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قُل كُلٌّ يَعمَلُ عَلَىٰ شَاكِلَتِهِ

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صَدَقَ ٱللَّهُ ٱلعَظِيم

سورة الإسراء الايه (٨٤-٨٥)

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List of Abbreviations

Abbreviation	Full name
2,4-D	2,4-dichlorophenoxyacetic acid
α	*Alpha*
BA	Banzyl adenine
B. ceries	*Bacillus cereus*
β	Beta
°C	Degree Celsius
CRD	Completely randomized design
C.f.u.	Cell factor unit
DDH_2O	Double distilled water
Dwt	Dry weight
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetate
G +ve	Gram positive
G-ve	Gram negative
G	Gram
Hrs	Hours
Kin.	Kinetin
LSD	Least significant difference
MS	Murashige and Skoog medium
M. chamomilla	*Matricaria chamomilla*
Min.	Minutes
MIC	Minimum Inhibitory Concentration
Mg	Milligram
N	Number of replicates
NAA	1-naphthalene acetic acid
NaOCl	Sodium hypochlorite
P. aureginosa	*Pseudomonas aureginosa*
P.B.S	Phosphate buffer saline
S. D	Standard deviation
S. aureus	*Staphylococcus aureus*
UV	Ultraviolet (light)
Wt	Weight

Effect of flowers and Callus extracts of *Matricaria chamomilla* L. on some bacteria causing eyes infections

Abstract:

In an attempt to treat infection of rabbit eyes using water and ethanolic extracts of chamomile (*M. chamomilla*) flowers compared to callus cultures extracts. Several experiments were carried out. Callus was induced and maintained on MS medium supplemented with 3.0 mg/l BA and 0.1 mg/l NAA initiated from seedlings. Plantlets regenerated on the same hormone combination after 40 days in culture.

Water and ethanolic extracts of *M. chamomilla* flowers and callus cultures were investigated for their antimicrobial activity. It contained resins, tannins, coumarines, phenols, flavonoids and very little amounts of glycosides.

Chamomile flowers water extract showed better inhibitory effect than ethanolic one against *Staphylococcus aureus* and *Bacillus ceries* and to a lesser extent to *Pseudomonas aeriginosa* and *Escherichia coli*. Callus extracts showed an inhibitory effect on the same bacterial isolates than flower extracts particularly on growth of *S. aureus* and *B. ceries*.

The Minimum inhibitory concentration (MIC) of the flower extracts showed that water extract had better activity than ethanolic one.

It has been noticed that all extracts of have a anti inflammatory effect at a concentration (40mg/ml). However, these extracts showed a antibacterial activity at a concentration 40mg/ml that applied on laboratory rabbit eyes.

CHAPTER TWO

Literature Review

CHAPTER THREE

Materials and Methods

CHAPTER FOUR

Results and Discussion

Conclusions and Recommendations

References

1- Introduction

Chamomile has been used medicinally for thousands of years, and is widely used in Europe. It is a popular treatment for numerous aliments, including sleep disorders, anxiety, indigestion, skin infections such as eczema, wound healing, infantile colic, and teething pains and eye infection (Hewitt, 2001).

German chamomile and Roman chamomile are the two major types of chamomile. They are believed to have similar effect on the body, although German chamomile may be slightly stronger. Extracts of Roman chamomile showed antitumor activity, and extracts of German chamomile contain several antibacterial, antifungal and antiseptic properties. It is used against different types of bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Micrococcus spp*. and *Pseudomonas aeriginosa*. Both types of chamomile contain minute amounts of a blue oil (azulene). This oil has neutralizing abilities on the toxins produced by various bacteria and therefore, assists in the healing process of wounds (Hewitt, 2001).

The production of secondary metabolites *in vitro* is possible through plant tissue culture. *In vitro* study holds a potential for the production of high-quality plant based medicines. This can be achieved through different methods including micropropagation of cell lines which are capable of producing high yield of secondary compounds. The accumulation of secondary products in plant cell cultures depends on many factors including the composition of the culture medium and environmental conditions (Murch *et al*., 2000).

Although chamomile is widely used, there is not enough reliable research in humans to support its use for many conditions. Despite its reputation as gentle medicinal plant, chamomile-tea helps in reducing the swelling and redness of eyes, and reduces inflammations, which are two common eye problems. Chamomile is also used in beauty products to make skin looks smoother and less puffy (Smith, 2006).

The aims of this work are:

1- Callus induction on chamomile explants.

2- Preparation of (water and ethanolic extracts) from chamomile flowers and callus cultures.

3-Determination of minimum inhibitory concentrations of chamomile water and ethanolic extracts

4- Examination of these extracts for their antibacterial activity against some pathogenic bacteria (*E. coli, S. aureus, P. aeruginosa, B. ceries*).

2. Litreture review

2.1-Herbal medicine

Medicinal plants are gifts of nature, used against various infections and diseases. In many parts of the world, herbs were used as food (vegetables) and flavors for centuries. Some herbal plants are considered as house medicines and played important role in nearly most cultures and all over the contents (Duke, 1985).

The herbs chemical components are the most important for pharmaceutical companies. Furthermore, peoples are interested in medicines prepared from plants due to their little side effects, cheap and almost available compared with synthetic drugs. This may be because of the low concentrations of the active compounds found in plants which the human body would need (Mackin, 1993).

2. 2-Chamomile plant

Family: Asteraceae

Genus: Matricaria

Species: Chamomilla

Common name: Chamomile, Bayboon, Wild Chamomile, Sweet False Chamomile, Matricaria Flos (Chakravarty, 1976).

Types: German Chamomile (*M. chamomilla)* and Roman Chamomile (*Anthemis nobilis).*

These two types have similar medicinal properties. The flower is the main medicinal part of the herb. Chamomile products can be administrated in many ways. The common ones are: oral, inhalation, and solution for bath and infusion (Newel *et al.,* 1990).

2.3-Description

Chamomile flowers are white Fig.(2-1), conical in shape with yellow centers and less than 3 centimeters in width. The plant grows vertically with light green stems. It reaches up to three feet height. Chamomile prefers sandy soil and full sun. They grow in late spring or early summer. Flowers are harvested throughout summer when they are fully open (Joe and Teresa, 1999; Hewitt, 2001).

Fig. (2-1): Flowers of chamomile (*Matricaria chamomilla* L.) used as a source for plant material (Khayyal *et al.*, 2001).

2.4-Distribution of chamomile

Roman chamomile is cultivated in Europe, especially in Belgium, France, and England (Simon *et al.,* 1984). German chamomile is cultivated in Germany, Hungary, Russia, Argentina, Slovakia and Poland, Egypt and Iraq. German chamomile is native to Europe and western Asia and North America (Chakravarty, 1976; Salamon, 1992).

2.5-History and popular Uses

Chamomile is one of the widely used and well-documented medicinal plant in the world. It is included in the pharmacopoeia of 26 countries. In Germany, more than 4,000 tons of chamomile flowers are produced each year (Salamon, 1992; Berry, 1995).

Chamomile flowers are used internally and externally to treat extensive list of conditions. It is used internally for relaxing sleep aid, stomach, bladder, kidney and spleen troubles, treatment for fevers, colds, hysteria, nightmares, asthma and hemorrhoids (Culpeper, 1976; Duke, 1985; Hewitt, 2001).

Chamomile flowers tincture have long been used for treating colic, convulsions, croup, diarrhea, indigestion, insomnia, infantile convulsions, toothache, bleeding, swollen gums, a folk cancer remedy, and many other uses (Leung and Foster, 1996).

Chamomile flowers are used externally for the treatment of inflammations and irritations of the skin and mucosa, including the mouth and gums, lumbago, wounds, eczema, gout, neuralgia, sciatica, rheumatic pain, conjunctivitis. It is used as a perfume, cosmetic and as hair tint and conditioner (Newel *et al.,* 1990; Smith, 2006).

Dried flowers of chamomile are used in herbal teas, flavoring beverages, candy, baked goods (Emongor *et al*., 1990). Chamomile tea is used to relieve spasms and inflammatory conditions of the gastrointestinal tract, as well as peptic ulcers (Mann and Staba, 1986; Der Marderosian and Liberti, 1988). It is estimated that over one million cups of chamomile tea are ingested world wide each day. It treats colic, croup and fever for children. It is thought that it reduces the reaction to allergens such as pollen or dust in sensitive individuals (Bartram, 1995). Chamomile essential oil is used for treating malaria and parasitic worm infection, cystitis, cold, and flu (Nemecz, 1998). Components in the essential oil of German chamomile help in smooth muscle relaxing activity (Tyler, 1999).

Extensive scientific research has confirmed many of the traditional uses, and established pharmacological mechanisms for the plant's therapeutic activity, including antipeptic, antispasmodic, antipyretic, antibacterial, antifungal, and antiallergenic activity (Qurban, 2002).

2.6-Plant secondary metabolites

Plants produce large diverse array of organic compounds that have no direct function in plant growth and development. These substances are known as secondary metabolites, secondary products, or natural products (Hartmann, 1996), while primary metabolites (proteins, carbohydrates and fats) are important in plant physiological process, such as growth and development (Mann, 1987).

The importances of the secondary metabolites in plants are: to protect the plants against being eaten by herbivores and against being infected by microbial pathogens. They serve as attractants for pollinators and as agents for plant competition. Plant secondary metabolites can be divided into three chemically distinct groups: Phenolics, Terpenes, and Nitrogen-containing compounds (Oomah, 2003).

2.6-1 Phenolic compounds

Phenolic compounds include a wide range of plant substances, which posses in common an aromatic ring that bears one or more hydroxyl subunit. Some phenolic substances tend to be water-soluble carboxylic acids and glycosides, some are soluble only in organic solvents and others are insoluble polymers, since they mostly occur with sugar as glycosides. The flavonoids are one of the largest groups monocyclic phenols among natural phenolic compounds, phenylpropanoids and phenolic quinones (Taiz and Zeiger, 2002).

The major groups of phenolic compounds are flavonols and flavones. Flavonols are very widely distributed in plants with a considerable range of flavonol glycosides. More than a hundred different glycosides of quercetin have been described. The most common flavones are apigenin and luteolin (Harborne, 1973).

2.6.2- Chamomile essential oil

The oil is extracted from flower heads by steam distillation. It changes in color from brilliant blue to deep green due to the terpenoid chamazulene, when flowers are fresh, but its color change over time to dark yellow. However, the oil does not lose its potency (Mann and Stapa, 1986).

The essential oil of chamomile flowers is water insoluble contains compounds responsible for many uses. The essential oil of Roman chamomile contains less chamazulene (Grieve, 1982; Hoffmann, 1995).

In addition to genetic differences in quality and quantity of essential oil and other plant components, as in most medicinal plants, are dependent upon a wide range of variables, such as environmental factors, cultivation practices, plant part, plant age, and post harvest handling (Simon *et al.*, 1984; Mann and Staba, 1986).

2.6.3 -Nitrogen compounds

They are substances containing one or more nitrogen atoms in combination as part of a cyclic system. The most common precursors of alkaloids are amino acids. Chemically, alkaloids are a very heterogeneous group, ranging from simple compound like coniine to the pentacyclic structure of strychnine.

Alkaloids are a large group of secondary products that exhibit important pharmacological properties (Goodwin and Mercer, 1983).

2.7-Chemical constituents

There are different classes of chemical constituents. They have been isolated and used individually in medical practices and cosmetics. About 120 secondary metabolites have been identified in chamomile, including 28 types of terpenoids, 36 flavonoids, and 52 additional compounds. All these constituents have a potential pharmacological activity. The most active chemical constituents found in chamomile are terpenoids, flavonoids, coumarins and spiroethers (Salamon, 1992).

2.7.1- Terpenoids

German chamomile contains alpha-bisabolol, alpha-bisabolol oxides A and B, and chamozulene sesquiterpens (Gardiner, 1999; Gyllenhaal, 2000).

Scientists believe that chamazulene has pharmacological activities such as anti inflammatory, antispasmodic, antimicrobial, and mild sedative effects (Mann and Staba, 1986; Der Marderosian and Liberti, 1988). Studies also showed that alpha-bisabolol has a protective effect against peptic ulcers, as well as antibacterial and antifungal. Alpha-bisabolol reduces fever and shortens the healing time of skin burns in laboratory animals (Der Marderosian and Liberti, 1988; Joe and Teresa, 1999). Stafford, (1991) isolated and purified chamazulene and a-bisabalol from callus cultures of *M. chamomilla*.

