growth, and determine the lethal concentration, then 0.1 ml aliquots of the suspected sublethal concentration was taken and diluted, then 0.1 ml of aliquots the proper dilution was taken and spread on nutrient agar plates and incubated overnight at 37ºC to assay for the cured colonies.

2.2.11.2 Curing of Plasmid DNA with Plant Extract

Alcoholic extracts of (*Harpagophytum procumbens* and *Hibiscus sabdariffa*) were used in an attempt to cure plasmids of *P. aeruginosa* RB19 and *E. coli* HB101 according to (Van and Wink, 2004). Aliquots of 0.1 ml of bacterial isolates were used to inoculate test tubes containing 5 ml nutrient broth, then tubes were incubated at 37ºC until mid log phase (O.D.600 about 0.4-0.5), then 0.1 ml of the fresh culture was used to inoculate test tubes containing 5 ml of nutrient broth with different concentrations (0, 50, 100, 200, 300, 400, 500, 750, 1000, 1250, 1500 mg/ml) of aqueous and methanolic extracts of the two plants to detect the ability for curing plasmids DNA of *P. aeruginosa* RB 19, and (0, 100, 200, 300, 400, 500, 750, 1000, 1250, 1500 mg/ ml) also from aqueous and methanolic extract at identical previously used concentration for curing plasmids DNA of *E. coli* HB 101, All tubes were incubated at 37Cº for 24-48 hrs with shaking.

The growth density of different tubes was measured visually and compared with control treatment 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 growth was considered as the minimum inhibitory concentration (MIC).

Samples of 0.1ml were taken from tubes containing the sub-inhibitory concentration of each plant extract that still allows bacterial growth and diluted appropriately, then 0.1 ml samples from the proper dilutions were taken and spread on nutrient agar plates, incubated overnight at 37ºC to detect the cured colonies, according to loose the antibiotic resistance by selecting 100 colonies of bacterial isolate after each treatment. These colonies were replica plated on nutrient agar plates (master plates) and on nutrient agar plates containing an antibiotic to which the original isolate is resistant (Van and Wink, 2004).

If a colony was able to grow on the master plate but not on the selective agar containing the appropriate antibiotic, this colony was regarded to be cured colony that may have lost its own plasmid responsible for resistance to the antibiotic. The percentage of cured cells was determined, and DNA was extracted to examine the plasmid Profile in comparison with the plasmid profile of the wild type.

3. Results and Discussion

3.1 Plants Extracts

3.1.1 *Harpagophytum procumbens*

3.1.1.1 Methanolic Extract

Results showed that the weight of the residue obtained after evaporation of methanol was 4.5 grams, the appearance of the extract was green color.

3.1.1.2 Water Extract

Results showed that the weight of the residue obtained after evaporation of water was 10g, the appearance of the extract was green color.

3.1.2 *Hibiscus sabdariffa*

3.1.2.1 Methanolic Extract

Results showed that the weight of the residue obtained after evaporation of methanol was 8.64g, the a appearance of the extract was red color.

3.1.2.2 Water Extract

Results showed that the weight of the residue obtained after evaporation of water was 16.0 3 g, the appearance of the extract was red color.

3.2 Detection of Some Active Compounds in the Plant Extracts

Using different chemical reagents and solutions for detection of various active compounds found in the *Harpagophytum procumbens* and *Hibiscus sabdariffa* Results were presented in table (3.1).

Result in table (3-1) indicated the presence of Flavonoids, Glycosides and Saponins in *Hibiscus sabdariffa* methanolic and water extracts, and the presence of Flavonoids, Glycosides in *Harpagophytum procumbens* methanolic and water extracts respectively. In other study (Seca *et al*., 2001) showed that there is a cardiac glycosides, flavonoids, saponins, anthocyanins, polyphenol and alkaloids in the aqueous –methanolic extract of *Hibiscus sabdariffa*, while (Blumenthal *et al*., 2000) refers to the presence of a group of compounds called the iridoid glycosides, which include harpagoside, and other compounds such as flavonoids and phytosterols, in the aqueous- methanolic extracts of *Harpagophytum procumbens*.

Secondary metabolites of different plants play an important role in the scientific research and the aspect of drug production, due to the availability of plants all around the world and their activity in many therapeutic sides (Uglyanista *et al*., 2000).

Table (3.1) Detection of some active compounds in *Harpagophytum procumbens* and *Hibiscus sabdariffa* Methanolic and water extract.

Active compound	Reagent used	Indication	Type of extract	Result of Detection	
				(1)	(2)
Flavonoids	Ethanol + KOH	Dark color	Methanol	positive	positive
Glycosides	Fehling reagent	Red precipitate	Water	positive	positive
Saponins	Ferric chloride	White precipitate	Water	negative	positive

(1): *Harpagophytum procumbens*

(2): *Hibiscus sabdariffa*

3.2.2 Detection of Flavonoids Compounds by TLC

The plate was examined under UV light to detect the separation of flavonoids compounds in the methanol extract samples as compared to the standard's flavonoids and the R_F value (Figure 3-1). Methanolic extracted of *Harpagophytum procumbens* contain kaempferol, Apigenin and chrysoeriol, while *Hibiscus sabdariffa* contain myricetin, azoleatin, vitexin and iso-orientin.

(1) (2) (3)

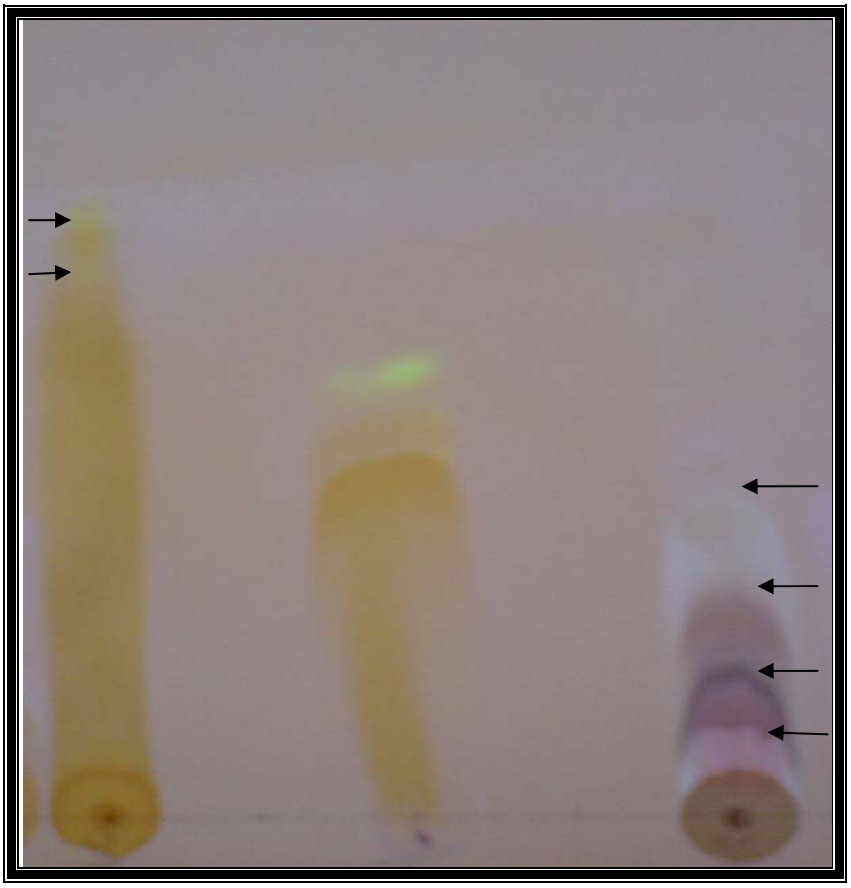


Figure (3-1) Thin layer chromatography of *Hibiscus sabdariffa* and *Harpagophytum procumbens* methanolic extracts

(1):*Harpagophytum procumbens* .

