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Synergetic Effect of *Chamomilla recutita* Callus and Flower Extracts and Surlactin Produced by *Lactobacillus acidophilus* on Eye Infectious Bacteria in Rabbits

A thesis

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Summary

The study included estimating the synergetic effect of *Chamomilla recutita* callus and flower extracts and surlactin produced by *Lactobacillus acidophilus* on some bacterial types causing eye infections as follows:

To study the effect of *C. recutita* extracts, callus was induced from chamomile seeds, and it was maintained on Murashige and Skoog, 1962 (MS) culture medium supplemented with 1.0mg/l Benzyl Adenin (BA) and 0.1mg/l 2,4-dichlorophynoxyacetic acid (2,4-D).

Results showed that flower (water and ethanolic) extracts have antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* which cause eye infections. The chemical constituents of these extracts were detected. Results revealed that they contain tannins, phenols, coumarins, flavonoids, resins and glycosides.

The results also showed that ethanolic extract of flowers has higher antibacterial activity than water extract against *P. aeruginosa* and *S. aureus*. Callus extracts (ethanolic and water) showed higher antibacterial activity against the same bacterial types.

Minimum inhibitory concentration (MIC) was determined for the extracts against the tested bacteria. Results showed that ethanolic extract has higher activity than water extract and the results varied according to the bacterial types.

Twenty five vaginal swabs from outpatients' healthy women were collected from Kamal Al-Samarai hospital, Baghdad to isolate and identify *L. acidophilus* from April 2007 to December 2007.

Three of the isolates were diagnosed as *L. acidophilus* which represented 20% of the total number of bacterial isolates, other *Lactobacillus* types represented 65% (20 isolates), while other bacterial types represented 15%.

The ability of *L. acidophilus* to produce surlactin was detected after measuring its biological activity to inhibit the adhesion of biofilm formed by bacteria (*P. aeruginosa*) to surfaces using test tube method.

It was found that all the isolates were able to produce surlactin but the activity of surlactin varied. Surlactin produced by isolates 1 and 13 was the most effective.

Biological applications of surlactin were studied by inhibiting the adhesion of pathogenic cells *P. aeruginosa* producing biofilm on industrial contact lenses. It was found that surlactin has the ability to inhibit the adhesion up to 60% and 55% for the isolates1 and 13 respectively. Antibacterial activity of surlactin was tested. Results revealed that it does not have this ability.

When administrated to rabbit's eyes, chamomile callus ethanolic extract (80mg/ml) had the ability to treat the infection in infected eyes; it also showed antibacterial activity when inoculated into rabbit eyes with infection caused by *P. aeruginosa*.

Surlactin showed an ability to treat the infection in rabbit eyes with *P. aeruginosa* while it did not show this ability against *S. aureus*. Additionally, it prevented the infection with *P. aeruginosa* when administrated to rabbit eyes inoculated with this bacteria only, while it showed no effect against *S. aureus*.

Synergetic effect of surlactin and chamomile ethanolic extract (80mg/ml) cured infections in rabbit eyes inoculated with *P. aeruginosa* at shorter period of time compared to surlactin and chamomile extract used separately, while they had less effect on *S. aureus*.

Finally, the synergetic effect of chamomile ethanolic extract and surlactin in inhibiting the adhesion of *P. aeruginosa* to synthetic contact lenses was determined. The inhibition percentage reached 50% when chamomile extract and surlactin extracted from isolate 1 were used, while it reached to 45% when surlactin extracted from isolate 13 and chamomile extract were used.

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List of Abbreviations

Abbreviation	Full name
2,4-D	2,4-dichlorophynoxyacetic acid
A	*Alpha*
B.	*Bacillus*
BA	Benzyl Adenin
°C	Degree Celsius
CRD	Completely Randomized Design
CFU	Colony Forming Unit
DDH_2O	Double Distilled Water
dwt	Dry weight
E.	*Escherichia*
EDTA	Ethylene Diamine Tetraacetate
g	gram
GRAS	Generally Regarded As Safe
hrs	hours
l	liter
L.	*Lactobacillus*

LAB	Lactic Acid Bacteria
LSD	Least Significant Differences
MS	Murashige and Skoog Medium, 1962
min.	minute
MIC	Minimum Inhibitory Concentration
mg	milligram
MRS	Man-Sharp-Rogosa
µ	microne
n	Number of replicates
N	Normality
NaOCl	Sodium hypochlorite
P.	*Pseudomonas*
PBS	Phosphate Buffer Saline
S.D.	Standard deviation
S.	*Staphylococcus*
UK	United Kingdom

Introduction

The herbal plants rich in secondary plant products are termed as "medicinal" or "officinal" plants. These secondary metabolites exert in general a profound physiological effect on the mammalian system, and thus are known as active principles of plant.

Chamomile is one of the most widely used and well-documented medicinal plants in the world. Chamomile (*Chamomilla recutita* L.) is a well-known medicinal plant in folk medicine cultivated all over the world.

Chamomile is used both externally and internally to treat an extensive list of conditions. Externally used for wounds, eczema, skin irritations, rheumatic pain, eye infections and leg ulcers. Internally used to treat anxiety, hysteria and nightmares (Martens, 1995).

Extracts of German chamomile contain several antibacterial, antifungal and antiseptic properties. It is used against different types of bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Micrococcus spp.*, *Streptococcus pyogenes* and *St. pneumonia*.

Lactic acid bacteria (LAB) are sometimes termed probiotics and are used as health adjuncts in food to provide a wide variety of health benefits. Currently probiotic preparations contain (*Lactobacillus acidophilus, L. plantarum, L. casei, L. fermentum, L. lactis,* and *L. brevis*) (Jean Penaud, 2002).

LAB secrete extra cellular secretions known as biological surface active agents (biosurfactants), surlactin is the most important one produced by *L. acidophilus* (Rhee and Park, 2001).

Most work on biosurfactants' applications has been focused on their use in environmental applications owing to their diversity, environmentally friendly nature, suitability for large-scale production and selectivity.

Despite their potential and biological origin only a few studies have been carried out on applications related to the biomedical field. Microbial surfactants have several advantages over chemical surfactants such as lower toxicity, higher biodegradability and effectiveness at extreme temperatures or pH values (Kosaric, 1992; Cameotra and Makkar, 1998).

The aims of the current work are:

1. Using tissue culture techniques to induce callus on explants taken from chamomile.
2. Preparation of water and ethanolic extracts from both chamomile callus and dried chamomile flowers.
3. Isolation and identification of *Lactobacillus acidophilus* from healthy women's vagina and detection of surlactin producing isolates.
4. Studying the effect of surlactin and chamomile extracts separately and together on pathogenic bacteria causing eye infection *in vitro* and *in vivo* in rabbits' eyes.
5. Studying the synergetic effect of surlactin and chamomile extracts on synthetic contact lenses.

1.2 Literature Review

1.2.1 Overview

Chamomile is one of the most widely used ingredients in herbal teas worldwide. It is one of the important herbal medicines as it is used for the treatment of many diseases (Simpson, 2001).

In Europe it is considered a "cure all", and in Germany it is referred to as "alles zutraut" meaning "capable of anything" (Berry, 1995).

In Germany, where chamomile sales exceeded $8.3 million in 1994, more than 4,000 tons of chamomile are produced yearly (Berry, 1995).

1.2.2 Nomenclature

The word chamomile is derived from the Greek *chamos* (ground) and *melos* (apple), referring to the plant's low growing habit and the fact that the fresh blooms are somewhat apple-scented (Smith, 1963).

According to Hill (1948), accurate identity of a plant under discussion as "chamomile" is hampered by the fact that the name has been applied to a dozen or more species in six genera of the aster family (Asteraceae); however, of all these plants, only two species are generally utilized in the herb trade and in broad folk usage.

C. recutita (L.) Rauschert is retained as the proper botanical name of the "common chamomile" of the European continent, better known as Hungarian or German chamomile, also known by the synonyms *Matricaria chamomilla* and *M. recutita* (Tucker, 1986; Tucker and Lawrence, 1987; Tucker *et al.*, 1989). Jeffery (1979) provided convincing evidence for elegating the genus name *Chamomilla* to synonymy with *Matricaria*.

1.2.3 History

Among the oldest known medicinal plants, chamomile was used by the Egyptians to cure ague and as an offering to their gods. The Arab herb physician Abul Abbas mentioned how the use of this plant spread from the Middle East to Spain. Hippocrates described this herb as helpful in the treatment of congestion and dysmenorrhea. Dioskurides, Galen and Plinius also recommended chamomile tea for inflammation of the mouth and sitz bath with chamomile tea or tincture added in cases of painful menstruation (Issac and Schimpke, 1965).