2.7.2- Flavonoids

Apigenin, luteolin, quercetin and other important flavonoids have been identified in German chamomile (Agullo and Gamet-Payrastre, 1997). Researchers believe that these constituents contribute to chamomile's antispasmodic, anti-inflammatory, and gastrointestinal relaxing effects. Research indicated different levels of inhibitory effects on certain malignant cell proliferation *in vitro* (Agullo and Gamet-Payrastre, 1997; Joe and Teresa, 1999).

Some alkylated flavonoids, such as chrysoplenin, chrysoplenol and jaceidin, also have been studied. These compounds have shown to possess anti-inflammatory and antispasmodic activities (Hoffmann, 1995; Gardiner, 1999).

2.7.3- Coumarins

Other classes of compounds have been identified. They are coumarins, herniarin and umbelliferone. These have anti-inflammatory properties.

2.7.4- Spiroethers (dicycloether)

Chamomile contains phenolic carboxylic acids such as vanillic, anisic, syringic and caffeic. There are other constituents like anthemic acid, anthemidine tannin and matricarin,. as well as polysaccharides (Salamon, 1992).

These active ingredients contribute to chamomile’s anti-inflammatory, antispasmodic, and smooth-muscle relaxing action, particularly in the gastrointestinal tract (Della Loggia and Dri, 1986).

2.8- *In vitro* production of secondary metabolites

Plant cell cultures have proved to be an important tool for the study of secondary products biosynthesis. The secondary products may not be synthesized during certain lifetime of the plant. For example, flower pigments are only produced at a specific developmental stage, while a number of types of chemicals are rapidly synthesized. These differences within plants have often made the biosynthesis study difficult. Even for those secondary products that are synthesized more or less continuously, the rate of synthesis is frequently very low (Ramawat, 2004).

The production of the secondary metabolites *in vitro* is possible through plant tissue culture (Barnum, 2003; Karam *et al.*, 2003). Theoretically by growing undifferentiated tissues *in vitro,* large amounts of biosynthetically active tissue could be generated. Thus, it would be possible to grow large quantities of biomass for the production of pharmaceuticals by fermentation using bioreactors (Stafford, 1991).

2.8.1-Callus cultures

Callus cultures are clumps of undifferentiated plant cells grown on nutrient media. The state of undifferentiated growth is maintained by the phytohormone balance, mainly auxins and cytokinins, added to the medium. In normal plant life, callus tissue is formed after wounding and this cell mass helps to close the wound rapidly. In *in vitro* culture a tissue is wounded and the induced callus is further subcultured on nutrient media (Ramawat, 2004).

Sateesh (2003) divided callus growth to:

a. Lag phase, where cells prepare to divide.
b. Exponential phase, where the rate of cell division is increasing.
c. Linear phase, where cell division slows but the rate of cells expansion increases.
d. Deceleration phase, where the rate of cell division and elongation decreases.
e. Stationary phase, where the number and size of cells almost remain constant. Cells are harvested at this stage.

The optimum conditions for callus formation as well as the suitable sterilizing procedures and nutrient media, have to be determined empirically (Haq, 1993).

The productions of secondary compounds are more stable in callus cultures than in the suspensions. The callus stock provides the material for the establishment of new suspensions. The degree of callus formation depends on the type of explants, plant species and plant growth regulators. Exogenous plant growth regulators (type, concentration, auxin to cytokinin ratio) are crusial for callus formation and this may depend upon the endogenous hormone content of the tissues under investigation (Pierik, 1987; Ramawat, 2004).

2.9-Enhancement of secondary metabolite production

One of the major limitations of plant cell culture systems is the variable production of secondary products in many *in vitro* cultures of medicinal and aromatic plants. Variability is also evident at the biochemical level between cells in order to produce secondary metabolites. The low production may involve competition between primary and secondary pathways for key intermediates (Kurz *et al*., 1988).

Different types of cell lines may be isolated from variant cell suspension cultures using screening and selection techniques. The definition of screening here is the analysis of large number of cells or cell lines to identify rare individuals with high production ability of secondary metabolites. The selection is defined as the application of an environmental factor, which allows the survival or growth of cells with desired traits, such as, resistance to antibiotics, salinity, heavy metals or herbicides (Ramawat, 2004).

2.9.1-Culture medium

Media components play an important role in stimulating the secondary metabolites production, e.g. many plant cells that grown in limited amount of nitrogen or phosphate gave enhanced yields of secondary metabolites (Zenk, 1977).

Sucrose and glucose are the preferred carbon source for plant tissue cultures. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. The maximum yield of rosmarinic acid produced by cell suspension cultures of *Salvia officinalis* was 3.5 g/L when 5% of sucrose was used but it was 0.7 g/L in the medium containing 3% sucrose (Whitaker, 1986).

2.9.2-Plant growth regulators

Growth regulators are required to induce callus tissues and to promote the growth of many cell lines. As an auxin, 2, 4-dichlorophenoxyacetic acid (2, 4-D) or naphthaleneacetic acid (NAA) are used. The concentration of auxins in the medium is generally between 0.1 to 50 mg/l. Kin. or BA as a cytokinin is occasionally required together with auxins for callus induction at concentrations of 0.1 to 10 mg/l. Other derivatives of auxin and kinetin are also used in some cases (Kuang and Cheng, 1981; Misawa, 1985).

Since each plant species requires different kinds and levels of growth regulators for callus induction, its growth and metabolites production, it is important to select the most appropriate combination (Zhao *et al*., 2001).

2.9.3-Physical factors

The effects of light, temperature, pH, and oxygen are all parameters that must be examined in the studies of secondary metabolites production.

Ramawat (2004) reported that blue light induced maximum anthocyanin formation in *Haplopappus gracilis* cell suspension cultures, whereas white light induced anthocyanin synthesis in *Catharanthus roseus* and *Populus spp*. In contrast to these, white or blue light completely inhibited naphthoquinone biosynthesis in callus cultures of *Lithospermum erythrorhizon*.

A temperature of 17- 25°C is normally used for induction of callus tissues and growth of cultured cells. But, each plant species may favor a different temperature. It was found that lowering temperature increased the total fatty acid content per cell (Toivonen, 1992).

The medium ph is usually adjusted between 5 and 6 before autoclaving and extremes of pH are avoided. The pH of the growth medium influences the production of phytochemicals in cultured cells. Cultures of *Daucus carota* produced less anthocyanin when grown at pH 5.5 than those grown at pH 4.5, since anthocyanin content decreased by 90% at pH 5.5 compared to tissues grown at 4.5 (Ramawat, 2004).

Each plant species has different optimized conditions both for growth of the cells and for production of useful products, so it is necessary to optimize the conditions in each case (Yamada *et al*., 1980).

2.9.4-Addition of precursors

Addition of appropriate precursors to the culture media or related compounds, sometimes stimulate secondary metabolites production. This approach is advantageous if the precursors are inexpensive. For example, phenylalanine is one of the biosynthetic precursors of rosmarinic acid (Zenk, 1977). Addition of this amino acid to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid.(Tabata *et al*., 1971) Addition of phenylalanine to the callus cultures of *Taxus cupsidata* cells stimulated the biosynthesis of the anticancer compound, taxol (Fett *et al*., 1995).

2.10- Eye infections

Eye infection is usually related to pinkeye (conjunctivitis), an inflammation of the sensitive mucous membranes that line the eyelids. Other causes of redness and irritation are a persistent scaliness on the eyelid edges called (blepharitits) and inflamed painful bumps at the base of the eyelashes known as (styes). A doctor should evaluate eyes that are red and painful to determine the proper course of treatment and rule out more serious ailments, such as glaucoma.

Viruses and bacteria cause eye infections. Inflammation and redness may also occur as a result of injuries to eye, allergies, or irritatants (such as smoke, make up, or chlorine in swimming pools).

2.11-Infectious eye diseases

1- Conjunctivitis is an inflammation of the conjunctiva, the membrane that lines the eyelid and wraps around to cover most of the eyes white. The eye may appear swollen and bloodshot; they are often itchy and irritated. The eyelids are apt to stick together after being closed for an extended period because the infected membrane is often filled with pus.

2 -Keratitis is an inflammation of the cornea, the transparent membrane that covers the colored part of the eye (iris) and pupil of the eye.

3- Dacryocystitis is an inflammation of the tear sac (lacrimal sac) at the inner corner of the eye.

Factors that contribute to eyes infection include bacterial infection, injury of the eye, fumes, smoke, contact lens solutions, chlorine from swimming pools, chemicals, makeup, or any other foreign substance that enters the eye (Babbar *et al* ., 1982).

2.12- Microorganisms cause eye infection

The eye and its associates are uniquely predisposed to infection by various microorganisms, if barriers of defense mechanisms are broken by a penetrating injury or ulceration, infection may occur. Infection can also reach the eye via the blood stream for another site of infection.

The microorganism infects the eye through many parts and cause diseases such as conjunctivitis, Keratitis, endophthalmitis or other disease of lacrimal apparatus. Number of bacteria can infect the eye, largely through conjunctiva (Tortora *et al.*, 1986).

So, many types of microorganisms infect it such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Corynebacterium diphtheriae*, *Haemophilus influenzae*, Escherichia *coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus spp*., and others, in addition to fungi such as *Candida albicans* and *Curvulania spp*.

The major related microorganisms to eye infection are discussed in some detail below.

2.12.1-*Staphylococcus aureus*

It is major cause of dacryocystitis and dacryoadenitis, conjunctivitis and Keratitis, (Baron *et al.,* 1994). *S. aureus* are G +ve cocci that occur in single, pair, tetrads, short chain, and irregular ``grape like `` clusters. They belong to family micrococcaceae (Kloos and Jorgensen, 1985). Members of this genus are facultative anaerobic (Harly and Prescott, 1996). They are non motile, non spore forming and are unencapsulated or have limited capsule (Kloos and Bannerman, 1999). They cause intraocular infection either by contamination of intraocular lens or direct intraocular inoculation.

2.12.2- *Escherichia coli*

It is found in conjunctiva of the eye (Prescott *et al.,* 1990); it infects the lacrimal sac causing dacryocystitis (Shah *et al.,* 2001). It was isolated from feces in 1885, and named by German bacteriologist T. Escherich. *Escherichia coli* is a common inhabitant of the large intestine of human and mammals. It is also found in the guts of birds, reptiles, amphibians and insects. The bacteria are excreted in great number with feces (Pyatkin and Kirvoshein, 1987; Manges *et al*., 2001). It occurs singly or in pairs, capsules or microcapsules in many strains, G-ve, small rods, motile by peritrichous flagella or non motile, facultativly anaerobe (Harey and Prescott, 1996).

2.12.3-*Pseudomonas aeruginosa*

It infects the eye causing many diseases such as Keratitis, endophthalmitis (Baron *et al*., 1994), and dacryoadenitis. It is one of the most common microorganisms encounted in hospital infection. It was found that 24% of patients carry *P. aeruginosa* and 38% become carriers during their stay in hospitals.

It causes several diseases and infections such as sever epidemic diarrhea of infant, ocular infection, burn infection, folliculitis, urinary tract infections and others (Tortora *et al*., 1986).

2.13- Treatment with chamomile

Studies on people are few. Studies on animals show antimicrobial activity of chamomile. It is effective against bacterial or fungal infections on the eyes (Popescu *et al.,* 1985).

Chamomile is used as dried extracts such as capsules or powders then used for external treatments as eye washes to treat infection. It is effective against both *Staphylococcus spp.* and *Streptococcus spp.* Which are the two major causes of conjunctivitis (Cummings, and Ullman, 1997).

The antibacterial effect of chamomile may depend on the concentration of chamazulen, bisabolol and bisabolol oxides in the extract, even at concentrations less than 100µg/mL (Korting and Schafer-Corting, 1993).

Chamomile is used as a skin wash or compress, It is use on skin is to increase wound healing and reduce inflammations such as those caused by allergies and pathogenic microorganism. Chamomile also makes an effective lotion for eczema. In Europe, chamomile is commonly used in skin care products to reduce cutaneous inflammation and other dermatological diseases (Popescu *et al.,* 1985; Merfort and Heilman 1994; Smith, 2006).