(2): Standard

(3): *Hibiscus sabdariffa*

K: Kaempferol; **A:** Apigenin**; Az:** Azaleatin; **M:** Myricetin; **V:** Vitexin; **I:** Iso-orientin.

3.3 Identification of bacterial Strains and isolates

3.3.1 Morphological and Cultural characteristics

E. coli HB101 was grows easily in all media at 37ºC. with a colony size of 1 to 1.5 mm in diameter, smooth, circular, glossy and

translucent. Also it cause blood hemolysin on blood agar. Colonies are pink on MacConkey agar because of the ability to ferment their lactose. These results are agreed with Collee *et al*., (1996). On the other hand *Pseudomonas aeruginosa* RB 19 was grow in all media at 37ºC it was non lactose ferment, Gram negative, motile and non- spore former. These results are agreed with Holt *et al*., (1994). These strains and isolates were further subjected to different biochemical tests mentioned later.

3.3.2 Biochemical tests

Biochemical tests were achieved to complete re-identification of both *E. coli* HB 101 and *P. aeruginosa* RB 19. Results mentioned in table (3.2) showed that *E. coli* HB 101 was G-ve, motile , and gave positive results for Catalase, indol, H_2S production, and methyl red, while it gave negative results for the urease, gelatinase and CO_2 production and voges- Proskaur test. On the other hand it was unable to grow on King A, King B and Cetrimide medium.

Results mentioned in table (3.2) showed that *P. aeruginosa* RB 19 was G-ve, motile , and gave positive results for catalase ,indol, H_2S , CO_2 production and voges- Proskaur test, while it gave negative result for urease. Also it was able to grow on King A, King B and Cetrimide agar medium.

All these results proved that these two strains as *E. coli* HB 101 and *P .aeruginosa* RB 19 respectively and as described by Collee *et al*., (1996).

Table (3.2) Morphological and biochemical characteristics of *E. coli* HB 101 and *P. aeruginosa* RB 19

Test		*E. coli* HB 101	*P. aeruginosa* RB 19
Gram stain		Negative	Negative
Motility		+	+
Catalase		+	+
Indol		+	+
Methyl red		+	+
Urease		-	-
Voges –Proskaur		-	+
Citrate utilization		-	+
Gelatin Hydrolysis		-	+
Growth on			
King A		-	+
King B		-	+
Cetrimide		-	+
TSI	H_2S	-	+
	CO_2	+	+
	Acid	A/A	K/K

*TSI : Triple Sugar agar (+): positive result

* A/A : acid/ acid (-) : Negative result

* K/K : alkaline / alkaline

3.4 Antibiotic Sensitivity

The standard disk diffusion method was used to determine the sensitivity of *E. coli* HB 101 and *P. aeruginosa* RB 19 to different antibiotics. Results in table (3.3) showed that *E. coli* HB 101 was resistant to ampicillin and tetracycline, while it was sensitive to erythromycin, trimethoprim, chloromphenicol, streptomycin, fusidic acid, vancomycin, imipenem, cephotaxime, cephalothin, cephalexin, gentamicin, amoxycillin, penicillin G, ticarpen and clavulanic acid. Result demonstrated in table (3.3) also showed that *P. aeruginosa* RB 19 was resistant to ampicillin, amoxycillin, chloromphenicol, penicillin G, cephalothin, cephotaxime, tetracycline, and clavulanic acid, while it was sensitive to erythromycin, trimethoprim, gentamicin, streptomycin, fusidic acid ticarpen, cephalexin, vancomycin, and imipenem.

In general it is documented that very few of the conventional antibiotics are active against *P. aeruginosa* RB 19. Many studies demonstrated a cluster of genes,which either plasmid or chromosomally encoded, responsible for antibiotic resistance in *P. aeruginosa* (Holloway *et al*., 1979; Padilla and Vasquez, 1993). From these results it is clear that *E. coli* HB 101 was resistant to two antibiotics, and sensitive to fifteen antibiotics. On the other hand *P. aeruginosa* RB 19 was resistant to eight antibiotics and sensitive for nine other antibiotics. These results were in agreement with Graafde *et al.*, 1994 who found the same pattern of antibiotic sensitivity.

Resistant to antibiotics may be caused by the antibiotic resistance genes; some of these genes may be located on chromosomal DNA, While others may be located on plasmid DNA as it was mentioned by (Jawetz *et al.*, 1998).

Table (3.3) Antibiotic Sensitivity of *E. coli* HB 101 and *P. aeruginosa* RB 19 to different antibiotics.

Antibiotic	**Code**	**Sensitivity of *E.coli* HB101**	**Sensitivity of *P. aeruginosa* RB 19**
Ampicillin	AMP	R	R
Chlaromphenicol	C	S	R
Cephotaxime	CE	S	R
Cephalothin	CK	S	R
Cephalexin	CL	S	S
Gentamicin	CN	S	S
Imipenem	IPM	S	S
Erythromycin	E	S	S
Fusidic acid	FA	S	S
Penicillin G	G	S	R
Trimethoprim	TM	S	S
Ticarpen	Tic	S	S
Tetracycline	TE	R	R
Vancomycin	VA	S	S
Streptomycin	S	S	S
Amoxycillin	AM	S	R
Clavulanic acid	C	S	R

S: sensitive

R: resist

The multidrug resistance of bacteria to several antibiotics may be due to the permeability of the outer membrane, which might prevent the entry of antibiotic into cell, or due to certain mutations that occur as a result of the over use and misuse of antibiotics, (Rasool *et al*., 2003).

As it was mentioned by Gazouli *et al*., (1998) ,plasmid encoding β-Lactamases in bacterial strains which cause the wide spread of Ampicillin resistance due to degradation of the antibiotic by β-Lactamases.