There are actually two herbs commonly called chamomile: Roman (common) chamomile (*Chamaemelum nobile*, *Anthemis nobilis*) and German (Hungarian, wild, scented mayweed) chamomile (*Chamomilla recutita*, *Matricaria recutita*) (Stray, 1992).

Today, chamomile is included in the 9 pharmacopoeia of 26 countries throughout the world (Salamon, 1992). German chamomile has a long tradition as a folk or domestic remedy used for a wide variety of purposes including inflammations, rheumatism and skin ailments.

A tea (infusion), decoction, or tincture has long been used for treating colic, diarrhea, fever, toothache, bleeding or swollen gums and a folk cancer remedy (Duke, 1985; Leung and Foster, 1996).

1.2.4 Description

German chamomile is a sweet-scented, smooth, branched annual growing to 75cm. in height. It is native to Europe and western Asia, and has become widely naturalized in the U.S. Roman chamomile is an aromatic, creeping perennial, growing to 30cm in height. It hails from the United

Kingdom (UK) and is widely grown in American herb gardens (Bailey Hortorium, 1976).

The leaves are finely divided, the lower ones grow in threes, the middle is paired and the upper is a single pinnate.

The mildly scented flowers are arranged in flower heads, which are convex when they first bloom and later become conical in shape. The head is surrounded by (12–18) tongue-shaped, white ray florets and the disk florets, the flowers are collected from May to July (Dtsch, 1965).

The flower heads of Roman chamomile are hemispherical and densely surrounded by silvery white florets. It is a low-growing plant, less than 25cm in height. The hairy and branched stems are covered with leaves divided into threadlike segments. This fineness gives the whole plant a feathery appearance (Fig. 1) (Stray, 1992).

Fig. (1): *C. recutita* (L.) Rauschert (Stray, 1992).

1.2.5 Chemical compounds of chamomile

C. recutita, the sun loving plant, is rich in active ingredients and has remained one of the most popular herbs since ancient times.

There are different classes of active constituents, which have been isolated and used individually in medical practice and cosmetics (Grieve, 1982; Petri and Lemberkovics, 1994; Hoffmann, 1995). About 120 secondary metabolite chemical constituents have been identified in chamomile, including 28 terpenoids, 36 flavonoids, and 52 additional compounds, all with potential pharmacological activity (Salamon, 1992).

This plant contains from (6-8)% flavonoids (Dölle *et al*., 1985; Bruneton, 1999; Hänsel *et al*., 1999), composed of flavone glycosides including luteolin glycosides, quercetin glycosides, and isorhamnetin (Bruneton, 1999), and up to 10% mucilage polysaccharides (Carle and Issac, 1985). Recent research indicates that they display more or less inhibitory effects on certain malignant cell proliferation *in vitro* (Agullo *et al.*, 1997).

In addition, *C. recutita* contains phenolic carboxylic acids such as vanillic, anisic, syringic and caffeic acids, and (0.24-1.9)% volatile oil, which is a wonderful blend of different individual oils. This oil, extracted from flower heads by steam distillation, can range in color from brilliant blue to deep green when fresh but fades over time to dark yellow, despite fading; the oil does not lose its potency. It contains α-bisabolol (up to 50%) chamazulene cyclic sesquiterpenes, which directly reduce inflammation and are mild antibacterials (Erazo and Garcia, 1997). The essential oil also contains bisabolol oxides, farnesene and spiro-ether, which have anti-inflammatory and antispasmodic actions (Grieve, 1982; Petri and Lemberkovics, 1994; Hoffmann, 1995).

In the major chamomile production countries (Argentina, Czech Republic, Germany, Hungary, Poland, and Slovakia), intensive plant improvement programs have been initiated to produce plants with high levels of defined chemical components (Salamon, 1992).

1.2.6 Uses of Chamomile

1.2.6.1 Pharmacological actions

According to German Commission E, chamomile is anti-inflammatory, muscle relaxant, antispasmodic, promotes wound healing, deodorant, antibacterial, bacteriostatic and stimulates skin metabolism (Blumenthal *et al.*, 1998).

1.2.6.1.1 Internal actions

i. Animal: Inhibits ulceration (Szelenyi, 1979), relaxes smooth muscle (Carle and Gomma, 1992) and depresses central nervous system (CNS) (Della Loggia *et al.*, 1982).

ii. Human: Sedative (Gould *et al.*, 1973; Mann and Staba, 1986; Bradley, 1992) and carminative (CCRUM, 1992).

1.2.6.1.2 External actions

i. Animal: Anti-inflammatory (Tubaro *et al.*, 1984; Carle and Gomaa, 1992; WHO, 1999).

ii. Human: Anti-inflammatory (Aertgeerts *et al.*, 1985), astringent, cooling (Nasemann, 1975) and promotes wound-healing (Glowania *et al.*, 1987).

1.2.6.2 Medicinal and other uses of chamomile

Chamomile (*C. recutita* L. Rauschert) is one of the important herbal medicines as it is used for the treatment of many diseases (Simpson, 2001).

German chamomile is considered the more potent of the two chamomiles. It has received more scientific evaluation, and is more widely cultivated than Roman chamomile.

It is believed to possess vulnerary, deodorant, bacteriostatic, antimicrobial, anticatarrhal and spasmolytic properties (Newall *et al.*, 1996; Blumenthal, 1998). Roman chamomile is believed to possess antiemetic, antispasmodic, and sedative properties (Newall *et al.*, 1996). Traditionally, it has been used for flatulent and nervous dyspepsia, motion sickness, nervous diarrhea, restlessness, anxiety, teething, nasal catarrh, dysmenorrheal and amenorrhea (Mills and Bone, 2000).

The pharmacological effect of chamomile is mainly connected with its essential oil for its spasmolytic and antimicrobial properties (Brunke *et al.*, 1992).

1.2.7 *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 different 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, 2008).

The production of secondary metabolites *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).

1.2.7.1 Callus cultures

Cell from any plant species can be cultured aseptically on or in a nutrient medium. The cultures are initiated by planting a sterilized tissue (explant) on an agar medium. Within (2-4) weeks, depending upon plant species, a mass of unorganized cells (callus) is produced. Such a callus can be subcultured indefinitely by transferring a small piece on to the fresh agar medium.

Callus cultures can be obtained from seedlings, young shoots or buds, root tips or developing embryos: fruits, floral parts, tubers and bulbs. Under the influence of plant growth regulators, the cells of the explant may be induced to divide to form a loose mass of cells (callus) (Ramawat, 2008).

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 the rate of cell division slows but the rate of cells expansion increases.
d. Deceleration phase, where the rate of cell division and elongation decrease.
e. Stationary phase, where the number and size of cells 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 production of secondary compounds is 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, 2008).

1.2.8 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 for key intermediates (Kurz *et al.*, 1988).

Different types of cell lines may be isolated from variant cell suspension cultures using screening 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, 2008).

1.2.9 Mechanism of action

Whole plant extracts of chamomile have demonstrated antispasmodic action, though the mechanism of action was unclear (Forster *et al*., 1980).

Antispasmodic effects are due mainly to chamomile's water-soluble constituents (Carle and Gomaa, 1991) such as the flavonoids apigenin and apigenin-7-O-glucoside and the volatile oil α-bisabolol, which act similarly to papaverine (Bruneton, 1999; WHO, 1999).

Sedative effects are attributed to the flavonoids, including apigenin, which acts as a ligand for the central benzodiazepine receptors. Apigenin competitively inhibits the binding of flunitrazepam, thus providing a molecular basis for possible weak CNS-depressing activity of water based preparations (e.g., teas) (Viola and Wasaowski, 1995).

Apigenin may be an anti-inflammatory constituent (Hadley and Petry, 1999), due to the water-soluble and lipophilic components. The flavones block the arachidonic acid pathway by inhibiting phospholipase A, cyclo-oxygenase, and lipoxygenase pathways. The volatile oil components, chamazulene and α-bisabolol, have also demonstrated anti-inflammatory action by interfering with 5-lipoxygenase and cyclo-oxygenase production (Carle and Gomaa, 1992).

The azulene components of the volatile oil have anti-allergenic and anti-inflammatory actions, though the mechanism of action was unclear (Farnsworth and Morgan, 1972).