2.14- Allergy of the chamomile plant

Chamomile is generally considered safe. However, when taken in high dosages, chamomile tea may cause vomiting (Gyllenhaal, 2000).

Hausen, (1979) examined 25 humans suffer from allergy caused by aster family members, and found that two of the patients (8 percent) were allergic to chamomile. Cross-reactions with other aster family members were also observed. Allergic reaction to chamomile is rare (Vallone *et al*., 2000). Individuals allergic happen due to the presence of lacton in chamomile. It causes allergic reaction in sensitive individuals (Hausen *et al.*, 1984). Chamomile is usually considered to be safe during pregnancy or breast-feeding (Reider *et al.*, 2000; Smith, 2006).

3.1-Materials:

3.1.1: Apparatus and equipments:

The following equipments and apparatus were used throughout the experimental work:

Apparatus	Company
Autoclave	Karl / Germany
Distillator	GFL /Germany
Electric balance	Mettler (Switzerland)
Hot plate with magnetic stirrer	Ikamag
Incubator	Sanyo / Japan
Laminar air flow cabinate	ESCO
Micropipettes	Brand / Germany
Oven	Gallenkamp / England
pH-meter	Metter Gmbh-Teledo / England
Refrigerater	Concord
Sensitive balance	Delta Range / Switzerland
Shaker incubator	Sanyo
Soxhlet	Electrothermal (England)
Water bath	Gallenkamp / England

Bacterial Isolates	Source
Bacillus ceries	Biotechnology Dept., College of Science Al-Nahrain University
Escherichia coli	Biotechnology Dept., College of Science Al-Nahrain University
Psedomonas aeruginosa	Biotechnology Dept., College of Science Al-Nahrain University
Staphylococcus aureus	Biotechnology Dept., College of Science Al-Nahrain University

Animals
(Nine) local rabbits of either sex, (6-8)months of age and weighting approximately (1.5-2)Kg were used in the experiment.

3.1.2: Chemicals:

Chemicals	Company
Acetic anhydride	BDH
Ammonia	BDH
Benzyladenine(BA)	BDH
Chloroform	BDH
Ethanol	BDH
Ferric chloride	BDH
Hydrochloride (HCL)	BDH

Chemicals	Company
Lead-acetate	BDH
Mercury chloride	BDH
Methanol	BDH
Sodium hydroxyl (NAOH)	BDH
Naphthaleneacetc acid (NAA)	BDH
Sodium chloride (Nacl)	Fluka
Sulphuric acid (H_2So_4)	BDH

3.1.3: Culture media:

3.1.3.1: Nutrient Agar

Medium	Company
Agar-Agar	Sleeze
Agar	Oxide / England
Nutrient broth	Oxide / England

3.1.3.2: MS (Murashige and Skoog, 1962) culture medium components.

Chemicals	Company
Ammonium nitrate	Mall
Boric acid	Merk
Cupric sulphate.$5H_2O$	BDH
Cobalt chloride.$6H_2O$	BDH
Calcium chloride anhydrate	Fluka
Ferrous sulfate.7 H_2O	BDH
Glycine	BDH
Magnesium sulphate anhydrate	Fluka
Myoinositol	BDH
Manganese sulphate.$4H_2O$	BDH
Molybdic acid (sodium salt).$2H_2O$	BDH
Nicotinic acid(free acid)	Kochligh
Pyrodoxine.HCl	BDH
Potassium phosphate monobasic	Fluka
Potassium nitrate	BDH

Chemicals	**Company**
Potassium iodide	Tetanal
Sodium ethylene diamine tetraacetate	Fluka
Thiamine. HCl	BDH
Zinc sulphate.$7H_2O$	BDH
Zinc sulphate.$7H_2O$	BDH

3.2: Methods

3.2.1: Plant material

Chamomile (*M. chamomilla)* dried flowers were bought from local market, seeds were obtained from dried flowers, sterilized and cultured on the nutrient medium.

3.2.2: Sterilization of Explants (Pierik, 1987)

Seeds are rinsed with tap water for 10 min. then transferred to laminar air flow-cabinet where submerged in sodium hypochlorite at different concentrations (0.0, 1.5, 3.0 or 6.0)% for 5 or 10 min. Seeds then rinsed with sterilized DDH_2O for three times. For each concentration 12 seeds were used and distributed into 4 culture jars.

3.2.3: Preparation of culture medium

MS (Murashige and Skoog, 1962) medium was prepared and used (Table 3-1). Sucrose 30 g/L, myoinositol 100 mg/L. Plant growth regulators (NAA and BA) at different concentrations were added. The pH was adjusted to 5.8 using NaOH or HCl (1N), and then 7g/l of the agar type (Agar-Agar) was added to the medium, placed on a hotplate magnetic stirrer till boiling. Aliquots of 10 ml were dispensed into (8 ×2.5) cm culture vessels. They left to cool at laboratory tempreture and become ready to culture seeds.

Table (3-1): MS (Murashige and Skoog, 1962) culture medium components

Components	Chemical formula	Weight (mg/l)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride anhydrate	$CaCl_2.2H_2O$	440
Magnesium sulphate anhydrate	$MgSO_4.7H_2O$	370
Potassium phosphate monobasic	KH_2PO_4	170
Micronutrients		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Manganese sulphate.$4H_2O$	$MnSO_4.4H_2O$	22.30
Zinc sulphate.$7H_2O$	$ZnSO_4.7H_2O$	8.60
Molybdic acid (sodium salt).$2H_2O$	$Na_2MoO_4.2H_2O$	0.25
Cupric sulphate.$5H_2O$	$CuSO_4.5H_2O$	0.025
Cobalt chloride.$6H_2O$	$CoCl_2.6H_2O$	0.025
Chelated Iron		
Sodium ethylene diamine tetraacetate	Na2-EDTA	33.6
Ferrous sulfate.7 H_2O	$FeSO_4.7H_2O$	27.8
Vitamins		
Thiamine.HCl (B1)	$Cl_2H_{17}C_1N_4OS.HCl$	0.1
Nicotinic acid(free acid)(B3)	$C_8H_{11}NO_3.HCl$	0.5
Pyrodoxine.HCl (B6)	$C_6H_5NO_2$	0.5
Glycine(free base)	$C_2H_5NO_2$	2.0
myoinositol	$C_6H_6(OH)_6$	100

3.2.4: Plant growth regulators

Different concentrations of the auxin NAA (0.0, 0.1, 0.2 or 0.3) mg/l and the cytokinin BA (0.0, 0.5, 1.0, 2.0 or 3.0) mg/l were prepared and added to the culture media as required before autoclaving (Zhao *et al*., 2001).

3.2.5: Media and instruments sterilization (Cappuecino and Sherman, 1987)

Culture media were sterilized by autoclaving at 121°C under (1.04 Kg/cm²) pressure, for 15 min., while glassware and other instruments either by autoclaving or using electric oven (180-200) °C for 2 hrs. The media were left at room temperature to cool and became ready to culture seeds.

3.2.6: Incubation of cultures

Surface sterilized seeds were inoculated into the culture vessels under aseptic conditions, placed in the incubator at 25°C for 16/8 hrs. Light/dark photoperiod using day light inflorescents at light intensity of 1000 lux.

3.2.7: Initiation of callus cultures

Different combinations of plant growth regulators were examined to determine the most effective one for callus initiation. Seeds were placed onto MS medium containing NAA and BA as in 2.2.4. Cultures were placed in the incubator. The response of these seeds to auxin and cytokinin combinations was evaluated after 21 days in culture to determine the proper combination for callus induction.

3.2.8: Maintenance of callus cultures

The initiated callus was removed from the explants using forceps and scalpel, and then pieces weighting approximately 50 mg were subcultured onto fresh medium supplemented with the same combinations of NAA and BA as in 2.2.4. Callus fresh weight was determined using a sensitive balance then oven dried at 40°C for 24 hrs. (Bos, 1997) for callus dry weight measurements.

3.3: Preparation of flowers extracts for antimicrobial activity

3.3.1: Water extracts (Swanston *et al*., 1990)

Plant powdered material was macerated with DDH**2**O in a ratio 1:5 (w/v), 50g of the powder was mixed with 250 ml DDH2O. The mixture macerated over night at room temperature.

The suspension was filtered through filter of gauze to get rid of the large particles then filtered through a filter paper (Whatman no.1). The filtrate was concentrated (100mg/l) using a rotary evaporator at 40°C.

3.3.2: Ethanolic extract (Harborne, 1973)

A quantity of 50g of flowers powder was extracted with 250 ml of 75% ethanol by soxhlet apparatus for 6 hrs at 40-60°C, and then the solvent was removed under reduced pressure by rotary evaporator at 40°C. The crude solid extract was kept in deep freeze until use.

3.4: Preparation of callus extracts for antimicrobial activity

The water and ethanolic extracts of callus that originally initiated from seedling explants grown on MS medium were prepared for antimicrobial activity as below:

3.4.1: Water extracts (Swanston *et al*., 1990)

The method used for flowers extraction was the same that used for callus extraction. The dried samples (10 g) were soaked with 50 ml DDH_2O. The mixture was left in a shaker incubator 100 r/min for 24 hrs, then filtered through a filter paper (Whatman no. 1). The filterate was concentrated using a rotary evaporator at 40°C until dryness.

3.4.2: Ethanolic extract (Harborne, 1973)

A quantity of 10 g of callus powder was mixed with 50 ml of 75% ethanol then placed in soxhlet apparatus for 6 hrs. at (40-60) °C. then the solvent was removed under reduced pressure by rotary evaporator at 40°C.

3.5: Detection of some active compounds of chamomile

3.5.1: Detection of tannins (Shihata, 1951)

A quantity of (10) g of the flowers powder was mixed with (50) ml DDH_2O using a magnetic stirrer. The mixture was left till boiling in a water bath for three minutes, then filtered through a filter paper (Whatman no. 1). The filtrate was treated with a few drops of 1% lead-acetate solution. The presence of viscous precipitate was an indication of the presence of tannins.

3.5.2: Detection of saponins (Stahl, 1969)

Aliquots of 5 ml plant extract were added to (1-3) ml of mercury chloride solution. A white precipitate was developed indicating the presence of saponins.

3.5.3: Detection of flavonoids (Harborne, 1973)

Ethanolic extracts of the plant material was partitioned with petrolium ether; the aqueos layer was mixed with the ammonia solution. The appearance of dark color was an indication for the presence of flavonoids.

3.5.4: Detection of glycosides (Shihata, 1951)

Equal amounts of water extract and Fehling's reagent (5ml) was mixed in a test tube, and then boiled in a water bath for 10 min. The formation of red precipitate indicated the presence of glycosides.

3.5.5: Detection of terpenes (Al-Abid, 1985)

One gram of ethanolic extract was precipitated in a few drops of chloroform, then a drop of acetic anhydride and a drop of concentrated sulphuric acid were added. Appearance of brown color indicated the presence of terpenes.

3.5.6: Detection of alkaloids (Stahl, 1969)

A quantity of 10 g of the powder plant material was added to 50 ml of 4% HCl in a steam bath, then 1 ml of the filtrate was treated with Mayer's reagent. The appearance of white precipitate was an evidence for the presence of alkaloids.

3.5.7: Detection of coumarins (Geissman, 1962)

A little amount of the dried water flowers extract was dissolved in some drops of 75% ethanolic alcohol in a test tube covered with filter paper, sprayed with NaOH (1%), then placed in a water bath till boiling. The filter paper was placed under UV light spectrum(336nm). The appearance of greenish-yellow color indicated the presence of coumarins.

3.5.8: Detection of resins (Shihata, 1951)

Aliquot of 50ml of ethanol (95%) added to 5 g of plant powder then placed in a water bath for 2 min., filtered using filter paper (Ederol No.2), Then 10 ml of diluted HCL (5%) was added to supernatant. The detection of resins was confirmed by the appearance of turbidity.

3.5.9: Detection of phenols (Harborne, 1973)

A quantity of (10) g of the plant powder was mixed with (50) ml DDH_2O using a magnetic stirrer. The mixture was left till boiling in a water bath for few min., then filtered, and the filtrate was treated with few drops of 1% ferric chloride solution. The development of greenish-blue precipitate was an indication of the presence of phenols.

3.6: Determination of MIC

The minimum inhibitory concentration was determined by assaying the ability of bacteria (*B. ceries, E. coli, P. aeruginosa* and *S. aureus*) to grow in broth cultures' containing different concentrations of the chamomile extracts.