3.5 Effect of Plant Extracts on Bacterial Growth

Methanolic extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens* were prepared from aerial parts of plants with different concentrations (50, 100, 200 ,300 ,400 ,500 , 750 ,1000, 1250, 1500 mg/ml). The antibacterial effect of any extract depends on type of extract , concentration and type of microorganism as it was mentioned by Rauha *et al.,* (2000).

Result stated in table (3.4) indicate that methanolic extracts of *Hibiscus sabdariffa* posses an inhibitory effect against *E. coli* HB 101 and *P. aeruginosa* RB 19 at various different concentrations. From these results, we find that crude methanolic extracts of *Hibiscus sabdariffa* show no effect on growth of *E .coli* HB 101 at the concentrations 50, 100, 200 mg/ml of the plant extracts, while there was no growth at the concentrations 300 400, 500, 750, 1000 ,1250 ,1500 mg/ ml.

On the other hand methanolic extracts of *Hibiscus sabdariffa* allow the growth of *P. aeruginosa* at the concentrations 50 mg/ml of the plant extracts , but there is no growth at the concentrations 100, 200, 300, 400,500 750 ,1000 ,1250 ,1500 mg/ml. Methanolic extracts of many

medicinal plants contains an active compounds such as flavonoids which have antimicrobial effect against many microorganisms and the ability to cure some bacterial plasmid (Cushnie, 2005).

Results listed in table (3.4) also showed that aqueous extracts of *Hibiscus sabdariffa* allowed growth of *E. coli* HB 101 and *P. aeruginosa* RB 19 only at these concentrations 50, 100,200,300 mg/ml but there is no growth at the concentrations of 400, 500,750, 1000, 1250, 1500 mg/ml. These results were agreed with these obtained by (Auddy *et al*., 2003) and Odigie *et al.,* (2003) found that both aqueous and methanolic extracts of *Hibiscus sabdariffa* have antimicrobial effect at the concentrations ranging between 100 and 1000 mg/ml when it was examined against *Staphylococcus aureus*, *Bacillus, stearothermo philus*, *Micrococcus luteus*, *Serratia mascences*, *Clostridium sporogenes*, *E. coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, and *Pseudomonas fluorescence*.

On the other hand Results mentioned in table (3.5) showed that methanolic extracts of *Harpagophytum procumbens* have an inhibitory effect against *E. coli* HB 101 and *P. aeruginosa* RB 19 at many concentrations. From these results it was also found that crude methanolic extracts of *Harpagophytum procumbens* allowed the growth of *E .coli* HB 101 at the concentrations 50,100, 200, 300 mg/ml but there is no growth at the concentrations 400, 500, 750, 1000, 1250, 1500 mg/ml respectively. while the effect of methanolic extract of *Harpagophytum procumbens* against *P. aeruginosa* RB 19 showed that the concentration 50 mg/ml has no effect on bacterial growth, while bacteria was very sensitive to high concentrations of the extract 100, 200, 300, 400, 500 , 750, 1000, 1250, 1500 mg/ml.

Table (3.4) Effect of methanolic and aqueous extract of *Hibiscus sabdariffa* on the growth of *E. coli* HB 101 and *P. aeruginosa* RB 19 .

Concentration (mg/ml)	*E. coli* HB 101		*P. aeruginosa* RB 19	
	Hibiscus sabdariffa extracts		*Hibiscus sabdariffa* extracts	
	Methanol	Water	Methanol	Water
0	+++	+++	+++	+++
50	+++	+++	±	+++
100	++	++	-	++
200	±	+	-	+
300	-	±	-	±
400	-	-	-	-
500	-	-	-	-
750	-	-	-	-
1000	-	-	-	-
1250	-	-	-	-
1500	-	-	-	-

(+++): very good growth ; (++): good growth ; (+): moderate growth
(±): slightly growth ; (-): no growth

These results was agreed with Neuwinger, (2000) and Powell,(2001). Who found that crude methanolic extracts of *Harpagophytum procumbens* has on inhibitory effect against gram negative bacteria. Anonymous, (2005) suggested that *Harpagophytum procumbens* was

widely used in South African traditional medicine for the treatment, management and /or control of a variety of human ailments, that's why this plant extract has many anti-inflammatory and antibacterial effects, especially as antibacterial at high concentrations ranging between 100 and 800 mg/ml (Raimondo *et al*., 2005).

On the other hand, results stated in table (3.5) showed that the aqueous extract of *Harpagophytum procumbens* has inhibitory effect against *P. aeruginosa* RB 19 at these concentrations 750, 1000, 1250, 1500, mg/ml. While *E. coli* HB 101 was resistant to the extract in all concentrations used. These results are incompatible with those obtained by (Raimondo *et al*., 2005),who found that aqueous extracts of *Harpagophytum procumbens* has an inhibitory effect on all pathogenic bacteria including *E.coli* and *Pseudomonas aeruginosa* at high concentrations of extracts ranging between 1000 and 2000 mg/ml.

In general, depending on the site of action, pharmaceutical studies of antimicrobials classified drugs into antimicrobial :

1-Drug that inhibit cell wall

2-Drug that inhibit nucleic acid synthesis

3-Drug that inhibit protein synthesis.

4-Drug affecting cytoplasm membrane (Laurence *et al.,* 1999).

Table (3.5) Effect of Methanolic and aqueous extracts of *Harpagophytum procumbens* on the growth *E.coli* HB 101 and *P.aeruginosa* RB 19

Concentration (mg/ml)	*E. coli* HB 101		*P. aeruginosa* RB19	
	H .procumbens extracts		*H .procumbens* extracts	
	Methanol	Water	Methanol	Water
0	+++	+++	+++	+++
50	+++	+++	±	+++
100	++	+++	-	+++
200	+	+++	-	+++
300	±	+++	-	++
400	-	+++	-	+
500	-	+++	-	±
750	-	+++	-	-
1000	-	+++	-	-
1250	-	+++	-	-
1500	-	+++	-	-

(+++): very good growth ; (++): good growth ; (+): moderate growth

(±): slightly growth ; (-): no growth

3.6 Extraction of Plasmid DNA

Plasmid DNA was extracted from both *E. coli* HB 101 and *P. aeruginosa* RB 19 according to the modified alkaline lysis method described by Maintains *et al.*,(1982). Result indicated in figure (3.1) showed that the bacterial strain of *E. coli* HB 101 has a single plasmid DNA of pBR322 that carries genes responsible for the resistance to Ampicillin and tetracycline , while *P. aeruginosa* RB19 has two plasmid DNA bands, the large plasmid pSR202 and small plasmid pSR101 as it was mentioned in previous study (Trevors,1986). (Gulig and Doyle, 1993).

From these results it could be concluded that alkaline lysis method is a suitable method for extraction of plasmid DNA by Lysozyme treatment as an important step in cell lysis, and potassium acetate was added to the lysis solution to allow precipitin of chromosomal DNA, and high molecular weight RNA- protein-membrane complexes (Maintains *et al*., 1982).