Azulene may prevent histamine discharge from tissue by activating the pituitary-adrenal system, causing the release of cortisone (Stern and Milin, 1956), or azulene may prevent allergic seizures caused by histamine release, activating cellular resistance and speeding the process of healing (Meer and Meer, 1960).

Chamomile extract accelerates wound-healing, reportedly by reducing inflammation and promoting tissue granulation and regeneration on topical application (Carle and Isaac, 1987).

1.2.10 Probiotics

1.2.10.1 Definition and history

The word probiotic is derived from the Greek and means "for life" (Lilley and Stillwell, 1965). Probiotics can be defined as organisms and substances which contribute to intestinal microbial balance (Fuller and Gibson, 1997; Guarner and Schaafsma, 1998).

From the past century the beneficial role of non pathogenic bacteria in the intestinal lumen was described and many clinical benefits to these specific non pathogenic organisms were studied like diarrhea treatment, antimicrobial activity, anticarcinogenic activity and others (Oyetayo and Oyetayo, 2005).

In the past century, they have undergone scientific scrutiny for their ability to prevent and cure a variety of diseases particularly in developing countries (Drisko *et al.*, 2003).

Lilley and Stillwell (1965) were the first who used the term (probiotic) to describe substances secreted by one microorganism to stimulate the growth of another, and thus was contrasted with the term antibiotic.

On the other hand, Fuller (1989) attempted to improve the definition of probiotics with the following distinction (a live microbial feed supplement which beneficially affects the host health by improving its intestinal balance).

Havenaar *et al.*, (1992) defined probiotics as a viable mono or mixed culture of microorganisms which is applied to animal or man, beneficially affects the host by improving the properties of the endogenous microflora. Furthermore, Salminen (1996) defined probiotics as live microbial culture or cultured dairy products which beneficially influence the health and nutrition of the host.

Probiotics are also termed as biotherapeutic agents because they are therapeutically used to modulate immunity, treat rheumatoid arthritis, prevent cancer, improve lactose intolerance and prevent or reduce the effect of atopic dermatitis, diarrhea, and constipation as well as candidiasis and urinary tract infection (Mercenier *et al.*, 2003).

The concept of probiotics progressed during the 20^{th} century, when Elie Metchnikoff hypothesized that the long and healthy lives of Bulgarian peasants were the outcome of their consumption of fermented milk products (Eduardo *et al.*, 2003).

At this time, Henry Tissier, a French paediatrician observed that children with diarrhea had in their stools a low number of bacteria characterized by a peculiar, Y-shaped morphology. These "bifid" bacteria were, on the contrary, abundant in healthy children (Oyetayo and Oyetayo, 2005).

The work of Metchnikoff and Tissier was the first to make scientific suggestions about the probiotic use of bacteria. The first clinical trials were done in the 1930s on the effect of probiotics on constipation (Koop-Hoolihan, 2001).

The mechanisms by which probiotics exert their effects on the host are still speculative (Koop-Hoolihan, 2001). Probiotics antagonize pathogens through production of antimicrobial and antibacterial compounds such as

cytokins and butyric acid (De Vuyst and Vandamme, 1994; Kailasapathy and Chin, 2000), improve immune function and stimulate immunomodulatory cells (Isolauri *et al.*, 1995; Rolfe, 2000), compete for binding and receptor sites that pathogens occupy (Fujinawa *et al.*, 1997; Kailasapathy and Chin, 2000), reduce gut pH by stimulating the lactic acid producing microflora (Langhendries *et al.*, 1995), or compete pathogens for available nutrients and other growth factors (Rolfe, 2000).

1.2.10.2 Probiotic microorganisms

Microbial probiotics are extremely safe and not associated with much significance for determined side effects, thus they are considered as (Generally Regarded As Safe) GRAS (McFarland and Elmer, 1995).

The most used probiotic microorganisms are lactic acid bacteria (LAB) including the genus *Lactobacillus*, especially *L. acidophilus*, *L. plantarum*, *L. rhamnosus*, and *L. fermentum* (Table 1) (Havenaar *et al.*, 1992; Greene and Klaenhammer, 1994).

In general, a group of requirements have been identified as important properties for *Lactobacilli* to be effective probiotic microorganisms (Salminen, 1994; Reid, 1999). These include adherence to cells (Ouwehand *et al.*, 1999), exclude or reduce pathogenic adherence to cells, persist and multiply, produce acids, hydrogen peroxide, bacteriocins antagonistic to pathogen growth (Reid and Burton, 2002), and resist vaginal microbicides including spermicides (Spanhaak *et al.*, 1998).

A beneficial association of microorganisms on the human host was suggested in the past, which proposed that vaginal bacteria produce lactic acid from sugars to prevent or inhibit the growth of pathogenic bacteria (Holzapfel *et al.*, 2001).

There are other types of microorganisms that are considered as probiotics such as *Streptococcus spp.*, *Enterococcus spp.***,** *Bacillus spp.* and *Saccharomyces spp.*

Table (1): Microorganisms considered as probiotics (Holzapfel *et al.*, 2001).

Lactobacillus **species**	***Bifidobacterium*** **species**	**Other Lactic Acid Bacteria**	**Microorganisms**
Lb. acidophilus	*B. adolescentis*	*Enterococcus faecalis2*	*Bacillus cereus var.* toyoi*2,3*
Lb. amylovorus	*B. animalis*	*Enterococcus faecium*	*Escherichia coli* strain nissle
Lb. casei	*B. bifidum*	*Lactococcus lactis4*	*Propionibacterium freudenreichii2,3*
Lb. crispatus	*B. breve*	*Leuconstoc mesenteroides*	*Saccharomyces cervisiae3*
Lb. delbruekii	*B. infantis*	*Pediococcus acidilactici4*	*Saccharomyces boulardii3*
Lb. gallinarum2	*B. lactis5*	*Sporolactobacillus inulinus2*	
Lb. gasseri and other	*B. longum*	*Streptococcus thermophilus4*	

1.2.10.2.1 History of LAB

There is a long history of health claims concerning living microorganisms in food, particularly LAB. In 76 before Christ, the Roman historian Plinius recommended the administration of fermented milk products for treating gastroenteritis (Schrezenmeir and Verse, 2001).

It was believed that intake of yoghurt containing *Lactobacilli* results in reduction of toxin producing bacteria in the gut and that result in increasing the longevity of the host (Abee *et al.*, 1995).

In 1926, it was found that *L. acidophilus* may survive in the human gut (Apella *et al.*, 1992; Reid, 1999), while the significant role of the intestinal micro flora to resist diseases was shown in 1954 (Agerholm-Larsen *et al.*, 2000; D'Souza *et al.*, 2002).

Over the last thirty years, intensified efforts to identify and characterize LAB have revealed their many critical roles in dairy foods (Salminen, 1994). Tissier (1984) recommended that administration of *Bifidobacteria* to infants suffering from diarrhea could suppress the putrefactive bacteria causing the disease, he added that *Bifidobacteria* were predominant in the gut flora of breast-fed infants.

1.2.10.2.2 Characteristics and requirements of LAB

LABS are "Gram-positive", non spore forming, catalase negative organisms that are devoid of cytochromes and of an anaerobic habit but are aerotolerant, acid-tolerant, and strictly fermentative (Holzapfel *et al.*, 1998).

Lactic acid is the major end-product of sugar fermentation (Axelsson, 1998). However, exceptions from this general description do occur because some species can form catalase or cytochromes on media containing hematin or related compounds (Whittenbury, 1964; Meisel *et al.*, 1994).

The production of a non heme catalase, called pseudocatalase, by some *lactobacilli* can also cause some confusion in the identification of LAB (Engesser and Hammes, 1994).

LAB are associated with habitats that are rich in nutrients such as various food products and plant materials. They can be found in soil, water, manure, sewage and silage and can ferment or spoil food; despite this,

particular LAB are inhabitants of the human oral cavity, the intestinal tract, and the vagina, and may have a beneficial influence on these human ecosystems (Holzapfel *et al.*, 2001), they have been used for centuries in fermentation processes and recently are gaining increased attention due to their probiotic properties (Altermann *et al.*, 2004).

1.2.10.3 *Lactobacillus* as probiotics

Lactobacillus is Gram-positive, rod in shape with a regular dimension of 0.5-1.5×10nm, single, paired, or small chain, non spore forming, facultatively anaerobic or microaerophilic, catalase negative and stable in acid media and salt (Stamer, 1979).