The following dilutions 10%; 20%; 30%; 40%; 50%; 60%; 70%; 80%; 90% were prepared in test tubes. They were inoculated with 0.1ml of bacteria (1.5×10^5 cfu/ml) then incubated at 37°C for 24hrs (Atlas *et al*., 1995).

Growth intensity of each tube was observed by inculcation on nutrient agar and incubation at 37°C (for 24hrs.) then recorded as light (+), medium(++), heavy(+++), and no growth (-). Growth was estimated by using spectrophotometer, and optical density (OD.600)nm for each dilution. Results were matched with the growth intensity mentioned by Midolo *et al.*, (1995).

The same procedure was repeated for ethanolic extracts. The lowest concentration of the extract that prevented growth of pathogenic bacteria was considered as the minimum inhibitory concentration.

3.7: Determination of the antibacterial activity of extracts (*in vitro*) (Nathan, 1978)

The activities of extracts were determined against target cells (bacterial isolates) *in vitro* by using modified agar diffusion method.

For water and ethanolic extract, the stock solution was prepared by dissolving (5) g of plant extract residue with (50) ml sterile DDH_2O. The extracts were prepared at different concentrations (0, 5, 10, 20 or 40) mg/ml.

The nutrient agar medium was mixed well and 20ml poured in Petri-dishes. The medium was inoculated with 0.1ml of (1.5×10^5 cfu/ml) target isolates of (*B. ceries, E. coli, P. aeruginosa* or *S. aureus*) by using sterile swabs.

Five evenly spaced wells 3mm in diameter were made in the agar of each plate with sterile cork borer. To identify the intrinsic extracts activity (water and ethanolic extracts for flower and callus cultures), one control well was filled with (100 µl) phosphate buffer saline. An equal volume of different concentrations (0, 5, 10, 20 or 40) mg/ml of the extracts was dispensed into each well (four replica plates were prepared for each agent). Test plates were then incubated at 37°C for 24 hrs. and zones of inhibition were measured using a ruler in millimeters.

A clear area indicated that the extract showed its antibacterial activity. This method was repeated twice for each extract.

3:8: The effect of chamomile extracts on rabbits eyes (*in vivo*) (George *et al.*, 1982)

Sample for injection: A liquot of 0.5 ml of water extract (40mg/ml) was mixed with 0.5 ml of nutrient broth media, then 0.1 ml of ($1.5*10^5$ cfu/ml) bacterial suspension (*B. ceries*,, *E. coli*, *P. aeruginosa* and *S. aureus*) was inoculated. As a control, a mixture was prepared individually, containing 0.5 ml of nutrient broth media, 0.5 ml of (P.B.S) and 0.1 ml of bacterial suspension. Samples and control were incubated at 37°C for 24 hrs.

Animals were administrated with an intrastromal injection of samples (0.1 ml/ left eyes). Right eyes for the same rabbits were injected with control sample. Results were recorded 24 hrs after injection.

3.9: Statistical analysis

A completely randomized design (CRD) was used. Least significant differences (LSD) were calculated. Means were compared at probability of 0.05. For secondary metabolite quantification, means were calculated and standard errors were computed for three sample replicates (Gomez and Gomez, 1984).

4.1- Sterilization of explants

NaOCl was used for explants sterilization (seedling) of *M. chamomilla*. Fig. (4-1) shows that the most effective concentration of NaOCl was 3% for 10 min that gave the highest percent (100%) survival. Increasing the concentration of NaOCl to 6% caused serious reduction in germination rate, whereas lowering the concentration led to high rate of contamination.

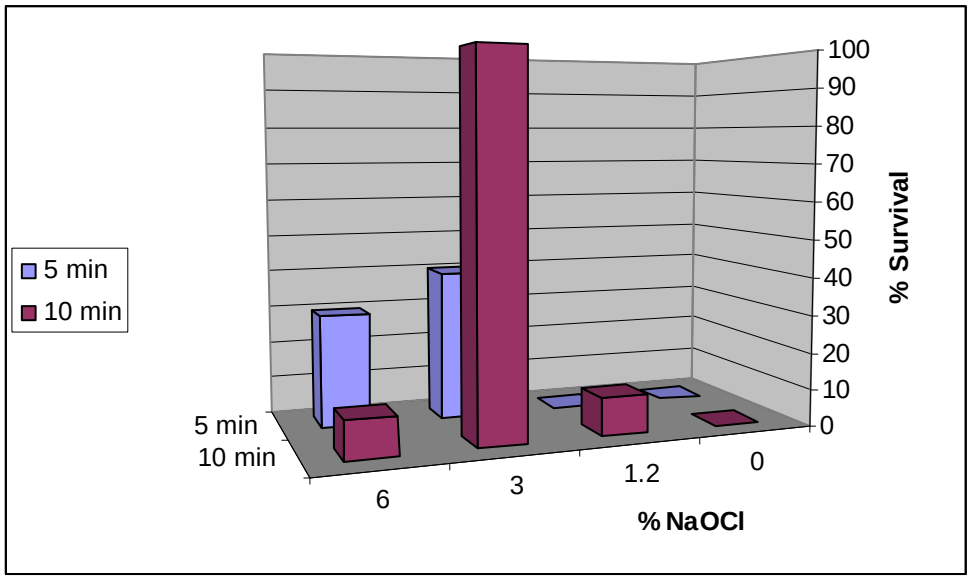


Fig. (4-1): Effect of different concentrations of NaOCl on explants survival at sterilization periods of 5 or 10 min. n= 4 * explants were seriously damaged

Treatment of explants with NaOCl was important to eliminate the contaminants. It is used widely in plant tissue sterilization. The selection of sterilizing material depends on the source of explants, roughness of its surface and other factors. The sterilization material should be easy to remove from explants when washed with sterilized DDH_2O (Yeoman and Macleod, 1977; Sateesh, 2003).

Pierik, (1987) referred to the importance of sodium hypochlorite for explants sterilization. Increasing the surface sterilization period and concentration often lead to serious reduction in survival rate. Optimization experiment is therefore necessary to achieve maximum survival rate with minimum contamination.

4.2- Induction of callus cultures

The effect of different concentrations of NAA and BA on the response (%) of callus induction on seedling is shown in table (4-1).

Table (4-1): Effect of different concentrations of NAA and BA on the response (%) of callus induction on *M. chamomilla* seedling explants (n= 4).

NAA (mg/l)	BA (mg/l)					Mean
	0.0	0.5	1.0	2.0	3.0	
0.0	0.0	32.6	35.2	78.6	85.7	46.4
0.1	8.2	38.0	67.0	96.4	100	61.9
0.2	12.2	12.5	20.6	32.7	44.8	24.6
0.3	8.5	8.8	12.1	0.0	0.0	5.9
Mean	7.2	23.0	33.7	51.9	57.6	
LSD 0.05	BA= 9.4 NAA= 12.7 BA× NAA= 17.1					

All concentrations of BA led to a significant increase in the percentage of explants showed callus induction (23.0, 33.7, 51.9and 57.6%) at the concentrations 0.5, 1.0, 2.0 and 3.0 mg/l respectively compered with control treatment (7.2%). Maximum callus response occurred (57.6)% at 3.0 mg/l BA, while minimum value was recorded at non-treated explants.

Addition of NAA at 0.1mg/l led to a significant increase in the percentage of explants showed callus induction reaching (61.9%). Percentage of explants that initiated callus decreased significantly when NAA was added at 0.2mg/l and 0.3 mg/l reached 24.6% and 5.9% respectively.

The interaction between the two growth regulators achieved 100% response in a combination of 3.0 mg/l BA and 0.1 mg/l NAA Fig. (4-2) and 96.4% when BA concentration was decreased to 2.0 mg/l and 0.1 mg/l NAA. While no callus induction was reported on untreated seedling explants and at high concentrations of growth regulators.

Callus induction requires a balanced ratio from auxin(s) and cytokinin(s) as stated by Skoog and Miller (1957). In a number of plant species callus induction favors higher auxins than cytokinins (Ramawat, 2004), but Murashige and Skoog, 1962 stated that seeds is a rich source of auxin so that a little amount of NAA was used.

M. chamomilla seedling may contain some levels of endogenous auxins that made a balanced ratio with the exogenous auxin and may be even cytokinin.

Establishment of a callus from explants was illustrated by Dodds and Roberts (1995) who divided the process into three developmental stages: induction, cell division and differentiation. The length of these phases depends mainly on the physiological status of the explant cells as well as the cultural conditions including the appropriate combination of plant growth regulators.

Fig. (4-2): Callus induction on seedling explants of *M. chamomilla* grown on MS medium containing a combination of 3.0 mg/l BA and 0.1 mg/l NAA, 21 days after culture.

4.3- Maintenance of callus cultures

All concentrations of BA led to a significant increase in callus fresh weight (149, 252, 503 and 679 mg) at the concentrations 0.5, 1.0, 2.0 and 3.0mg/l respectively (Table 4-2).

Addition of NAA at 0.1mg/l caused a significant increase in callus fresh weight (688.8mg). Callus fresh weight decreased significantly when concentrations of NAA increased more than 0.1 mg/l reaching to 182.2 and 70.2 for the concentrations 0.2 and 0.3 mg/l respectively.

Table (4-2): Effect of different concentrations of NAA and BA on callus fresh weight (mg) initiated on seedling explants of *M. chamomilla* grown on a maintenance medium. Initial weight was 50 mg. (n= 4).

NAA (mg/l)	BA (mg/l)					Mean
	0.0	0.5	1.0	2.0	3.0	
0.0	0.0	172	198	840	780	398
0.1	127	174	532	983	1628	688.8
0.2	127	134	152	191	307	182.2
0.3	109	116	126	0.0	0.0	70.2
Mean	91.0	149	252	503	679	
LSD 0.05	BA= 48 NAA= 60 BA× NAA=78					

The interaction between the two growth regulators resulted in maximum callus production (1628mg) at the combination of 3.0 and 0.1 mg/l BA and NAA respectively Fig. (4-3) and (983mg) when BA was decreased to 2.0 mg/l and 0.1 mg/l NAA. This combinations was significantly higher than all other interactions.

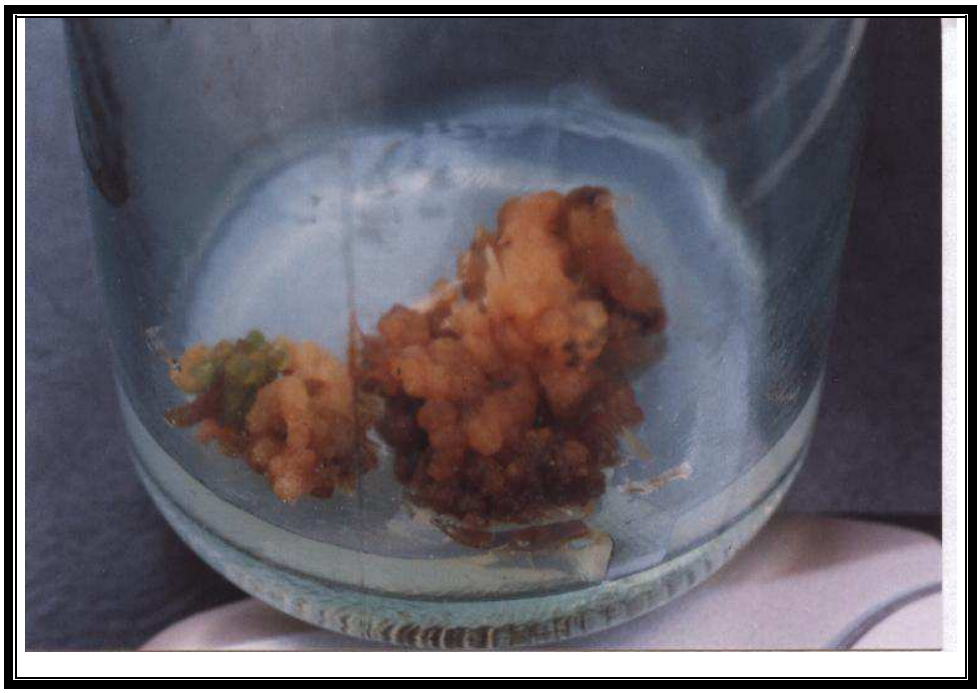


Fig. (4-3): Callus cultures initiated on seedling explants cultured on MS medium containing 3.0mg/l BA and 0.1 mg/l NAA 21 days in culture.