3.7 Curing of Plasmid DNA

In order to examine the ability of methanolic and aqueous extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens* as a curing agents, curing of plasmid DNA from both strains *E.coli* HB101 harboring pBR322 and *P. aeruginosa* RB 19 harboring two plasmids was achieved according to (Trevors, 1986) by using these plants extracts in addition to Ethidium bromide as a standard curing agent as follows.

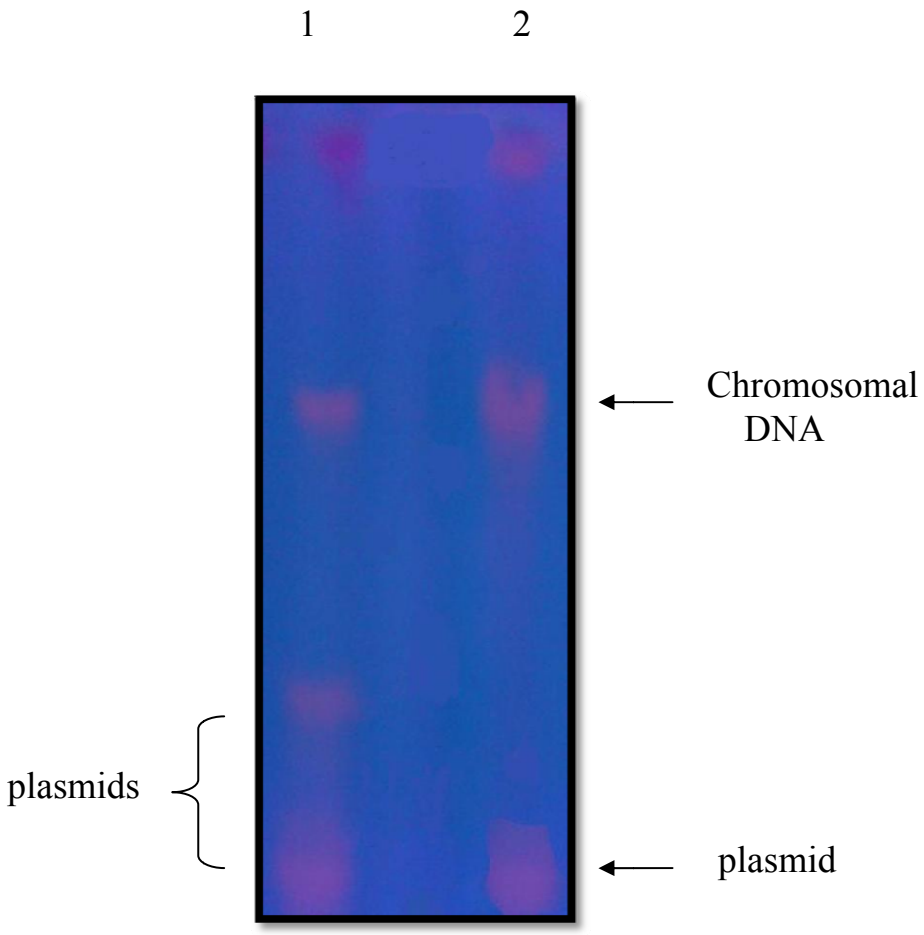


Figure (3.2) Plasmid profile of *P .aeruginosa* RB 19 and *E.coli* HB 101 On agarose gel (0.7%) after electrophoresis at 70 V for 3 hours

(1) :*P. aeruginosa* RB 19

(2): *E. coli* HB 101

3.7.1 Curing by Ethidium bromide

In order to cure *P. aeruginosa* RB 19 and *E.coli* HB 101 plasmids by using ethidium bromide as a curing agent. The mode of action of ethidium bromide in curing of plasmid DNA is the inactivation of replication of plasmid DNA during cell division without any effect on the chromosomal DNA replication, which leads to the presence of plasmid- less cells in the next generations. Furthermore, ethidium bromide was a good agent in curing of plasmid DNA, if it is compared with other physical and chemical agents (Hohn and Korn, 1969).

Fresh culture of *P. aeruginosa* RB 19 and *E. coli* HB 101 were used to inoculate nutrient broth containing gradual concentrations of ethidium bromide, and incubated for 24 hrs at 37ºC to determine the sub- lethal concentration.

Results indicated in table (3.6) showed that, the sub-lethal concentration of ethidium bromide that allows *P. aeruginosa* RB 19 growth was 1mg/ml and the sub-lethal concentration of ethidium bromide that allows *E. coli* HB 101 growth was 1.5 mg/ml. From the culture containing the sub-lethal concentration of ethidium bromide, serial dilutions were done and 100 µl of the appropriate concentration was spread on nutrient agar plates, and incubated at 37ºC for 24 hrs for each strain. Then one hundred of the resultant colonies were replica plated on nutrient agar plates containing different antibiotics (Ampicillin, tetracycline, Cephalexin) to examine the resistance of both strains to these antibiotics after curing with ethidium bromide.

Table (3.6) Effect of Ethidium bromide in different concentrations on bacterial growth of *E.coli* HB 101 and *P.aeruginosa* RB 19 after incubation at 37ºC for 24 hrs.

concentration (mg/ml)	**Growth of *E. coli* HB 101**	**Growth of *P. aeruginosa* RB 19**
0.1	+++	+++
0.2	+++	+++
0.3	+++	+++
0.4	+++	+++
0.5	+++	+++
0.6	+++	+++
0.7	+++	+++
0.8	+++	++
0.9	++	+
1	+	±
1.5	±	-
2	-	-
2.5	-	-

(+++):very good growth;(++): good growth ;(+): moderate growth; (±):slight growth; (-): no growth

Results indicated in table (3.7) showed that most of these colonies were still resistant to these antibiotics, while few of them were unable to grow on nutrient agar plates containing ampicillin, tetracycline, cephalexin. These results may be due to plasmid curing caused by the effect of ethidium bromide as a curing agent Bouanchaud *et al*., (1969).

Table (3.7) Pattern of the resistance to antibiotics by *E.coli* HB 101 and *P.aeruginosa* RB 19 after curing with ethidium bromide

Antibiotics	**Pattern of Resistance**			
	***E. coli* HB 101**		***P.aeruginosa* RB 19**	
	Before curing	After curing	Before curing	After curing
Ampicillin	Resist	Sensitive	Resist	Sensitive
Tetracycline	Resist	Sensitive	Resist	Sensitive
Cephalothin	Sensitive	Sensitive	Resist	Sensitive

From the results mentioned in table (3.7) it could be concluded that resistance traits to ampicillin, tetracycline in *E. coli* HB 101 were located on plasmid DNA (plasmid encoded phenotype). Also the resistance of *P. aeruginosa* RB 19 to tetracycline, ampicillin and cephalexin were located on plasmid DNA (plasmid encoded traits).

In order to confirm these results , cells suspected to be cured were taken and grown in nutrient broth for 24 hrs at 37ºC with

shaking (150 rpm), and then plasmid profile was examined by extraction using alkaline lysis procedure.