The *Lactobacilli* include over 25 unique species. The first level of differentiation is based on end-product composition. Some are homo-fermentatives, while others are hetero-fermentatives. The former are classified as organisms that produce over 85% lactic acid as their end-product from glucose. The latter include organisms that produce approximately 50% lactic acid as an end-product with considerable amounts of carbon dioxide, acetate, and ethanol (Chakraborty, 1996).

The next major criterion for distinguishing *Lactobacilli* is the production of gas from carbon source including glucose and gluconate. In addition there is a great degree of diversity in the ability of various *Lactobacillus spp.* to ferment pentose sugar including ribose and xylose (Batt, 1999).

Lactobacillus may be found in a number of fermented food products and the occurrence of it contributes to the preservation, nutrition availability, and flavor. Dairy products are produced by using *Lactobacillus* either alone or in combination with other LAB (Alm, 1982). Also it can be

found in wet places of the human body like the mouth, intestinal tract, and vagina (Baily *et al.*, 1999).

Lactobacillus spp. inhibit the activities and proliferation of pathogenic bacteria by several ways such as production of lactic acid, bacteriocins, hydrogen peroxide, and other metabolites, for example *L. acidophilus* is naturally occurring bacteria that reinforce protective mucosal surface and prevent enhancement and attachment of harmful microorganisms and allergens (Sanders and Klaenhammer, 2001).

It was found that *Lactobacillus* has the ability to produce biological surface active agents known as biosurfactants which have the ability to inhibit the adhesion of pathogenic bacteria causing infections (Velraeds *et al.*, 1998; Velraeds *et al.*, 2000).

1.2.10.3.1 *Lactobacillus acidophilus*

L. acidophilus is the most commonly used probiotic, or "friendly" bacteria. This type of friendly bacteria lives in the intestines and vagina and protects against the entrance and proliferation of pathogenic microorganisms that can cause disease. This is accomplished through a variety of mechanisms. For example, the breakdown of food by *L. acidophilus* leads to production of lactic acid, hydrogen peroxide, and other byproducts that make the environment hostile for undesired organisms. *L. acidophilus* also produces lactase, the enzyme that breaks down milk sugar (lactose) into simple sugars. People who are lactose intolerant do not produce this enzyme (Hammes and Vogel, 1995).

Studies report few side effects from *L. acidophilus* when used at recommended doses. Some experts recommend limiting the daily dose to fewer than 10 billion living *L. acidophilus* organisms to reduce the side

effects (Adolfsson *et al.*, 2004). Oral supplements of *L. acidophilus* in human have resulted in synthesis of B-complex vitamins and adsorption of calcium amilelioration of diarrhea, constipation and immunity activation (Reddy *et al.*, 1983).

1.2.11 Surface active agents (Surfactants)

Surface active agents are generally considered as dissolving, moisturizing, foam forming and conditioning substances. They are used in many industries especially detergents and cosmetics as they are considered as substances that influence the surface tension.

They vary in their chemical composition. They could be glycolipids, lipopeptides, fatty acids or glycoproteins (Balaguer, 1998). They are produced either chemically or biologically by many microorganisms.

The molecule of these agents consists of two chemical groups, the first is polar, water soluble; therefore, considered as hydrophilic group, while the second group is non polar, hydrocarbonic chain and considered as hydrophobic. The presence of this molecule in a mixture of water and oil is considered as a bridge between them (Velraeds *et al.*, 1996).

1.2.11.1 Biosurfactants

Biosurfactants are produced by many microorganisms especially those found as normal flora in the gastrointestinal tract, the urogenital tract, the skin and the eye.

They interfere with substances grouped on surfaces especially moist and air exposed surfaces, as a result they remove those groups, break them and may take their places; therefore, they were used in many industries

and medicine to reduce infection and preserve vitality of some substances (Millsap *et al.*, 1997; Morris *et al.*, 1999; Van Hoogmoed *et al.*, 2000).

It was found that *St. thermophillus* used in the production of dairy products has the ability to produce biosurfactants, also *St. mitis* isolated from the mouth has the ability to produce these substances which act as inhibitors to the adhesion of pathogenic *St. mutans* in mouth (Velraeds *et al.*, 1996; Van Hoogmoed *et al.*, 2000).

Many other microorganisms like *Nocardia spp., B. subtilis* and some types of *Candida lipolytica* also produce biosurfactants which vary in their chemical composition and effect; therefore, they were divided into two classes according to their molecular weight. The first includes molecules with low molecular weight and effect on surface tension while the second includes high molecular weight polymers which have the ability to bind strongly to surfaces (Critchly and Douglas, 1987).

Biosurfactants produced by *Lactobacillus spp.* are considered the most important biologically active substances produced by these bacteria and have special importance compared with biosurfactants produced by other microorganisms because of the low toxicity and the ability to biodegrade many substances so they are used in treating sewage, in addition to their important medical applications to reduce microbial infection (Granato *et al.*, 1999; Rhee and Park, 2001).

Biosurfactants produced by *Lactobacillus spp.* are called surlactins or what is known as surface lactins, it is thought that the reason for this naming is due to their chemical nature (mucoproteins) (Velraeds *et al.*, 1998).

1.2.11.2 Uses of surlactin

Surlactins are used in cosmetic industries and detergent industries (Kim *et al.*, 2000), they have ability to emulsify hydrocarbons; therefore, they were used to protect and clean the environment from pollution by many hydrocarbonic materials (Swarajit and Randhir, 2004).

Many researchers found that surlactin produced by *L. acidophilus* can be used in medical field because they are multi-functional substances, as antimicrobial, antitumor, antimutagenic agents and immunomodulators (Eijsink *et al.*, 1998).

1.2.12 Microorganisms causing eye infection

The eye is infected by a number of organisms that enter through many parts and cause diseases such as conjunctivitis, keratitis, endophthalmitis and other diseases. The microorganisms that cause these diseases are *S. aureus*, *St. pneumoniae*, *P. aeruginosa*, *Corynebacterium diphtheriae*, *Klebsiella pneumoniae*, *E. coli* and *Neisseria gonorroeae* (Tortora *et al.*, 1986).

1.2.12.1 Genus *Staphylococcus*

Gram positive cocci which possess no flagella and do not form spores. Facultative anaerobic and catalase positive which distinguish them from *Streptococcus*, *Enterococcus* and *Lactococcus* species despite they are also Gram positive cocci but obligate fermenters that lack the enzyme catalase (Carrity, 2001). On blood agar, pathogenic *Staphylococcus* causes hemolysis of the erythrocytes, rabbit and sheep erythrocytes are the most sensitive to Staphylococcal haematoxin (Jensen and Wright, 1989).

Several species of *Staphylococcus* are notable for their medical significance and even they are typically part of the normal flora, they can cause infection in people who have underlying medical problems (Nester *et al.*, 2001).

S. aureus was discovered by Robert Koch (1878) and it is a major cause of conjunctivits and keratitis (Baron *et al.*, 1994).

One of the most identifying characteristics of *S. aureus* is production of coagulase (coagulates plasma), fermentation of mannitol and the ability to grow under quite high salt concentrations (Nester *et al.*, 2001).

Virulence factors of *S. aureus* are capsules (inhibit phagocytes) and production of α, β, γ and σ hemolysis (characterized by lethal, hemolytic and necrotic activity) and leucocidin (destroying leucocytes) (Kingsbury and Wanger, 1991).

1.2.12.2 Genus *Pseudomonas*

Gram negative rod shaped bacteria, non spore former, aerobic although some species are facultative anaerobes. They grow in soil, marshes and coastal marine habitats, as well on plants and animal tissues. Almost all strains are motile by means of a single polar flagellum, and some strains have two or three flagella. *P. aeruginosa* is Gram negative rod shaped bacterium. It forms biofilms on wet surfaces such as those of rocks and soil. The emergence of *P. aeruginosa* as a major opportunistic human pathogen during the past century may be a consequence of its resistance to the antibiotics and disinfectants that eliminate other environmental bacteria. *P. aeruginosa* is now a significant source of bacteremia in burn victims, urinary-tract infections and hospital-acquired pneumonia in patients. It is also the predominant cause of morbidity and mortality in cystic fibrosis

patients (Forbes *et al.*, 2002). It is a major cause of nosocomial infections and an occasional cause of community acquired infections including eye infections from contaminated contact lens solutions, keratitis and endophthalmitis and dacryocytitis (Baron *et al.*, 1994; Hartikainen *et al.*, 1997).

It produces pigments, its cultures have distinct fruity odor, and oxidase positive serve (Brook *et al.*, 1998).