Dry weights of callus cultures initiated from germinated seed explants are shown in (Table 4-3).

Table (4-3): Effect of different concentrations of NAA and BA on callus dry weight (mg) initiated on germinated seed explants of *M. chamomilla* and grown on a maintenance medium (n= 4).

NAA (mg/l)	BA (mg/l)					Mean
	0.0	0.5	1.0	2.0	3.0	
0.0	0.0	15.0	16.3	46.6	48.3	25.2
0.1	5.3	15.5	32.7	112.0	141.0	61.3
0.2	6.5	7.0	13.2	15.2	23.0	13.0
0.3	4.5	5.7	6.6	0.0	0.0	3.4
Mean	4.1	10.8	17.2	43.5	53.1	
LSD 0.05	BA= 4.6 NAA= 7.2 BA× NAA=9.3					

The highest dry weights of explants were exhibited by the combination of (3.0 mg/l BA and 0.1mg/l NAA) and (2.0 mg/l BA and 0.1 NAA). These weights were (141.0 and 112.0 mg) respectively which they were significantly higher than all other treatments.

According to the results stated above, callus was induced on germinating seed explants then maintained for many subcultures on MS medium containing 3.0 mg/l BA and 0.1 mg/l NAA for subsequent experiments.

Increasing the levels of the two plant growth regulators suppressed callus growth. The increase of callus mass is important as a source for the production of secondary metabolites since they are proportionally related (Ramawat, 2004).

Regenerated shoots from callus cultures was noticed when some callus cultures left on maintenance medium for 40 days without subculture are shown in (Table 4-4).

Table (4-4): Effect of different concentrations of NAA and BA on regeneration of *M. chamomilla* callus culture.

NAA (mg/l)	BA (mg/l)				
	0.0	0.5	1.0	2.0	3.0
0.0	-	-	+	+	+
0.1	-	-	+	+	+
0.2	-	-	-	+	-
0.3	-	-	-	-	-

+ : Regenerated shoots from callus culture

- : no regeneration

Many concentrations of BA regenerated shoots from callus at the concentrations (1.0, 2.0 and 3.0 mg/l). While no shoots regeneration was reported when NAA interacted with 0.0 and 0.5 mg/l BA.

The interaction between the two growth regulators BA and NAA regenerate shoots from callus in the combinations (1.0, 2.0 and3.0 mg/l) of BA and (0.0, 0.1 and 0.2mg/l) of NAA Fig.(4-4).

Fig. (4-4): *M. chamomilla* shoots regenerated from callus cultures after 40 days in culture.

Shoot proliferation may due to the continuous depletion of growth regulators over time. This may led to a new balanced ratio suitable for plant regeneration Fig. (4-5).

Fig. (4-5): *M. chamomilla* shoots regenerated from callus cultures after 55 days in culture.

Kintzios and Michaelakis, (1999) reported that chamomile explants responded to callus induction and thereafter plantlet regeneration from somatic embryos *in vitro* on a MS medium supplemented with BA and NAA.

4.4- Detection of some active compounds in chamomile flowers

The pH of water and ethanolic extracts was 6.26 and 5.76 respectively indicating the acidity of the water and etnanolic extracts.

Results obtained by chemical detection indicated the presence of flavonoids, tannins, resins, phenols, alkaloids, cumarins, glycosides and terpenes. Saponins were not detected which are displayed in Table (4-5).

Table (4-5): Detection of some secondary metabolites in *M. chamomilla*.

Type of secondary metabolite	Results of detection (flowers)	
	Water extract	Ethanolic extract
Tannins	+ve	+ve
Saponins	-ve	-ve
Flavonoids	+ve	+ve
Glycosides	+ve	+ve
Terpenes	+ve	+ve
Resins	+ve	+ve
phenoles	+ve	+ve
Alkaloids	-ve	+ve
Coumarins	+ve	+ve

+ve indicates the presence of secondary metabolite.

-ve indicates the absence of secondary metabolite.

4.5- Determining minimum inhibitory concentration (MIC) for *M. chamomilla* extracts

Minimum inhibitory concentration (MIC) is the least concentration that prevents growth of bacteria after incubation at 37°C for 24 hrs.

There are many factors that influence MIC estimation, volume of bacterial inoculums (the value of MIC increased upon increasing this volume), pH, temperature and nature of cell membrane (Nikaido, 1989).

4.5.1- Water extract

Table (4-6) displays MICs of the water extract of chamomile flowers for four types of bacteria. Results of the table declared that the first two concentrations (10% and 20%) had no observed effect against different types of bacteria but heavy growth of bacteria was noticed after incubation. Growth decreased at the following two concentrations (30% and 40%) for all bacterial types

At the concentration of 50%, the situation was different since no growth observed for *S. aureus* and *B. ceries* only but the other two types of bacteria showed light growth. The last four concentrations of water extract (60%, 70%, 80%, 9%) were enough to retard any growth of *E-coli, B. ceries, S. aureus* and *P. aeruginosa.*

Table (4-6): Minimum inhibitory concentrations (MIC) of *M. chamomilla* flowers water extract against tested bacteria.

Type of bacteria	Concentration of extract									
	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%
E- coil	+++	+++	+++	+++	++	+	–	–	–	–
B. ceries	+++	+++	+++	++	+	–	–	–	–	–
S. aureus	+++	+++	+++	++	+	–	–	–	–	–
P. aeruginosa	+++	+++	+++	+++	++	+	–	–	–	–

- : no growth
+ : light growth
++ : medium growth
+++ : heavy growth

4.5.2- Ethanolic extract

Table (4-7) shows MIC of the ethanolic extract of chamomile flower for the four types of bacteria. Results of the table indicated that the first two concentrations (10% and 20%) had no effect against bacteria while heavy growth of bacteria was noticed after incubation. However, the growth was reduced at the following two concentrations (30% and 40%) for all bacterial types.

The situation was different at the concentration 50% since no bacterial growth was observed. The last four concentrations of ethanolic extract (60%, 70%, 80%, 90%) were sufficient to cease growth of *E-coli, B. ceries, S. aureus* and *P. aeruginosa.*

Table (4-7): Minimum inhibitory concentrations (MIC) of *M. chamomilla* flowers ethanolic extract against tested bacteria.

Type of bacteria	Concentration of extract									
	0%	10%	20%	30%	40%	50%	60%	70%	80%	90%
E- coil	+++	+++	+++	++	+	–	–	–	–	–
*B cerie*s	+++	+++	+++	++	+	–	–	–	–	–
S. aureus	+++	+++	+++	++	+	–	–	–	–	–
P. aeruginosa	+++	+++	+++	++	+	–	–	–	–	–

- : no growth
+ : light growth
++ : medium growth
+++ : heavy growth

4.6- Effect of water and ethanolic extracts on the growth of some bacteria (*in vitro*)

Water and ethanol extracts taken from dried flower material and dried callus were investigated for their antimicrobial activity against (*E-coli, S. aureus, B. ceries* and *P. aeruginosa*).

4.6.1-Effect of water extract

Results displayed in table (4-8) indicate that high concentrations of chamomile water extract (20 and 40mg/ml) have inhibitory effects against Gram-positive bacteria (*S. aureus)* with (16.3 and 18.2)mm inhibition zones diameter respectively, while 13.6mm was recorded in the concentration 10 mg/ml. Slight inhibition was observed at the concentration of 5mg/ml Fig.(4-6 a). While *B. ceries* gave 13.5mm in a concentration of 20mg/ml, 18mm at a concentration of 40mg/ml, and the concentration (10mg/ml) showed 13.0mm and slight inhibition was observed at a concentration of 5mg/ml Fig. (4-6 b).

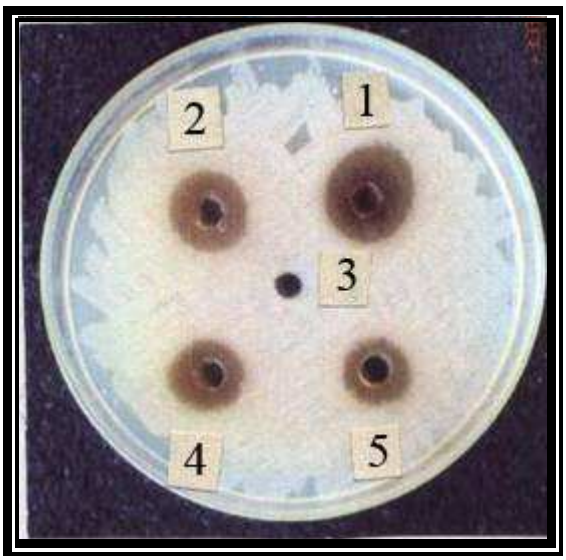
The inhibition ability was more pronounced against *S. aureus*, whereas it showed less activity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*).
In *E coli*, the high concentrations of water extract (20 and 40)mg/ml showed (15 and 16)mm inhibition zones subsequently and 13.0mm in the concentration 10 mg/ml. Slight inhibition was observed at the concentration of 5mg/ml Fig. (4-6 c). While *P. aeruginosa* showed a slight inhibition at 5mg/ml. The concentration 10 mg/ml showed 13.3mm inhibition zones, the inhibition zones at (20 and 40)mg/ml were (15.4 and 16.3)mm of the water extract subsequently Fig. (4-6d).

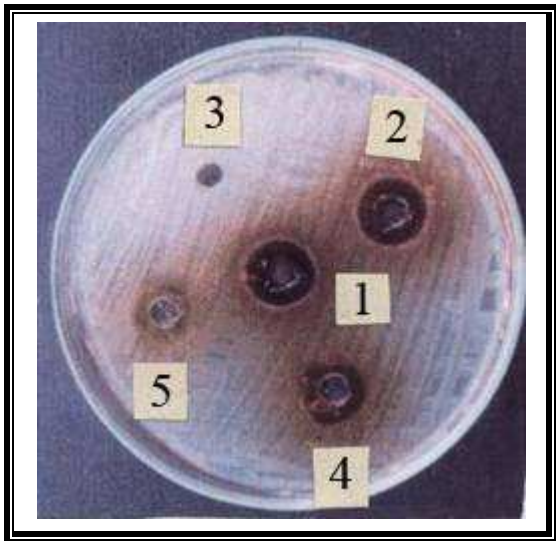
Table (4-8): Diameter of inhibition zones caused by *M. chamomilla* flowers water extracts at various concentrations on some G+ve and G-ve bacteria.

Conc. mg/ml dwt	Diameter of inhibition zone (mm) of bacterial isolates ± S.D.			
	S. aureus	***E. coli***	***B. ceries***	***P. aeruginosa***
40	18.2±0.75	16.0±0.96	18.0±0.20	16.3±0.84
20	16.3±0.24	15.0±0.65	13.5±0.62	15.4±0.44
10	13.6±0.47	13.0±0.81	13.0±0.70	13.3±0.79
5	slight inhibition	slight inhibition	slight inhibition	slight inhibition
Control	**-ve**	**-ve**	**-ve**	**-ve**

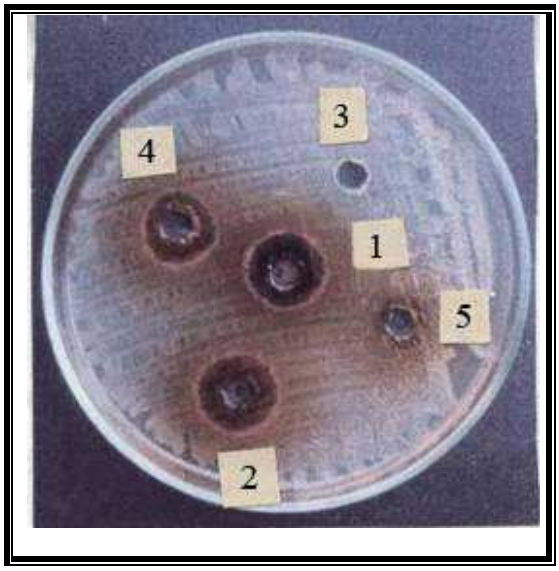
-ve: no activity was observed.