Results indicated in figure (3.2) and (3.3) showed that cured cells have lost its plasmid DNA after electrophoresis on agarose gel *P.aeruginosa* RB19 had lost the small plasmid which encodes for antibiotic resistance to ampicillin, tetracycline and cephalexin, while *E. coli* HB 101 had lost its plasmid DNA as it was mentioned in table (3.7),which means that the wild- type of *E .coli* HB 101 was harbur of a single plasmid conferring the resistance for ampicillin and tetracycline antibiotics.

3 .7.2 Curing by Plant Extracts

P. aeruginosa RB 19 and *E. coli* HB 101 were treated with methanolic and aqueous extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens*, in an attempt to examine the role of these plant extracts as curing agents for bacterial plasmids.

Result shown in table (3.4) indicate that the highest concentration of *Hibiscus sabdariffa* methanolic extract which allows *E. coli* HB 101 growth was 200 mg/ml, but the highest concentration of *Hibiscus sabdariffa* aqueous extracts that allows *E. coli* HB 101 to grow was 300mg/ml. On the other hand the highest concentration of *Harpagophytum procumbens* methanolic extract that allows *E. coli* HB 101 growth was 300 mg/ml while the aqueous extract of *Harpagophytum procumbens* has no effect on the growth of *E. coli* HB 101 after incubation with gradual concentrations of aqueous extracts of *Harpagophytum procumbens.*

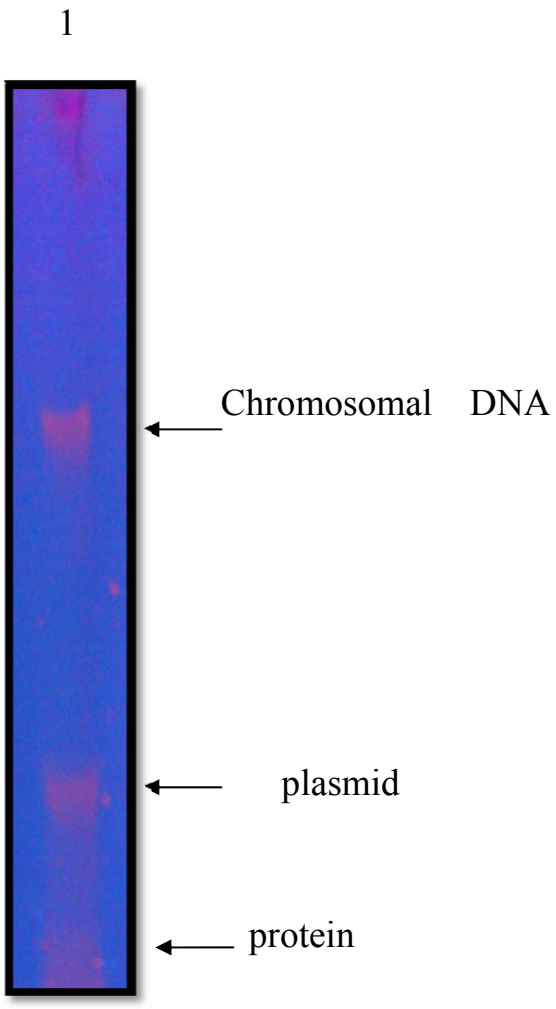


Figure (3.3) Plasmid profile of cured *P. aeruginosa* RB 19 using ethidium bromide after electrophoresis on agarose gel 0.7%(w/v); Voltages 70 V for 3 hours .

1: Cured cells

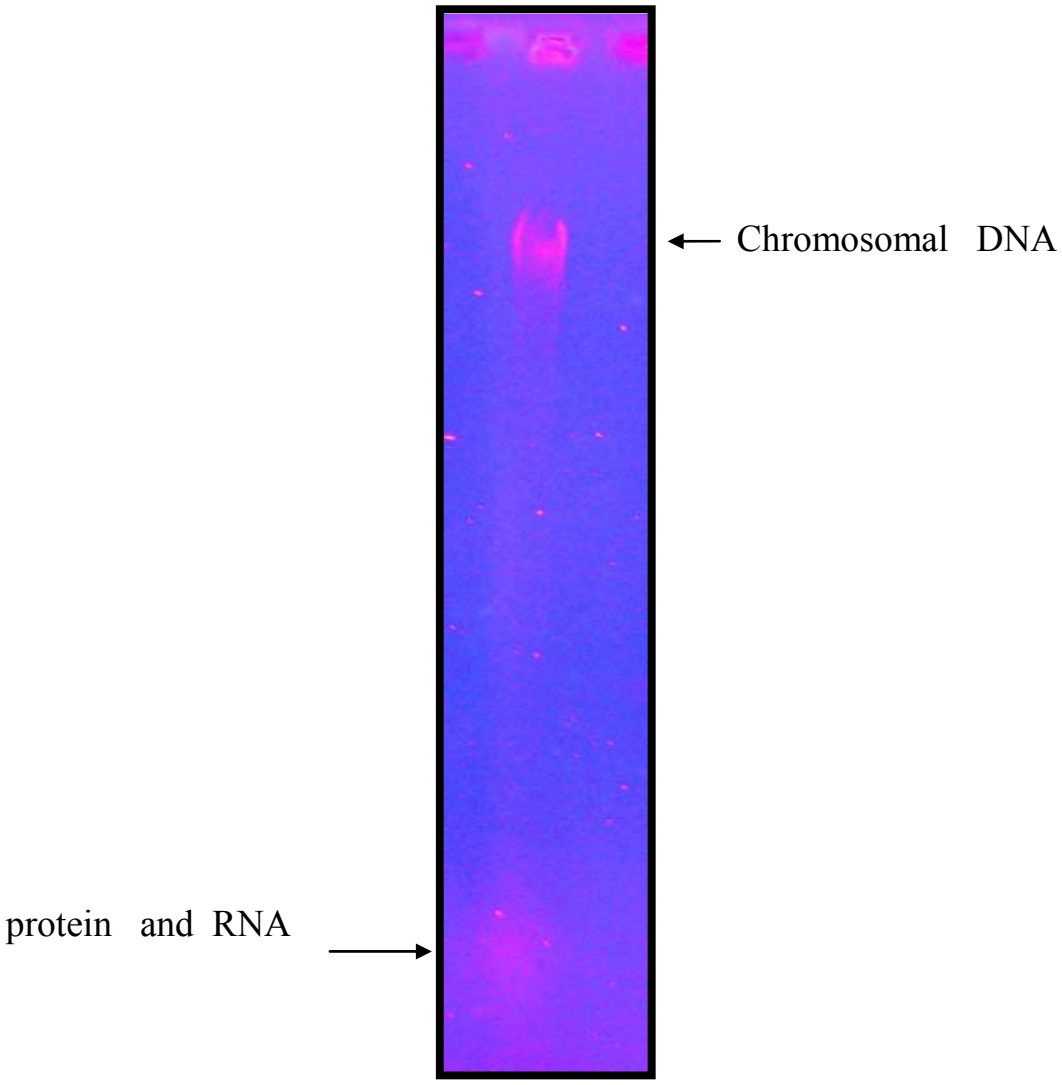


Figure (3.4) plasmid profile of *E. coli* HB 101 using ethidium bromide after electrophoresis on agarose gel (0.7%), 70 V for 3 hours.

as it was recorded in table (3.5).From the sublethal concentrations,100µl of the appropriate dilution was taken and spread on nutrient agar plates, then 100 bacterial colonies of each plant extracts treatment were examined on a medium containing a specific antibiotics (ampicillin, tetracycline, cephalothin) in order to determine the cured colonies, which cannot grow on this antibiotics containing media.