It has many virulence factors such as exotoxins which have toxic effect on corneal tissue (Ijiri *et al.*, 1993). Also it has the ability to produce proteolytic enzymes and hemolysin that destroy cells and tissues.

1.2.13 Treatment with chamomile

Studies on animals show antimicrobial activity of chamomile. It is effective against bacterial or fungal infections on the eyes. Chamomile is used as dried extracts such as capsules or powders then used for external treatments as eye washes to treat infection (Popescu *et al.*, 1985; Al-Azawy, 2007).

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 chamazulin, 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 used on skin to increase wound healing and reduce inflammations such as those caused by allergies and pathogenic microorganisms, 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; Merfert and Heilman 1994; Smith, 2006).

2.1 Materials

2.1.1 Apparatus and equipments

The following equipments and apparatuses were used to perform the study:

Apparatus	Company (origin)
Anaerobic jar	Rodwell (U.K.)
Autoclave	Gallenkamp (U.K.)
Balance	Ohans (France)
Compound light microscope	Olympus (Japan)
Distillator	GFL (Germany)
Electrical balance	Mettler (Switzerland)
Electrical incubator	Gallenkamp (Germany)
Electrical incubator	Sanyo (Japan)
Electrical oven	Gallenkamp (Germany)
Glass Pasteur pipettes	John poulten Ltd. (England)
Laminar air flow cabinet	ESCO
Millipore filter unit (0.22µm)	Millipore and Whatman (England)
Micropipette	Oxford (U.S.A.)
pH-Meter	Meter GmbH-Teledo (England)
Refrigerator	Concord
Rotary evaporator	Gallenkamp (Germany)
Sensitive balance	Delta Range (Switzerland)
Shaker incubator	Sanyo (Japan)
Soxhlet	Electrothermal (England)
Spectrophotometer	Apel (Japan)
Vortex	Stuart Scientific Co. Ltd. (England)
Water bath	Gallenkamp (England)

2.1.2 Chemicals

Chemicals used in this study were classified according to the manufacturing companies, as follow:

Chemicals	Company (Origin)
2,4-Dichlorophynoxyacetic acid, Agar-agar, Calcium Carbonate, Cobalt chloride.6H_2O, Cupric Sulphate.5H_2O, Ethanol, Ferrous sulphite.7H_2O, Glucose, Glycerol, Glycine, Hydrochloric acid, Iodine, Lactose, Lead acetate, Maltose, Mannitol, Manganese Sulphate.4H_2O, Mercury chloride, Molbdic acid (sodium salt).2H_2O, Myoinositol, Peptone, Potassium Nitrate, Pyridoxine.HCl, Raffinose, Sodium Chloride, Sodium Hydroxide, Sucrose, Thiamine.HCl, Xylose, Zinc Sulphate.7H_2O.	BDH (England)
Meat extract, Yeast extract.	Biolife (Italy)
Acetic hydride, Calcium chloride anhydrate, Crystal violet, Ferric chloride, Hydrogen peroxide, Magnesium sulphate anhydrate, Magnesium sulphate (hydrate), Manganese sulphate (hydrate), Pteroleum ether, Potassium phosphate monobasic, Sodium ethylene diamin tetraacetate, Sulphuricacid.	Fluka (Switzerland)
Boric acid, Sodium acetate trihydrate, Triammonium citrate.	Merek (Germany)
Tween-80.	Sigma (U.S.A.)

2.1.3 Culture media

2.1.3.1 Ready to use (powdered) media

Media	Company (Origin)
Blood Agar Base	Oxoid (England)
Brain Heart Infusion Agar	Oxoid (England)
Litmus Milk Broth	Biolife (Italy)
Nutrient Agar	Biolife (Italy)
Nutrient Broth	BDH (England)

2.1.3.2 Laboratory-prepared media

The following media were prepared in the laboratory:

* Fermentation Medium
* Gelatin Medium
* Man-Rogosa-Sharp (MRS) broth
* MRS-$CaCO_3$ agar
* Murashige and Skoog Medium, 1962 (MS)
* Starch agar

2.1.4 Stains

Stain	Company (Origin)
Crystal violet	Fluka (Germany)
Safranin	Fluka (Germany)

2.1.5 Solutions and reagents

2.1.5.1 Ready to use solutions and reagents:

Solutions and Reagents	Company (Origin)
Fehling's Reagent	Fluka (Germany)
Phenol Red Reagent	Fluka (Germany)
Catalase Reagent	Local market
Kovacs Reagent	BDH (England)
Mayer's Reagent	Fluka (Germany)

2.1.5.2 Laboratory prepared solutions and reagents:

2.1.5.2.1 Laboratory prepared solutions and reagents

- **Normal saline solutions**

It was prepared by dissolving 0.85g of NaCl in 100ml of distilled water. The pH was adjusted to 7.0 and then sterilized by autoclaving as in (2.2.2.1) (Atlas *et al.*, 1995).

- **Oxidase reagent (Baron *et al.*, 1994)**

A solution of (1%) N, N, N, N-tetramethyl-p-phnylene dihydrochloride was prepared in sterile distilled water was used for oxidase production.

- **Phosphate buffer saline (PBS) pH 7.2**

It was prepared as mentioned by Atlas *et al.*, (1995) as follows:

Solution (A): Dissolving 13.9g of sodium phosphate dihydrate (NaH_2PO_4) in 1000ml of distilled water.

Solution (B): Dissolving 17.79g of sodium phosphate monohydrate ($NaHPO_4$) in 1000ml of distilled water. While the final solution was prepared by adding 13ml of solution (A) and 87ml of solution (B) and 100ml of distilled water, the final volume was 200ml. Then it was sterilized by autoclaving as in (2.2.2.1).

2.1.6 Synthetic soft contact lenses

Synthetic soft contact lenses (By Fusion^{TM}, made in USA) were purchased from pharmacies.

2.1.7 Rabbits

Fourteen rabbits (local and albino) from either sex, (6-8) months of age weighing approximately (1.5-2Kg) were obtained from the National Center for Drug Control and Research, Baghdad.

2.1.8 Plant material

Chamomile (*C. recutita*) dried flowers were purchased from local market. Seeds were obtained from the dried flowers, sterilized and cultured on the nutrient medium.

2.1.9 Bacterial isolates

A total of 25 vaginal samples were obtained from healthy premenopausal women in Kamal Al-Samarai hospital, Baghdad for isolation and identification of *L. acidophilus* by the gynecologist doctor**.**

The target pathogenic bacteria (*S. aureus* and *P. aeruginosa*) which are isolated from wound infections were obtained from Al-Nahrain University, Biotechnology department.

2.2 Methods

2.2.1 Sterilization of seeds (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.

2.2.2 Sterilization (Baily *et al.*, 1999)

Three methods of sterilization were used as required:

2.2.2.1 Moist heat sterilization

Media and solutions were sterilized by autoclaving at 121°C ($1.04kg/cm^2$) for 15 minutes unless otherwise mentioned.

2.2.2.2 Dry heat sterilization

Electric oven was used to sterilize glass-wares and other instruments at 160-180°C for 2-3 hrs.

2.2.2.3 Filtration

Millipore filters (0.22µm) were used to sterilize some sugars, chamomile extracts and surlactin.

2.2.3 Media preparation

2.2.3.1 Ready to use powdered media

The media listed in (2.1.3.1) were prepared according to the information fixed on their containers by the manufacturer. After pH was adjusted, they were sterilized in the autoclave as in (2.2.2.1) unless otherwise stated.

2.2.3.2 Laboratory prepared media

2.2.3.2.1 Fermentation medium

It was prepared according to Forbes *et al.*, (1998) by using sterilized MRS broth after substituting glucose and meat extract by 1% of each of the autoclaved sugars (lactose, fructose, raffinose, maltose, sucrose and mannitol) or filtrated sugar (xylose). After adding 0.004% of chlorophenol red reagent, the pH was adjusted to (6.2-6.5).

2.2.3.2.2 Gelatin medium (Baily *et al.*, 1999; Baron *et al.*, 1994)

Gelatin 12% (w/v) was dissolved in MRS broth medium, and then sterilized by autoclaving as in (2.2.2.1).

2.2.3.2.3 Man-Rogosa-Sharp broth medium (MRS)

This medium was prepared as described by Deman *et al.*, (1960) by dissolving the following ingredients:

Peptone	**10g**
Beef extract	**10g**
Yeast extract	**5g**
Glucose	**20g**
Tween-80	**1ml**
K2HPO4	**2g**
Sodium acetate hydrate	**5g**
Triammonium citrate	**2g**
MgSO4.7H2O	**200mg**
MnSO4.4H2O	**50mg**

All dissolved in 1000ml of distilled water. Then pH was adjusted to 6.0 before autoclaving.