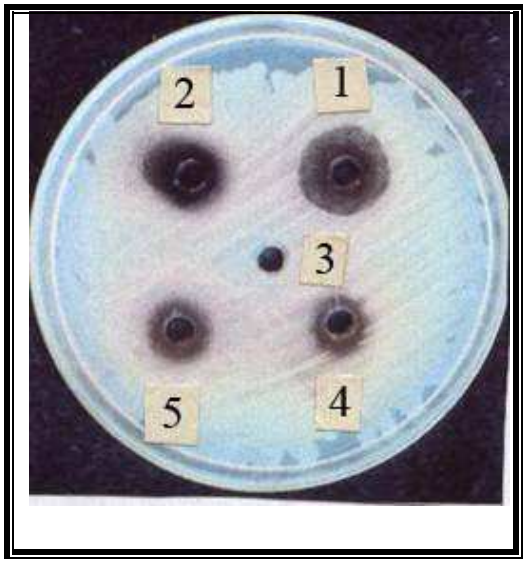
Values: are mean of 3 replicates ± S.D.





-a- **-b-**





-a- **-b-**

Picture (4-6):Effect of *M. chamomilla* flowers water extract on the growth of *a- S. aureus* , b- *B. ceries*., c- *E. coli.* and d- *P. aeruginosa*.

1= 40 mg/ml, 2= 20 mg/ml, 3= control(P.B.S.), 4= 10 mg/ml, 5= 5 mg/ml.

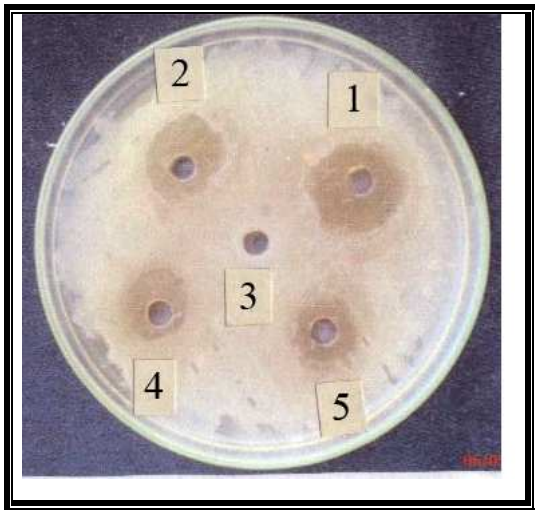
Water extract of chamomile callus (Table 4-10) showed antimicrobial action against all tested microorganisms at the concentrations (20 and 40) mg/ml. The largest inhibition zones was observed against *S. aureus* recording 24.6mm in diameter at 40mg/ml whereas, decreased to 20.8mm at the concentration of 20mg/l. The lower concentrations of extracts (5 and 10mg/ml) *S. aureus* showed (14.3 and 16.9) mm respectively Fig. (4-7 a). *B. ceries* was inhibited at extract concentrations (5, 10, 20 and 40mg/ml). They showed (14.8,18.3, 20.0 and 24.3)mm inhibition zones diameter respectively Fig. (4-7b), followed by *E. coli* which showed (17.1 and 19.0)mm inhibition zones at (20 and 40)mg/ml subsequently. Inhibition zones of (12.0 and 15.0)mm were observed at the concentrations of 5 and 10mg/ml respectively Fig.(4-7 c). While *P. aeruginosa* showed (13.0 and 16.0) mm inhibition zones in diameter at concentrations (5 and 10)mg/ml and at concentrations (20 and 40)mg/ml the inhibition zones were (17.3 and 19.2)mm respectively Fig. (4-7 d).

Table (4-9): Diameters of inhibition zones caused by *M. chamomilla* water extracts of callus cultures at various concentrations on some G +ve and G-ve bacteria.

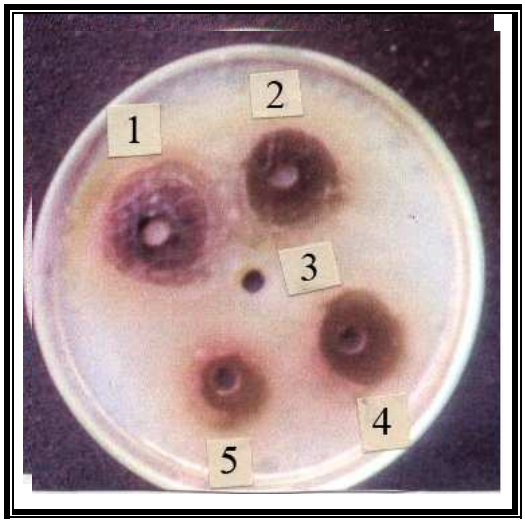
Conc. mg/m dwt	Diameter of inhibition zone (mm) of bacterial isolates ± S.D.			
	S. aureus	***E. coli***	***B. ceries***	***P. aeruginosa***
40	24.6±0.32	19.0±0.87	24.3±0.94	19.2±0.74
20	20.8±0.09	17.1±0.62	20.0±0.65	17.03±0.69
10	16.9±0.75	15.0±1.31	18.3±0.53	16.0±1.10
5	14.3±0.68	12.0±0.93	14.8±0.41	13.0±0.77
Control	**-ve**	**-ve**	**-ve**	**-ve**

-ve: no activity was observed.

Values: are mean of 3 replicates ± S.D



-a-



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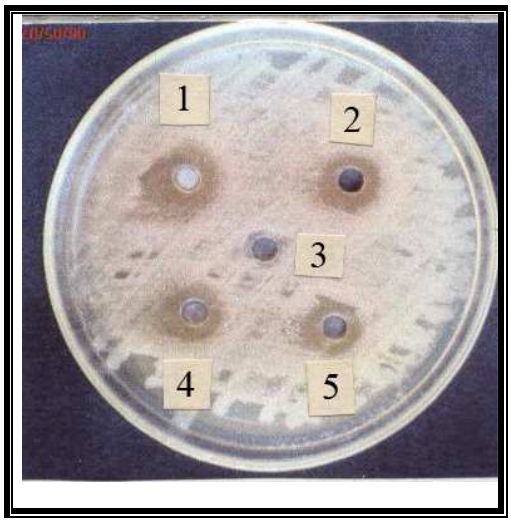

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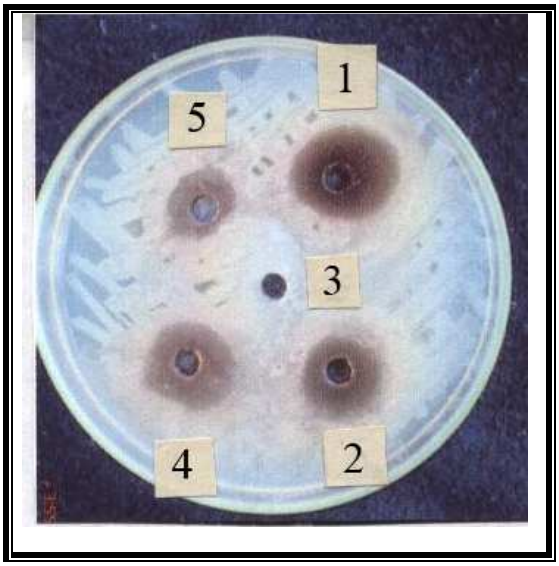


-d-

Fig.(4-7):Effect of *M. chamomilla* water extract on the growth of

a- S. aureus* , b- *B. ceries*., c- *E. coli.* and d- *P. aeruginosa

Extract was taken from callus cultures initiated from germinating seed explants.

1= 40 mg/ml, 2= 20 mg/ml, 3= control(P.B.S.), 4= 10 mg/ml, 5= 5 mg/ml.

The results agree with Korting and Schafer-Corting (1993) who regarded G+ve bacteria especially *B. subtilis* and *S. aureus* are sensitive to *M. chamomilla* water extract, G-ve bacteria *E. coli* and *P. aeruginosa* were relatively less sensitive. The antibacterial effect may depend on the concentration of chamazulen, bisabolol and bisabolol oxides (A and B) in the extract. Even at concentrations, lower than 100-µg/mL. a-bisabolol and its spiro-ether were effective antibacterial agents (Smith, 2006).

In addition Smith, (2006) referred that chamomile has antibacterial constituents such as chamazulen, bisabolol and bisabolol oxides.

The resistance of G-ve bacteria could be due to the permeability barrier provided by cell membrane (Adwan and Abu-Hasan, 1998).

The results are also in agreement with Mann and Staba (1986) who showed the role of chamomile action in preventing infections and promotion of wound healing by fighting infection causing bacteria such as *S. aureus*.

Essawi and Srour (2000) mentioned that the hole plate diffusion method is preferred over disk diffusion one. Therefore, this method was used for this purpose.

4.6.2- Effect of ethanolic extract

Chamomile ethanolic extract exhibited antibacterial activity against microorganisms at the concentrations (20 and 40) mg/ml. The diameter of the inhibition zones against *S. aureus* was 15.3mm at 40mg/ml. whereas, decreased to 12.6mm at the concentration of 20 mg/l. The lower concentrations of extracts, (10 and 5 mg/ml), *S. aureus* showed 10.8 mm and slight inhibition respectively Fig.(4-8 a). Results in table (4-10) show that *B. ceries* was inhibited at extract concentrations (10, 20 and 40mg/ml). It showed 12.3, 14.3 and 16.4mm inhibition zones diameter Fig. (4-8 b).

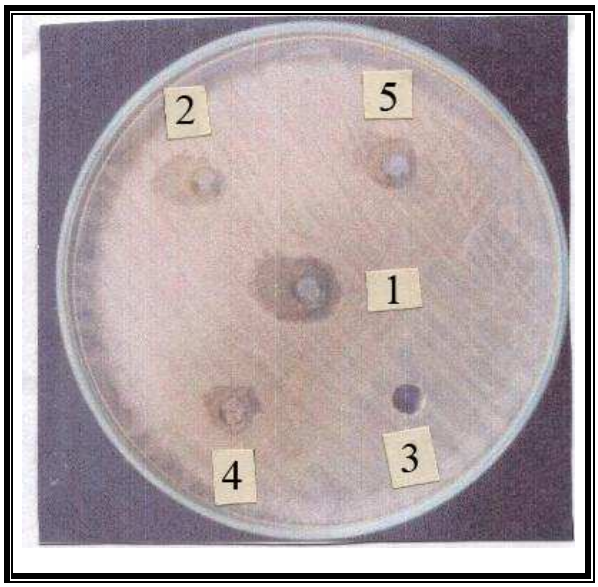
E. coli showed (11 and 14.2)mm inhibition zones at (20 and 40) mg/ml respectively. Slight inhibition was observed at the concentration 10 mg/ml Fig. (4-8 c). While *P. aeruginosa* showed slight inhibition at 10mg/ml and inhibition zones at (20 and 40)mg/ml subsequently were (12.0 and 14.0)mm of the chamomile ethanolic extract Fig.(4-8 d).

Table (4-10): Diameter of inhibition zones caused by *M. chamomilla* flowers ethanolic extracts at various concentrations on some G+ve and G-ve bacteria.

Conc. mg/ml dwt	Diameter of inhibition zone (mm) of bacterial isolates ± S.D.			
	S. aureus	***E. coli***	***B. ceries***	***P. aeruginosa***
40	15.3±0.62	14.2±1.4	16.4±0.82	14.0±0.71
20	12.6±0.61	11.0±0.81	14.3±0.49	12.0±0.94
10	10.8±0.23	slight inhibition	12.3±0.62	slight inhibition
5	slight inhibition	-ve	slight inhibition	-ve
Control	**-ve**	**-ve**	**-ve**	**-ve**

-ve: no activity was observed.

Values: are mean of 3 replicates ± S.D.