Results in table (3.8) showed that there are a number of colonies which were failed to grow on medium containing ampicillin, tetracycline, cephalothin, these result indicated that, methanolic extract of *Hibiscus sabdariffa* and that of *Harpagophytum procumbens* was responsible for losing *E. coli* HB 101 plasmid which carries resistance genes for ampicillin and tetracycline, with figure (3.5), while the aqueous extracts of *Hibiscus sabdariffa* had no effect on the plasmid of *E. coli* HB 101 after incubation with gradual concentrations of the aqueous extracts *Hibiscus sabdariffa.*

The elimination of *E. coli* HB 101 plasmid by alcoholic extract could be attributed mainly to the presence of flavonoids, detected in this extract which have curing activity as it was mentioned by Beg and Ahmed (2000) who found that alcoholic extract of *Plumbago zeylanica* (root), which tested against multidrug- resistance clinical isolates of *Salmonella paratyphi, Staphylococcus aureus,* and *Escherichia coli*, exhibited strong effect against all the tested bacteria. Phyotochemical analysis of crude extract of this plant revealed the presence of flavonoids, saponins and naphthoquinone.

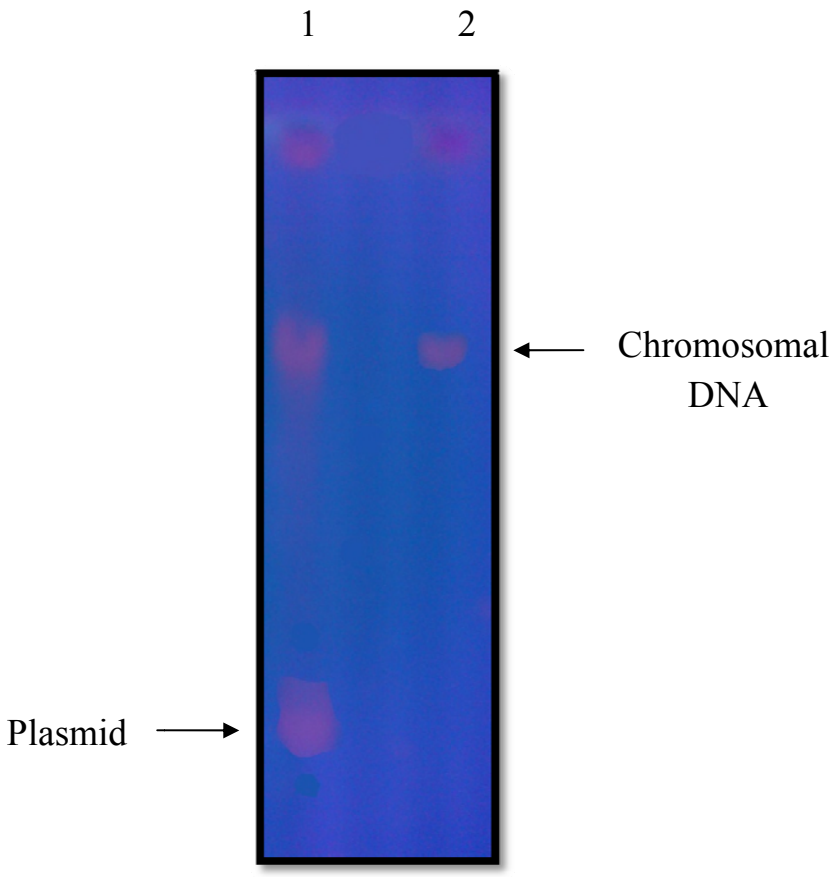


Figure (3.5) plasmid profile of wild- type and cured of *E.coli* HB 101 using plant extracts after electrophoresis on agarose gel (0.7%), 70 V for 3 hours.

1: wild- type

2: cured cells after treatment with *Hibiscus sabdariffa* methanolic extracts

Curing of plasmid from *E. coli* HB 101 was indicated by determining the loss of resistance in the cured derivative culture. Plasmid curing by plant extracts may be caused by the effect of plant active compounds on bacterial cell membrane and on plasmid – specified pili on the cell membrane which may cause damage of these pili and losing of plasmid from the cell or may be caused by the inhibitory effect of plant active compounds on plasmid replication enzymes which cause the multiplication of the bacterial cell without plasmid replication (Van and Wink *et al.*, 2004).

Table (3.8) Percentage of *E.coli* HB 101 cured colonies after treatment with *Hibiscus sabdariffa* and *Harpagophytum procumbens* methanolic extracts.

Plant extracts	Cured colonies (%)
Hibiscus sabdariffa	37
Harpagophytum procumbens	25

(1) (2)

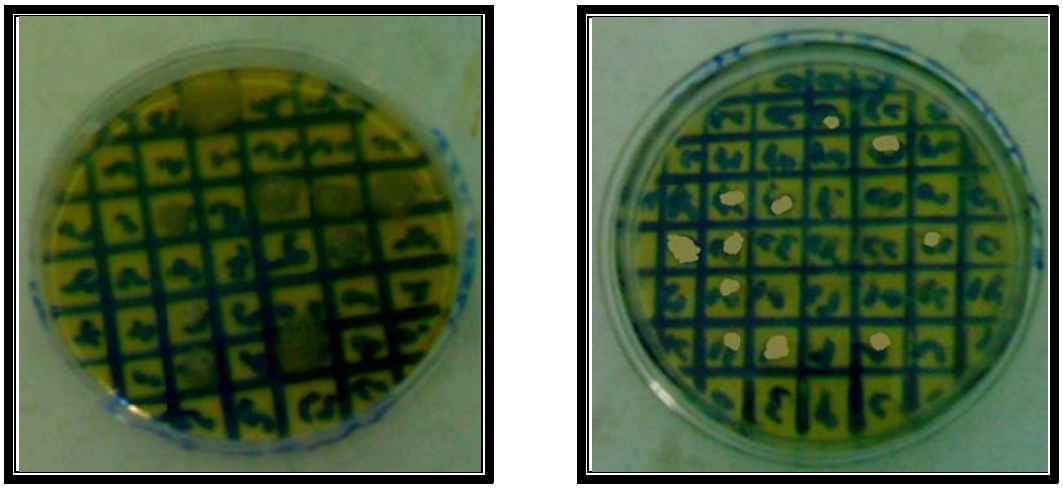


Figure (3.6) cured and wild- type of *E. coli* HB101 on nutrient agar plates containing ampicillin and tetracycline after incubation at 37ºC for 24hrs