2.2.3.2.4 MRS-$CaCO_3$ agar (1988 ،القصاب)

This medium contains all MRS broth components plus 1.5% (w/v) agar and 1gm of $CaCO_3$ dissolved in 1000ml of distilled water.

2.2.3.2.5 Murashige and Skoog, 1962 (MS) Medium

MS medium was prepared and used (Table 2). Sucrose 30g/l, Myoinositol 100mg/l. Plant growth regulators (2,4-D and BA) at different concentrations were added. The pH was adjusted to 5.8 using 1N NaOH or 1N HCl, and then 7g/l of the agar type (Agar-Agar) was added to the medium, placed on a hotplate magnetic stirrer till boiling.

All dissolved in 1000ml of distilled water and then sterilized by autoclaving as in (2.2.2.1).

Table (2): MS culture medium components (Murashige and Skoog, 1962).

Components	Chemical formula	Weight (mg/l)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride hydrate	$CaCl_2.2H_2O$	440
Magnesium sulphate hydrate	$MgSO_4.7H_2O$	370
Potassium phosphate monobasic	KH_2PO_4	170
Micronutrients		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Manganese sulphate.4Hydrate	$MnSO_4.4H_2O$	22.30
Zinc sulphate.$7H_2O$	$ZnSO_4.7H_2O$	8.60
Molybdicacid(sodiumsalt).$2H_2O$	$Na_2MoO_4.2H_2O$	0.25
Cupric sulphate.$5H_2O$	$CuSO_4.5H_2O$	0.025
Cobalt chloride.$6H_2O$	$CoCl_2.6H_2O$	0.025
Chelated Iron		
Sodiumethylenediaminetetraacetate	Na_2EDTA	37.3
Ferrous sulphate.$7H_2O$	$FeSO_4.7H_2O$	27.8
Vitamins		
Thiamine.HCl (B1)	$C_{12}H_{17}C1N_4OS.HCl$	0.1
Nicotinic acid (free acid) (B3)	$C_8H_{11}NO_3$	0.5
Pyridoxin.HCl (B6)	$C_6H_5NO_2.HCl$	0.5
Glysine (free base)	$C_2H_5NO_2$	2.0
myoinositol	$C_6H_6(OH)_6$	100

Aliquots of 10ml were dispensed into (8×2.5) cm culture vessels. They were left to cool at room temperature and become ready to culture seeds.

2.2.3.2.6 Starch agar (Atlas *et al.*, 1995)

This medium was prepared by dissolving 10g of soluble starch, 3g of beef extract and 12g of agar in 1000ml of distilled water, gently heated and brought to boiling, then autoclaved as in (2.2.2.1).

2.2.4 Plant growth regulators (Zhao *et al.*, 2001)

Different concentrations of the auxin 2,4-D (0.0, 0.1, 0.5 or 1.0) mg/l and the cytokinin BA (0.0, 0.5, 1.0 or 2.0) mg/l were prepared and added to the MS medium as required before autoclaving.

2.2.5 Incubation of chamomile 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 inflorescent at light intensity of 1000 lux (Ramawat, 2008).

2.2.6 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 2,4-D and BA as in (2.2.4). Cultures were placed in the incubator at 25°C for 16/8 hrs light/dark. 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 (Ramawat, 2008).

2.2.7 Maintenance of callus cultures

The initiated callus was removed from explants using sterilized forceps and scalpel, pieces were weighed using a sensitive balance, then pieces weighing approximately 50mg were subcultured onto fresh medium supplemented with the same combinations of 2,4-D and BA as in (2.2.4). Callus fresh weight was determined using a sensitive balance then oven dried at 40°C for 24hrs for callus dry weight measurements (Bos, 1997).

2.2.8 Preparation of flower extracts for antibacterial activity

2.2.8.1 Water extract (Swanston *et al.*, 1990)

Dried flowers were macerated with DDH_2O in a ratio 1:5 (w/v), (50g) of the powder was mixed with 250ml DDH_2O. The mixture was left in a shaker incubator for 24hrs at room temperature.

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

2.2.8.2 Ethanolic extract (Harborne, 1973)

A quantity of (50g) of flowers powder was mixed with 250ml of 75% ethanol by soxhlet apparatus for 6hrs 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.

2.2.9 Preparation of callus extracts for antibacterial activity

The water and ethanolic extracts of callus that originally initiated from seed explants grown on MS medium were prepared for antibacterial activity as follows:

2.2.9.1 Water extract (Swanston *et al.*, 1990)

The same method which was used for flowers extraction was followed for callus extraction. The dried samples (10g) were soaked with 50ml DDH_2O.

The mixture was left in a shaker incubator for 24hrs and then filtered through a filter paper (Whatman no. 1). The filtrate was concentrated using a rotary evaporator at 40°C until dryness.

2.2.9.2 Ethanolic extract (Harborne, 1973)

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

2.2.10 Detection of some active compounds of chamomile

2.2.10.1 Detection of tannins (Shihata, 1951)

A quantity of (10g) of the flowers powder was mixed with (50ml) DDH_2O using a magnetic stirrer. The mixture was left till boiling in a water bath for a few minutes, and 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.

2.2.10.2 Detection of saponins (Stahl, 1969)

Aliquot of 5ml crude flower water extract was added to (1-3)ml of mercury chloride solution. A white precipitate was developed indicating the presence of saponins.

2.2.10.3 Detection of flavonoids (Harborne, 1973)

Flower ethanolic extract was partitioned with petroleum ether. The aqueous layer was mixed with the ammonia solution. The appearance of dark color was an indication for the presence of flavonoids.

2.2.10.4 Detection of glycosides (Shihata, 1951)

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

2.2.10.5 Detection of terpenes (Al-Abid, 1985)

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

2.2.10.6 Detection of alkaloids (Stahl, 1969)

A quantity of (10g) of powdered flowers was added to 50ml of 4% HCl in a steam bath, and then 1ml of the filtrate was treated with Mayer's reagent. The appearance of white precipitate was an indication for the presence of alkaloids.

2.2.10.7 Detection of coumarins (Geissman, 1962)

A little amount of the dried water flower 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 (336 nm). The appearance of greenish-yellow color indicated the presence of coumarins.

2.2.10.8 Detection of resins (Shihata, 1951)

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

2.2.10.9 Detection of phenols (Harborne, 1973)

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

2.2.11 Determination of minimum inhibitory concentration (MIC) of chamomile extracts (Ericsson and Sherris, 2000)

The minimum inhibitory concentration was determined by assaying the ability of bacteria (*P. aeruginosa* and *S. aureus*) to grow in broth cultures containing different concentrations of chamomile flower extracts (water and ethanolic extracts). The following dilutions 0:10; 1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1 were prepared in test tubes as water extracts: nutrient broth. They were inoculated with 0.1ml of pathogenic test bacteria (1×10^5 CFU/ml) then incubated at 37°C for 24hrs. Growth intensity of each tube was observed by inoculation on nutrient agar and incubation at 37°C for 24hrs and then recorded as light (+), medium (++), heavy (+++) and no growth (-). Growth was estimated by measuring optical density (O.D 600) nm for each dilution. Results were matched with growth intensities mentioned by Midolo *et al.*, (1995). The same procedure was repeated for ethanolic extracts. The lowest concentration of the extract that prevented the growth of pathogenic bacteria was considered as the minimum inhibitory concentration.

2.2.12 Determination of the antibacterial activity of chamomile extracts *in vitro* (Nathan, 1978)

The activity of chamomile extracts was determined against pathogenic bacterial isolates (*S. aureus* and *P. aeruginosa*) *in vitro* by using agar diffusion method.

For water extracts, the stock solution was prepared by dissolving (5g) of plant extract residue with 50ml sterile DDH_2O. The extracts were prepared at different concentrations (20, 40, 60 and 80) mg/ml.

Brain Heart agar diffusion medium was mixed well and 20ml poured in Petri-dishes. The medium was inoculated with 0.1ml of (1×10^5 CFU/ml) target isolates of (*P. aeruginosa* and *S. aureus*) by using sterile swabs.

Five evenly spaced wholes 3mm in diameter were made in the agar of each plate with a sterile cork borer. To identify the intrinsic activity of extracts (water and ethanolic for flower and callus cultures), one control well was filled with (100µl) PBS.