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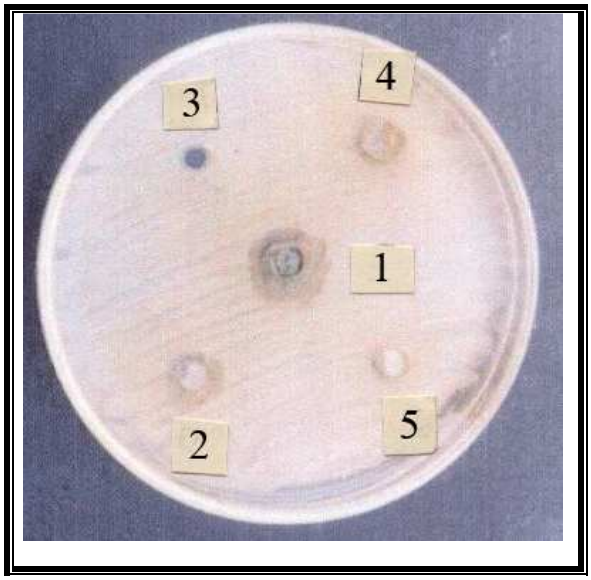

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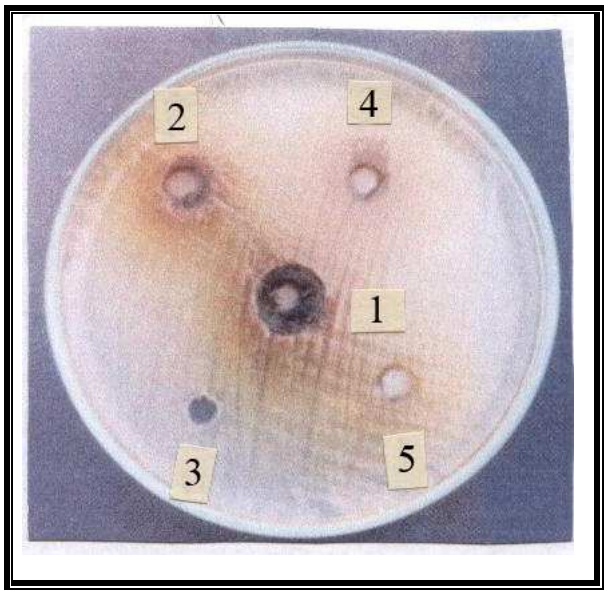

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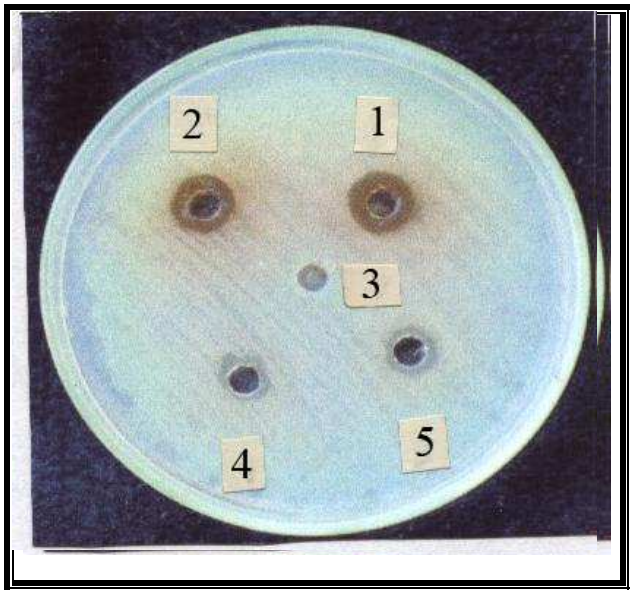


-d-

Fig. (4-8): Effect of *M. chamomilla* flowers ethanolic extract on the growth of *a- S. aureus* , b- *B. ceries.*, c- *E. coli.* and d- *P. aeruginosa*

1= 40 mg/ml, 2= 20 mg/ml, 3= control(P.B.S.), 4= 10 mg/ml, 5= 5 mg/ml.

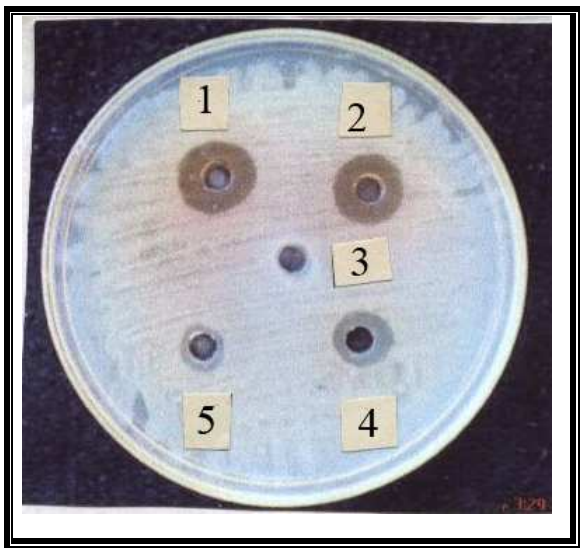
Results displayed in table (4-11) confirmed the activity of chamomile ethanolic callus extract against all tested microorganisms**.** The ethanolic extract (20 and 40mg/ml) has inhibitory effects against (*S. aureus)* with (17.8 and 23.5)mm inhibition zones diameter respectively, and showed (14.2 and 16.0) mm inhibition zones at concentrations (5 and 10)mg/ml respectively Fig. (4-9 a). While *B. ceries* gave 19mm in the concentration of 20mg/ml, 23.3mm at the concentration of 40mg/ml. The lowest concentration (5mg/ml) showed 13.5mm and at the concentration 10mg/ml gave15.9 mm Fig. (4-9 b). Whereas it showed less activity against *E. coli* which showed (16.2 and 18.6)mm inhibition zones at (20 and 40) mg/ml. Meanwhile (12 and 14.3) mm were observed at the concentrations of (5 and 10) mg/ml respectively Fig. (4-9 c). *P. aeruginosa* showed (11.5 and 15)mm at (5 and 10)mg/ml and inhibition zones at (20 and 40)mg/ml were (16 and 19)mm respectively of the water extract Fig. (4-9 d).

Table (4-11): Diameter of inhibition zones caused by *M. chamomilla* ethanolic callus extracts at various concentrations on some G+ve and G-ve bacteria

Conc. mg/ml dwt	**Diameter of inhibition zone (mm) of bacterial isolates ± S.D.**			
	S. aureus	***E. coli***	***B. ceries***	***P. aeruginosa***
40	23.5±0.72	18.6±0.78	23.3±0.69	19.0±0.90
20	17.8±0.47	16.2±0.82	19.0±0.61	16.0±0.81
10	16.0±0.16	14.3±0.92	15.9±0.55	15.0±1.08
5	14.2±0.80	12.0±0.71	13.5±0.35	11.5±0.70
Control	**-ve**	**-ve**	**-ve**	**-ve**

-ve: no activity was observed.

Values: are mean of 3 replicates ± S.D.



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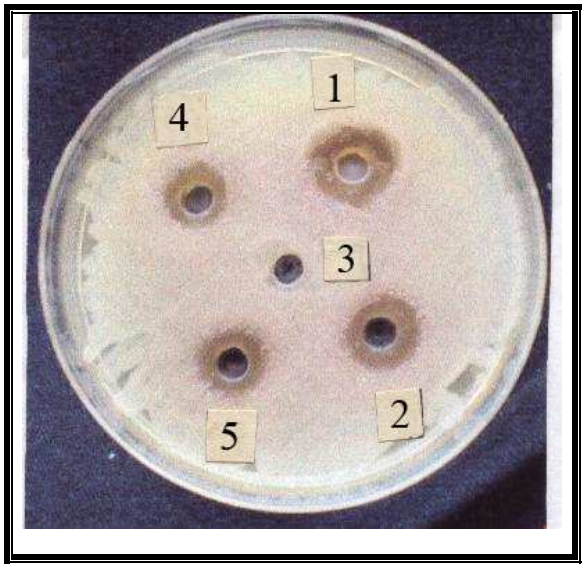

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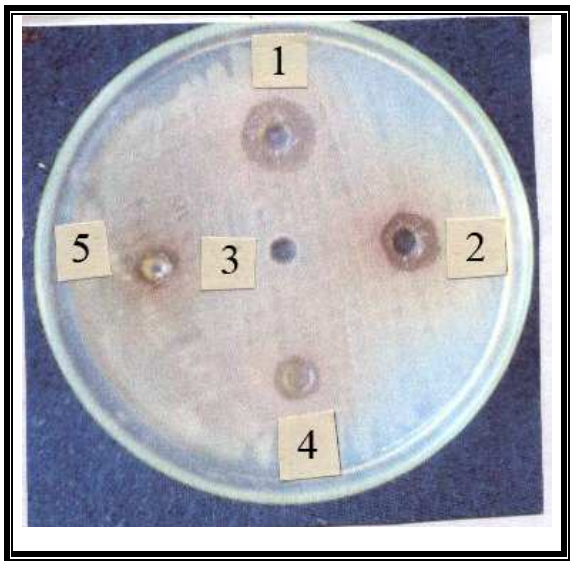

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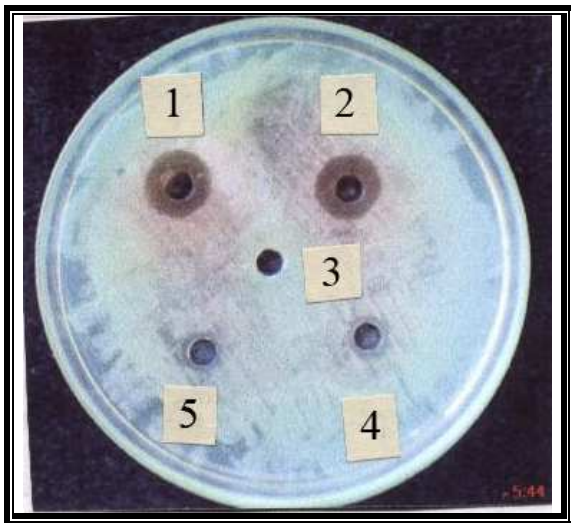

-d-

Fig.(4-9): Effect of *M. chamomilla* ethanolic extract on the growth of *a- S. aureus* , b- *B. ceries.*, c- *E. coli.* and d- *P. aeruginosa*

Extract was taken from callus cultures initiated from germinating seed explants.

1= 40 mg/ml, 2= 20 mg/ml, 3= control(P.B.S.), 4= 10 mg/ml, 5= 5 mg/ml.

It appears that water extract is more efficient than ethanolic. The reason may be due to the compounds already extracted by water particularly flavanoids.

This results disagree with Al-naymi, (2005) who reported that ethanolic extracts of *M. chamomilla* flowers have higher activity than water ones.

It is clear from the data presented in tables (3-9, 3-10, 3-11 and 3-12) that among the four tested microorganisms, *S. aureus* was the most susceptible microbe to the four extracts.

Furthermore, our results are in agreement with Mann and Staba, (1986) who showed that *M. chamomilla* water and ethanolic extracts are widely used as anti-inflammatory and antibacterial activity.

Previous studies showed that chamazulene and α- bisabolol are antibacterial thus, chamomile reduces inflammations (Der Marderosian and Liberti, 1988; Chevallier, 1996).

Although *M. chamomilla* contains many active compounds, most studies attributed the antimicrobial activity in chamomile to terpene compounds (Mann and Staba, 1986; Der Marderosian and Liberti, 1988; Tyler, 1999; Gyllenhaal, 2000).

Al-naymi, (2005) suggested that the activity of *M. chamomilla* could be attributed to the existence of chamazulen, α- bisabolol (sesquiterpenes) that showed high inhibition activity against *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Micrococcussp.*, and *Candida albicans.*

4.7- The effect of *M. chamomilla* extracts on rabbits eye (*in vivo*).

The ethanolic extract of chamomile showed obvious destroying effect on *S. aureus* cells in the rabbit eyes after injection at a concentration (40mg/ml) compared with controls Fig. (4-10).

Fig. (4-10): Appearance of rabbit's eye after administration of chamomile ethanolic extract at a concentration 40 mg/ml after one day

Water extract of chamomile showed a better effect than ethanolic one on damaging *S. aureus* cells in the rabbit eyes when a sample is injected at the concentration 40 mg/ml Fig. (4-11). Callus water extract was the best when adminstrared on the eyes at the same concentration Fig. (4-12). The control showed swallowing, closed eyes and filled with pus cells Fig. (4-13).