(1)- Nutrient agar containing ampicillin
(2)- Nutrient agar containing tetracycline

P. aeruginosa RB19 was selected to perform the curing experiment with methanolic and aqueous extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens*. Result indicated in table (3.4) showed that the highest concentration of *Hibiscus sabdariffa* methanolic extracts that allows *P.aeruginosa* RB19 growth was 50 mg/ml, and the highest concentrations of *Hibiscus sabdariffa* water extract that allows *P.aeruginosa* RB 19 growth was 300 mg/ml, on the other hand the highest concentration of *Harpagophytum procumbens* methanolic extract that allows *P. aeruginosa* RB19 growth was 50 mg/ml, and the highest concentration of *Harpagophytum procumbens* aqueous extracts that allows *P. aeruginosa* RB19 growth was 500mg/ml.

From culture medium containing these concentration aliquots of

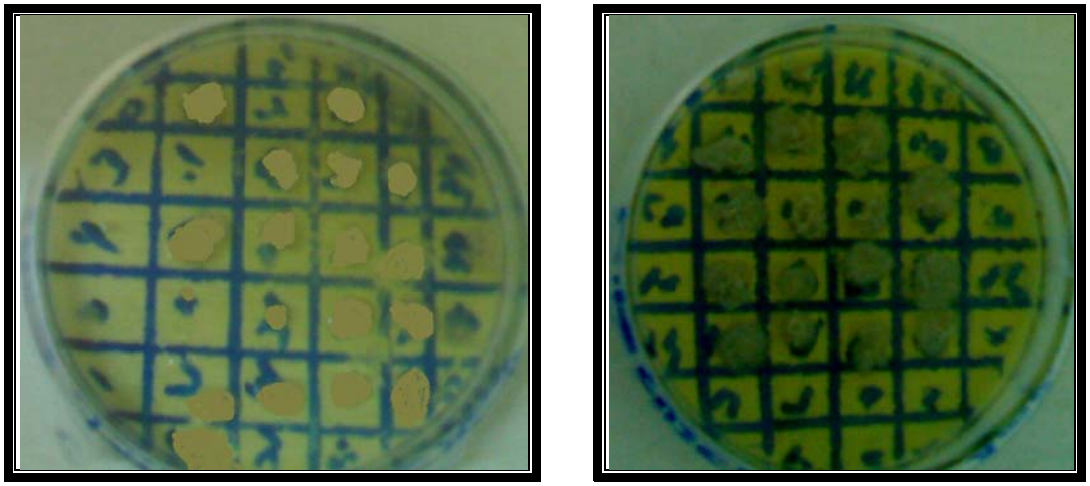
100µl were taken, diluted, and then 100µl of the appropriate dilution was taken and spread on nutrient agar plates and incubated at 37ºC for 24hrs, then 100 colonies treated with each plant extracts were replica plated on a media containing a specific antibiotics (ampicillin, tetracycline, cephalothin) in order to determine the cured colonies, which cannot grow on medium containing these antibiotics.

percentage of bacterial colonies, which lost the resistance to antibiotics after treatment with *Hibiscus sabdariffa* methanolic extract and *Harpagophytum procumbens* methanolic extract are mentioned in table (3.9).These results indicated that, methanolic extract of *Hibiscus sabdariffa* and methanolic extract of *Harpagophytum procumbens* has the ability to cure the small plasmid (pSR 101) of *P.aeruginosa* RB19 which carries the resistance genes for antibiotic resistance. The elimination of *P. aeruginosa* RB19 small plasmid could be attributed mainly to the presence of flavonoids, glycosides and saponins, which have curing activity and as it was mentioned by (Beg and Ahmed, 2000).

Table (3.9) percentage of *P. aeruginosa* RB 19 cured colonies after treatment with *Hibiscus sabdariffa* and *Harpagophytum procumbens* methanolic extracts.

Plant extracts	Cured colonies (%)
Hibiscus sabdariffa	28
Harpagophytum procumbens	35

This is study which referred to the ability of methanolic and aqueous extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens* to eliminate (cure) plasmid from *E. coli* HB 101and *Pseudomonas aeruginosa* RB19.



1 2

Figure (3.7) cured and wild- type of *P.aeruginosa* RB19 on nutrient agar plates containing ampicillin and cephalothin after incubation at 37ºC for 24 hrs .

(1) -nutrient agar containing ampicillin
(2)- nutrient agar containing cephalothin

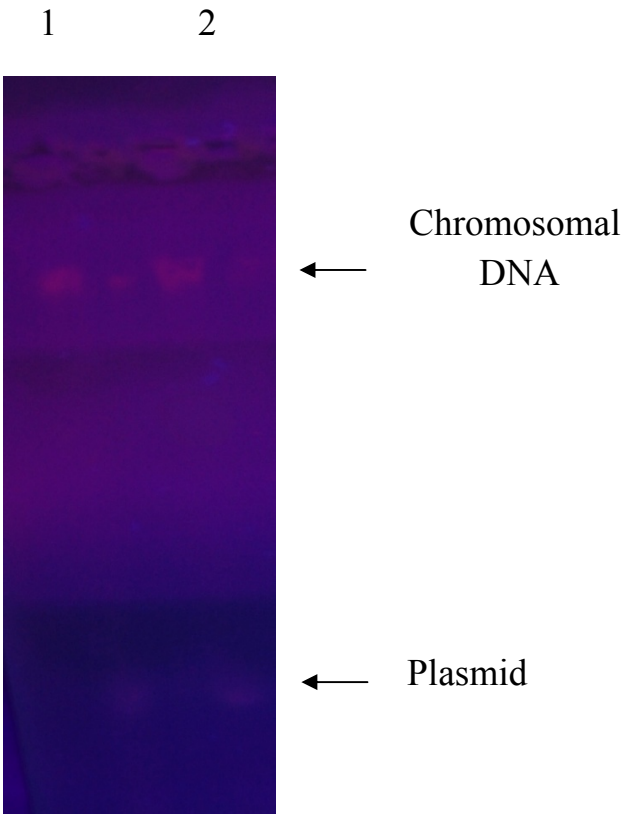


Figure (3.8) Plasmid profile of cured *P. aeruginosa* RB 19 using plant extracts after electrophoresis on agarose gel (0.7%), 70 V for 3 hours.

1: cured cells after treatment with *Harpagophytum procumbens* methanolic extracts

2: cured cells after treatment with *Hibiscus sabdariffa* methanolic extracts .