Equal volumes (100µl) of different concentrations (20, 40, 60 or 80) mg/ml of the extracts were dispensed into each well (four replica plates were prepared for each agent).

Test plates were then incubated at 37°C for 24hrs 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 three times. The results were compared to positive control where antibiotic solution (chloramphenicol) was used against the pathogenic bacteria.

2.2.13 Isolation of *Lactobacillus acidophilus*

L. acidophilus isolates were isolated from the vagina of healthy premenopausal women by the gynaecologist doctor in Kamal Al-Samarai hospital, Baghdad.

2.2.14 Identification of *Lactobacillus acidophilus* isolates

2.2.14.1 Microscopic examination (Harely and Prescott, 1996)

A loop full of suspected bacteria was fixed on a microscopic slide, and then stained with Gram stain to examine cells shape, Gram reaction, grouping and non-spore forming phenomena.

2.2.14.2 Biochemical tests

2.2.14.2.1 Catalase test (Atlas *et al.*, 1995)

A loop full of each of the suspected isolates was transferred to a sterile glass slide and (2-3) drops of (3%) H_2O_2 was added. Positive result was observed through formation of gas bubbles indicating the ability of bacteria to produce catalase enzyme.

2.2.14.2.2 Oxidase test (Atlas *et al.*, 1995)

A clump of suspected colonies from bacterial growth was picked up with a sterile wooden stick and smeared on a filter paper and was moistened with a few drops of freshly prepared oxidase reagent. An immediate color change to deep blue indicates a positive result.

2.2.14.2.3 Gelatinase test (Baron *et al.*, 1994)

Gelatin agar medium was used to detect gelatin liquefaction in tubes, by stabbing 1% of suspected isolates, and incubation at 37°C for 48hrs. After that, they were placed in the refrigerator at 4°C for 30 min. This test was performed to demonstrate the ability of isolates to hydrolyze gelatin.

2.2.14.2.4 Acid production and clot formation test (Kandler and Weiss, 1986)

Tubes containing 10ml of litmus milk medium were inoculated by 1% of the suspected bacterial culture and then incubated at 37°C for 48 hrs to detect color change. Curd production and pH decrease indicating positive result.

2.2.14.2.5 Carbohydrate fermentation test (Atlas *et al.*, 1995)

Tubes containing fermentation media were inoculated with 1% of suspected bacterial isolates and incubated with the positive control tube (fermentation medium only) and the negative control tube (containing MRS broth) at 37°C for 5 days. Change of the color to red indicates (alkality) while yellowish indicates (acidity). Production of CO_2 is considered as positive result.

2.2.14.2.6 Growth on nutrient agar (Atlas *et al.*, 1995)

Bacterial isolates were cultured on nutrient agar then incubated at 37°C for 24 hrs, growth is considered a positive result.

2.2.14.2.7 Growth at 45°C and 15°C

Tubes containing 10ml MRS broth were inoculated with 1% of suspected bacterial isolate then incubated at 15°C and 45°C for 24 hrs. After incubation at 37°C growth was observed in the tubes and compared with control ones.

2.2.15 Maintenance of bacterial isolates

Maintenance of bacterial isolates was performed according to Conteras *et al.*, (1997) as follows:

2.2.15.1 Daily working culture

Culture media broth were inoculated with bacterial isolates and incubated at 37°C for 24hrs. The tubes were then kept in refrigerator at 4°C and activated weekly.

2.2.15.2 Stock culture

Bacterial isolates were cultured in broth culture media for 24 hrs at 37°C. Then 1ml of fresh preparation of bacterial growth was added to Bejo bottles containing 20% glycerol, and then stored at -20°C.

2.2.16 Preliminary detection of *Lactobacillus acidophilus* ability to produce surlactin

The method of Velraeds *et al.*, (1998) was used to separate the surlactin from other metabolites produced by *L. acidophilus* (isolates 1 and 13) especially in the logarithmic phase including bacteriocins, hydrogen peroxide and enzymes.

This method involves preparing activated culture of *L. acidophilus* in MRS broth (prepared as in 2.2.3.2.3) for 18hrs at 37°C with (5-10)% CO_2.

Then it was centrifuged at 6000 rpm at 4°C for 30 min., supernatant was discarded, the cells precipitate was suspended in PBS then washed twice by sterile distilled water, the final precipitate was suspended in 2ml of PBS and left in the incubator at 25°C for 2 hrs. Thereafter, it was centrifuged at 6000 rpm at 4°C for 10 min. The supernatant was taken and the biological activity of surlactin was measured as follows:

2.2.16.1 Inhibition of biofilm adhesion in test tubes

P. aeruginosa was used as a target to study the activity of surlactin. A modified method of Christensen *et al.*, (1982) was used to detect the biofilm production by culturing 5ml of nutrient broth with *P. aeruginosa* and incubating at 37°C for 48hrs, the contents of the tube were discarded carefully and 1% of crystal violet was added to the tube and left for 15min

and then the dye was removed and the tubes were left to dry at room temperature (20-25) °C. Formation of biofilm as a layer on the inner membrane of the tube was noticed by nacked eye in comparison with negative control (nutrient broth without *P. aeruginosa*).To determine the ability of *L. acidophilus* to produce surlactin by observing its influence in preventing the adhesion of biofilm of the producing bacteria in test tubes:

250µl of surlactin extracts (prepared as in 2.2.16) was added to 5ml of activated culture of *P. aeruginosa* for 18hrs grown in nutrient broth. The combination was incubated at 37°C for (24-48)hrs. The contents of the tubes were discarded very carefully and tubes were stained with 1% crystal violet. Results were observed by naked eye in comparison to negative control (without *P. aeruginosa*) and to positive control (with *P. aeruginosa* but without surlactin).

2.2.17 Extraction and purification of surlactin produced by *Lactobacillus acidophilus*

The procedure of Velraeds *et al.*, (1998) was used to extract and purify surlactin from *L. acidophilus*. The isolates (1 and 13) were reactivated in MRS broth at 37°C for 18hrs in the presence of (5-10) % CO_2, and then centrifuged at 6000rpm for 30 min at 4°C. One ml of the precipitated bacterial cells were inoculated in 25ml of MRS broth and incubated in the same conditions with shaking at 160 rpm for 18hrs in order to reach the logarithmic phase. Then the culture was centrifuged at 6000 rpm for 30 min at 4°C. The precipitate was washed twice with PBS and left to suspend for two hrs at 25°C with light stirring. Then the suspension was centrifuged at the same speed for 10 min. and the supernatant was filtered by Millipore

filter paper 0.22µm and the product was reserved to study its biological influences.

2.2.18 Biological and medical applications of surlactin

2.2.18.1 Testing antibacterial activity of surlactin

To detect the antibacterial activity of purified surlactin, agar diffusion method was used according to Nathan *et al.*, (1978) as follows:

Nutrient agar plates were prepared then inoculated with 0.1ml of (1×10^5 CFU/ml) of activated pathogenic bacteria (*S. aureus* and *P. aeruginosa*) by diffusion method and three replica plates were made for each isolate.

Three holes of 3mm in diameter were made with equal distances using sterilized cork borer. Equal volumes of (100µl) of surlactin, primary filtrate of *L. acidophilus* and chloramphenicol as positive control were added into the holes. The plates were incubated at 37°C for (18-24)hrs. Results were observed by the formation of inhibition zones around the holes.

2.2.18.2 Inhibition of pathogenic bacterial adhesion to the industrial contact lenses

The method of Kamil, (2005) was used to study effect of surlactin on the adhesion of pathogenic bacteria (*P. aeruginosa*) to the contact lenses. 10ml of nutrient broth containing surlactin (500µg/ml) was inoculated with 0.1ml of (1×10^5CFU/ml) of activated bacterial growth of *P. aeruginosa*. Sterilized contact lenses were added to the culture and left for 48hrs at 37°C then washed with weak flow of sterilized distilled water and left to dry at

room temperature. They were stained with 1% crystal violet for 15min. Lenses were rewashed to remove extra dye. Results were observed by naked eye in comparison with the control (contact lenses and bacterial culture without surlactin). Absorbency was measured at 550nm to determine the growth intensity of each case.

2.2.19 The antibacterial activity of chamomile callus ethanolic extract and surlactin (*in vitro*)

To detect the antibacterial ability of chamomile extract and purified surlactin together, agar diffusion method was used according to Nathan *et al.*, (1978) as follows:

Nutrient agar plates were prepared then inoculated with 0.1ml of (1×10^5 CFU/ml) activated pathogenic bacteria (*S. aureus* and *P. aeruginosa*) by diffusion method and three replica plates were made for each isolate.