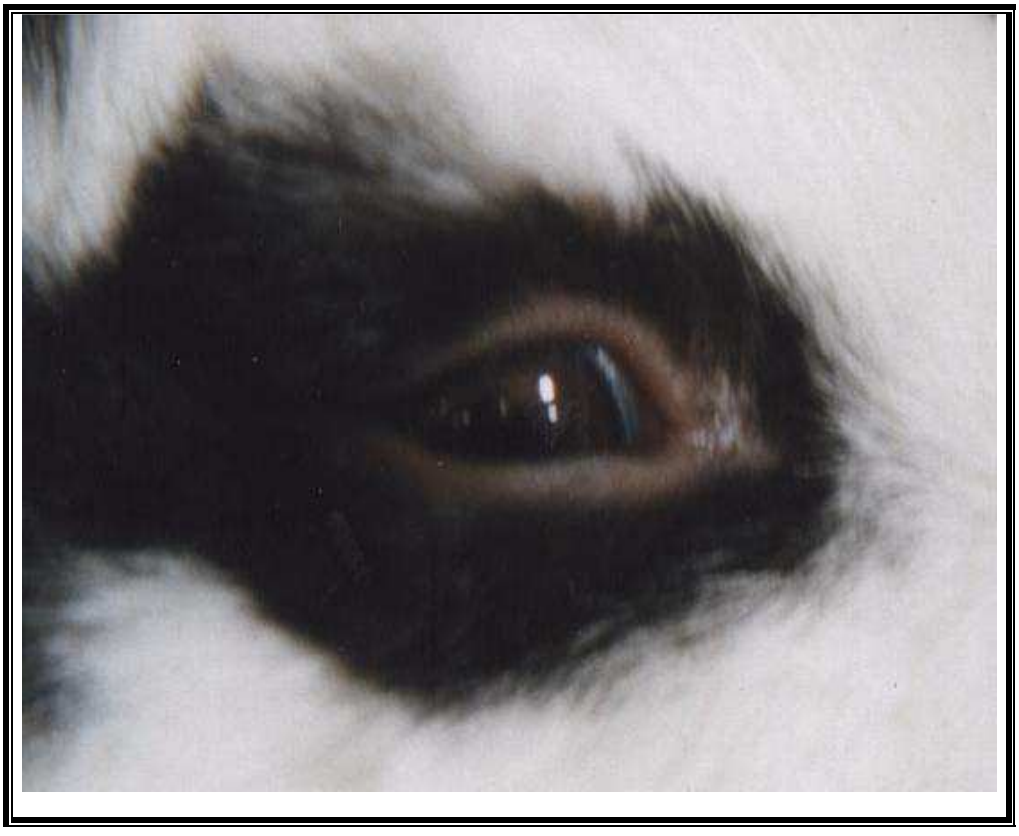

Fig. (4-11): Appearance of rabbits eye after administration of chamomile water extract at a concentration of 40 mg/ml after one day

Fig. (4-12): Appearance of rabbits eye after inject with 40 mg/ml of chamomile callus extract after one day

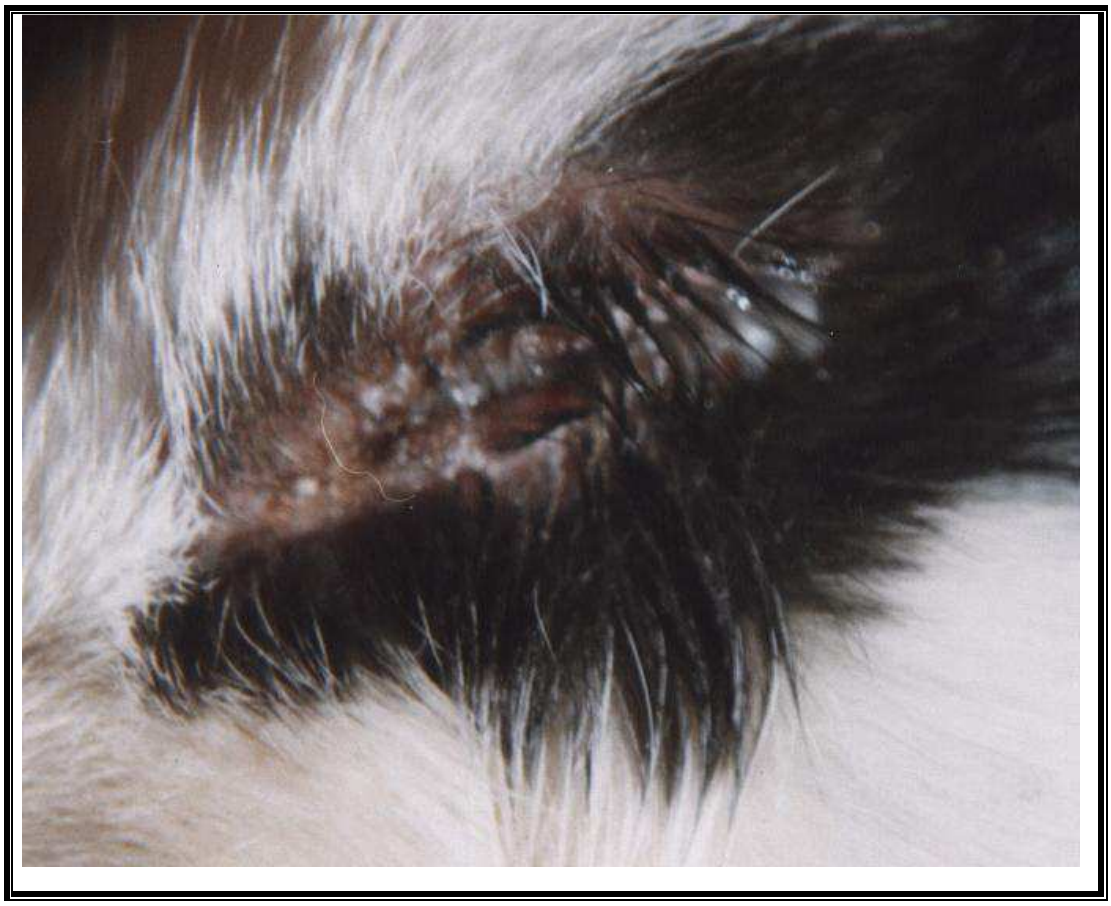

Fig. (4-13): Rabbit eye injected with *S. aureus* and P.B.S. (as a control)

Our results agree with Smith, (2006) that showed that chamomile tea help to relieve eyes redness and swollen eyes. Chamomile contains azulene, an anti-inflammatory compounds. Alpha-bisabolol has also been shown to shorten the healing time of skin burns in laboratory animals (Der Marderosian and Liberti, 1988).

Merfort and Heilman (1994) conducted *in vivo* study on nine female volunteers. They stated that chamomile (that contains flavons, apigenin, luteolin and apigenin glycoside) absorbed at the skin surface and penetrated into deeper skin layers. This observation supports their use as topical antiphlogistic agents to treat inflammations in deep tissues such as cornea.

The effect of alcoholic extract of *M. chamomilla* was tested in mice skin. It has anti-inflammatory activity. The response was observed when chamomile extract was used to reduce swelling. Both bisabolol and the flavonoids are nearly active in reducing inflammations in animals (Joe and Teresa, 1999).

Conclusions

1. The best concentration for *M. chamomilla* seedling surface sterilization was 3% of NaOCL for 10 min.
2. Callus cultures can be induced and maintained on MS medium supplemented with 3.0 mg/l BA and 0.1 mg/l NAA using germinating seeds as a source of explants.
3. Plant tissue culture techniques are potential source for the production of secondary metabolites.
4. *M. chamomilla* plantlets are regenerated from callus cultures grown on MS medium supplemented with 3.0 mg/l BA and 0.1 mg/l NAA.
5. Water extracts can be used against all tested microorganisms at concentrations (40 and 20)mg/ml. The largest inhibition zone was observed against *S. aureus* and *B. cereus.* Callus water extract showed a better inhibition activity than flower extracts.
6. High concentrations of chamomile ethanolic flower extract (20 and 40mg/ml) have inhibitory effects against (*S. aureus* , *B. cereus*, *E. coli* and *P. aeruginosa.*) bacteria. Callus ethanolic extract has a better inhibition activity than flower extracts
7. Water, ethanolic and callus extracts are potential cure for eyes infection and inflammation in rabbits.

Recommendations

1. Investigation of other medicinal plants as a source for phytochemicals using tissue culture techniques.
2. Using genetic engineering approaches for the manipulation of genes responsible for higher production of secondary metabolites.
3. Application of plant cell bioreactors for mass production of secondary metabolites.
4. Purification of the target secondary metabolite then examination for antimicrobial activity to find out which compound is the most effective.
5. Examination of extracts on other types of pathogenic bacteria and other kinds of infections.

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تأثير مستخلصات أزهار نبات البابونج *Matricaria chamomilla* L. و الكالس على بعض البكتيريا المسببه لالتهابات العيون

الملخص:

في محاولة لمعالجه أصابه عيون الأرانب باستخدام المستخلص المائي والكحولي لإزهار نبات البابونج *Matricaria chamomilla* ومقارنته مع مستخلصات نسيج الكالس. تم تنفيذ عدد من التجارب لهذا الغرض.

استحث الكالس و أديم على وسط MS المجهز ب ٣,٠ ملغم/لتر من BA و٠,١ ملغم/لتر من NAA باستعمال البذور النباتية للبابونج كمصادر لنشوء الكالس. وظهرت النموات الخضرية من الكالس بعد 40 يوم من زراعته وضهرت النباتات بعد ٥٥ يوما من زراعه الكالس.

أشارت النتائج إلى أن المستخلصين المائي والكحولي لإزهار البابونج و الكالس له فعاليه مضادة للبكتيريا و تم الكشف عن مكوناتها الكيميائية ووجد إن المستخلصات قد احتوت على الراتنجات ،التانينات ،الكومارينات ،الفينولات و الفلافونات و كذلك الكلايكوسيدات و لكن بكميه اقل.

كما وأوضحت النتائج بأن المستخلص المائي للازهار قد أعطى أعلى فعاليه تثبيطية من الكحولي في نمو أنواع البكتيريا *Staphylococcus aureus* و*Escherichia coli* و بنسبة تثبيط أقل لكل من *Pseudomonas aeriginosa* و*Bacillus ceriese*. اظهر مستخلص الكالس فعاليه تثبيطية أعلى من مستخلصات الأزهار و لنفس العزلات البكتيرية

تم في هذه الدراسة أيضا تحديد قيمه التركيز المثبط الأدنى MIC للمستخلصات النباتية على جميع أنواع البكتريا المدروسة أظهرت النتائج بأن فعاليه المستخلص المائي أعلى من الكحولي للأزهار و قد تباينت النتائج تبعا لاختلاف نوع المستخلص و نوع البكتيريا.

لوحظ إن لكل المستخلصات النباتية لأزهار البابونج تأثيرات مضادة للالتهابات عند إعطائها بجرعة٤٠ ملغم/مل، كما أظهرت هذه المستخلصات فعلا مضادا للبكتيريا مما أدى إلى عدم أصابه الأرانب التي حقنت عيونها بالمستخلصات بتركيز 40 ملغم/ مل.

CHAPTER ONE

Introduction

الأهداء

ألى القلب الذي اتسع لكل الحنان....
والدتي الحبيبة

ألى رمز الشموخ الذي علمني الاصرار على التفوق....
والدي الحبيب

ألى سندي الدائم ....
زوجي العزيز

ألى من كانوا خير معين لي....
أخواني

اهدي لهم ثمرة جهدي المتواضع

نور

Ministry of Higher Education and

Scientific Research

Al-Nahrain University

College of Science

Effect of Flowers and Callus extracts of *Matricaria chamomilla* L. on some bacteria causing eyes infections

A thesis

Submitted to the College of Science, Al-Nahrain University

in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biotechnology

By

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B.Sc., Biotechnology, College of Science, 2004

Al-Nahrain University

October **2007**

Shawal **1428**

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الإحيائية

تأثير مستخلصات أزهار نبات البابونج *Matricaria chamomilla* L و الكالس على بعض البكتيريا المسببة لالتهابات العيون

رسالة
مقدمة الى كلية العلوم – جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل
نور سعد لطيف العزاوي
بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠٤

Acknowledgments

Praise to ***Allah*** *the first of all cause, the glorious creator of the Universe for his mercy and kindness, and blessing upon Mohammed prophet of Allah and upon his family. I wish to express my deepest thanks to my supervisor* ***Dr. Kadhim M. Ibrahim*** *for the great help and advice during this work. I would also express my thanks to* ***Dr. Munira C.H. Ismail*** *for her support. My deep gratitude and appreciation to all staff of Biotechnology department at Al- Nahrain University. Grateful thanks to my* ***mother*** *for her help and support all the time.* Special *thanks are also due to* ***my family*** *and every one gave me a hand to complete this work.*

Noor