Conclusions

1- Methanolic and water extracts of *Hibiscus sabdariffa* and *Harpagophytum procumbens* have inhibitory effect against *P.aeruginosa* RB19 ,while methanolic and water extracts of *Hibiscus sabdariffa* and methanolic extract of *Harpagophytum procumbens* have inhibitory effect against *E.coli* HB101.There inhibitory effect could be attributed to flavonoids, glycosides and saponins active compounds.

2- *Hibiscus sabdariffa* and *Harpagophytum procumbens* methanolic extracts were effective curing agents in elimination of plasmid responsible for antibiotics resistance in *P. aeruginosa* RB19 and *E. coli* HB101.

3- Methanolic extract of *Hibiscus sabdariffa* was more efficient than methanolic extract of *Harpagophytum procumbens* for curing *E.coli* HB 101 plasmid DNA.

4- Methanolic extract of *Harpagophytum procumbens* was more efficient than methanolic extract of *Hibiscus sabdariffa* in curing *P. aeruginosa* RB19 plasmid DNA .

Recommendations

1- Future work is needed for separation and purification of active compound of the plant used in this study.

2- Using new technique of biotechnology for production of there natural curing agents.

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الخلاصة

أستهدف البحث دراسة فعالية المستخلصات المائية و الكحولية للأجزاء الهوائية لنباتات شاي كجرات و مخالب الشيطان ضد نمو الأحياء المجهرية خارج الجسم الحي وقدرتها على تحييد البلازميدات لنوعين من البكتريا *E.coli* HB 101 و*P.aeruginosa* RB19 بينت نتائج الفحص الكيميائي للمستخلصات النباتية أحتواء المستخلص الميثانولي لنباتات شاي كجرات على الفلافينويدات و المستخلص المائي لشاي كجرات على الكلايكوسيدات و السابونيات و المستخلص الميثانولي لمخالب الشيطان على الفلافينويدات وأحتواء و المستخلص المائي لمخالب الشيطان على الكلايكوسيدات أوضحت النتائج خارج الجسم الحي أن المستخلص المائي و الميثانولي لشاي كجرات و المستخلص الميثانولي لمخالب الشيطان لهما فعالية ضد النمو البكتيري لنوعي *E.coli* HB 101 و *P. aeruginosa* RB 19 بينما المستخلص المائي لمخالب الشيطان لة فعالية ضد نمو بكتريا *P.aeruginosa* RB 19 ولكن ليس لة اي تأثير ضد نمو *E.coli* HB 101 أختبرت حساسية *P.aeruginosa* RB 19 و *E.coli* HB 101 ضد المضادات الحيوية المختلفة ووجدت قابلية *E.coli* HB 101 لمقاومة امبسلين ، تتراسايكلين بينما حساسة fusidic acid, erythromycin, trimethoprim, chloromphenicol, streptomycin, vancomycin, cephotaxime, cephalothin, cephalexin, gentamicin, amoxycillin, imipenem, penicillin G, ticarpen, clavulanic acid. بينما *P.aeruginosa* RB19 مقاومة الى ampicillin, amoxycillin, chloromphenicol penicillin G, cephalothin, cephotaxime, tetracycline, clavulanic acid. وبينما كانت حساسة الى erythromycin, trimethoprim, gentamicin, streptomycin fusidic acid, ticarpen, cephalexin, vancomycin, and imipenem.

درس النسق البلازميدي لعزلة *P.aeruginosa* RB 19 و *E.coli* HB 101 باستخلاص الدنا الكلي بطريقة التحلل القاعدي ثم الترحيل على هلام الا كاروز وقد اظهرت النتائج ان *E.coli* HB 101 تحتوي على بلازميد صغير وعزلة *P.aeruginosa* RB 19 تحتوي بلازميد كبير و بلازميد صغير ولدى اجراء عملية تحييد الدنا البلازميدي باستخدام مادة بروميد الايثيديوم لمعرفة دور البلازميدات في ضراوة هذة العزلات، ومقاومتها لمضادات الحياة.واظهرت نتائج التحييد الحصول على عدد من المستعمرات التي فقدت قابليتها على مقاومة المضادات الحيوية لكلا نوعي من البكتريا بعد معاملتها مع بروميد

الايثيديوم اظهرت النتائج ان عدد من المستعمرات التي فقدت قابليتها على مقاومة امبسلين، تتراسايكلين، سيفالوثن للعزلة *P.aeruginosa* RB 19 بينما *E.coli* HB 101 فقدت قابليتها لمقاومة امبسلين، تتراسايكلين ولدى اجراء عملية تحييد الدنا البلازميدي باستخدام المستخلصات النباتية لشاي كجرات و مخالب الشيطان ضد *P.aeruginosa* RB19 *E.coli* HB 101 فقد اظهرت النتائج ان العديد من المستعمرات فقدت قابليتها على مقاومة المضادات الحيوية، مما يشير الى قابلية المستخلصات النباتية على تحييد البلازميدات البكتيرية وان المستخلص الميثانولي لشاي كجرات هو افضل في تحيد بلازميد *E.coli* HB101 وفقدان قابليتها لمقاومة امبسلين، تتراسايكلين و المستخلص الميثانولي لمخالب الشيطان هو افضل في فقدان *P.aeruginosa* RB 19 قابليتها على مقاومة امبسلين, تتراسايكلين، سيفالوثن .

بِسْمِ ٱللَّهِ ٱلرحمَن ٱلرَّحِيمِ

وَأنزَلَ الله عَلَيْكَ ٱلكِتَابَ وَٱلحِكْمَةَ وَعَلَّمَكَ

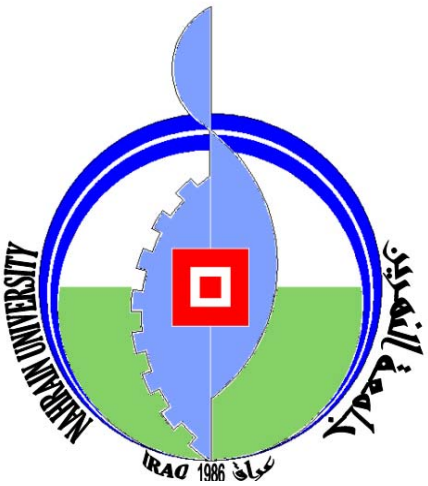
مَالم تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللهِ عَلَيْكَ عَظِيماً

صَدَقَ ٱللَّهُ ٱلعَظِيم

سورة النساء

أية 113

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم



تقييم تأثيرات بعض المركبات النباتية الفعالة على البلازميدات البكتيرية

رسالة

مقدمة الى كلية العلوم – جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

هبه محمد جواد احمد القرةغلي

بكالوريوس تقانة احيائية / كلية العلوم

جامعة النهرين 2006

بأشراف

الأستاذ الدكتورة خلود وهيب السامرائي **الأستاذ المساعد الدكتور حميد مجيد**

ذو الحجة 1430

كانون الأول 2009