Five holes of 3mm in diameter were made with equal distances using sterilized cork borer. Equal volumes of 50µl of chamomile callus ethanolic extract with different concentrations (20, 40, 60 and 80) mg/l were added with 50µl of surlactin into the holes, and the fifth well was filled with (100µl) of PBS as control. The plates were incubated at 37°C for (18-24) hrs. Results were observed by the formation of inhibition zones around the holes.

2.2.20 The effect of chamomile ethanolic extract and surlactin on bacteria causing eye infections in rabbits' eyes (*in vivo*) (George *et al.*, 1982)

Callus ethanolic extract (80mg/ml) was used since results showed that it was the most effective one among other extracts.

Fourteen rabbits were used in this experiment; they were classified into seven groups each group consisted of a male and a female.

Four injection samples were prepared:

Control Sample: Aliquot of 0.5ml of nutrient broth was mixed with 0.5ml PBS and 0.1ml of bacterial suspension (*P. aeruginosa* and *S. aureus*).

Sample (A): Aliquot of 0.5ml of callus ethanolic extract (80mg/ml) was mixed with 0.5ml of nutrient broth. Then 0.1ml of (1×10^5 CFU/ml) bacterial suspension (*P. aeruginosa* and *S. aureus*) was inoculated.

Sample (B): Aliquot of 0.5ml of surlactin was mixed with 0.5ml of nutrient broth and then 0.1ml of (1×10^5 CFU/ml) bacterial suspension (*P. aeruginosa* and *S. aureus*) was inoculated.

Sample (C): Aliquot of 0.5ml of callus ethanolic extract (80mg/ml) and 0.5ml of surlactin were mixed with 0.5ml of nutrient broth, and then 0.1ml of (1×10^5 CFU/ml) bacterial suspension (*P. aeruginosa* and *S. aureus*) was inoculated.

Four groups of rabbits were inoculated with 0.1ml of control sample, the 5^{th}, 6^{th} and 7^{th} groups were inoculated with 0.1ml of samples A, B and C respectively (right eye with sample containing *P. aeruginosa* and left eye with sample containing *S. aureus*).

Results were calculated after 24hrs of inoculation for all groups. Group 2 and 3 were administrated with 0.1ml of callus ethanolic extract

(80mg/ml) and surlactin respectively, while group 4 was administrated with 0.1ml of callus ethanolic extract (80mg/ml) and surlactin together, results were noticed daily.

2.2.21 Effect of chamomile callus ethanolic extract and surlactin in inhibiting the adhesion of pathogenic bacteria of industrial contact lenses

The method of Kamil, (2005) was used to study the effect of chamomile ethanolic extract and surlactin on industrial contact lenses. Ten ml of nutrient broth containing 0.5ml of surlactin and 0.5ml of chamomile callus ethanolic extract (80mg/ml) was inoculated with 0.1ml of (1×10^5 CFU/ml) activated *P. aeruginosa*. Sterilized eye lenses were added to the culture and left for 48hrs at 37°C then washed with weak flow of sterilized distilled water and left to dry for (10-15min) at room temperature (20-25)°C. They were stained with 1% crystal violet for 15min, lenses were rewashed to remove extra dye. Results were observed by naked eye in comparison with the positive control (eye lenses and bacterial culture without extracts) and negative control (eye lenses and extracts without bacterial culture). Absorbency was measured at 550nm to determine the growth intensity of each case.

2.2.22 Statistical analysis

A completely randomized design (CRD) was used. Least significant differences (LSD) of the means were calculated, means were compared at probability of ≤0.05 (Gomez and Gomez, 1984).

3.1 Sterilization of explants

Sodium hypochlorite (NaOCl) was used for explants sterilization (seeds) of *C. recutita*. Fig. (2) shows that the most effective concentration of NaOCl was 3% for 10 min that gave the highest percentage (100%) of non-contaminated explants. Increasing the concentration of NaOCl to 6% caused reduction in survival rate, whereas lowering the concentration led to high rate of contamination.

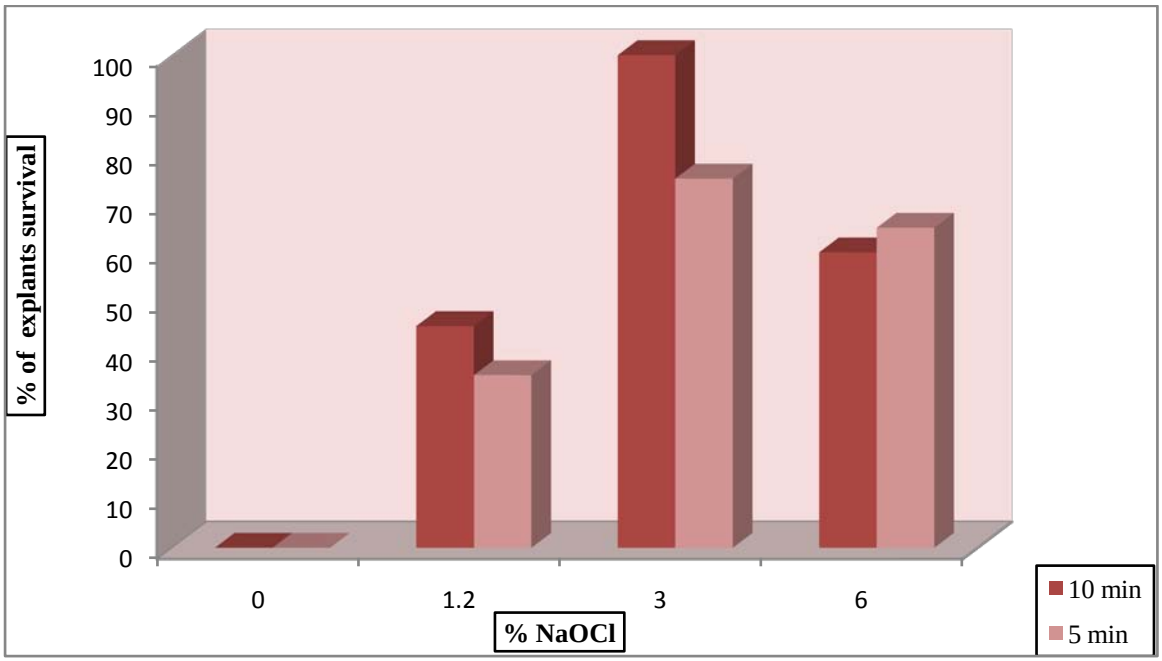


Fig. (2): Effect of different concentrations of NaOCl on survival of explants at sterilization periods of 5 and 10 minutes.

Treatment of explants with NaOCl was important to eliminate the contaminants. It is used widely in plant tissue sterilization. The selection of sterilization 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 growth rate. Optimization experiment is therefore necessary to achieve maximum survival rate with minimum contamination.

3.2 Induction of callus cultures

The effect of different concentrations of 2,4-D and BA on the response (%) of callus induction on seed explants is shown in table (3).

Table (3): Effect of different concentrations of 2,4-D and BA on the response (%) of callus induction on *C. recutita* seed explants, (n=4).

2,4-D (mg/l)	BA (mg/l)				Mean
	0.0	0.5	1.0	2.0	
0.0	**4.1**	**16.66**	**25**	**30**	**18.94**
0.1	**8.33**	**80**	**100**	**83.33**	**67.91**
0.5	**15**	**61.66**	**65**	**63.33**	**51.24**
1.0	**23.33**	**26.66**	**28.33**	**0.0**	**19.58**
Mean	**12.69**	**46.24**	**54.58**	**44.16**	
LSD ≤0.05	BA=10.2	2,4-D=13.1		BA×2,4-D=17.8	

n= number of replicates

All concentrations of BA led to a significant increase in the percentage of explants that showed callus induction (46.24, 54.58 and 44.16) at the concentrations (0.5, 1.0 and 2.0)mg/l respectively as compared with control treatment (12.69%). Addition of 2,4-D at 0.1mg/l led to a significant increase in the percentage of explants showed callus induction reaching (67.91%). Percentage of explants that initiated callus decreased dramatically when 2,4-D was added at 0.5 and 1.0mg/l reaching (51.24 and 19.58%) respectively.

The interaction between growth regulators achieved 100% response in a combination of 1.0mg/l BA and 0.1mg/l 2,4-D (Fig. 3) and 83.33% when BA concentration was increased to 2.0mg/l and 0.1mg/l 2,4-D. While no callus induction was reported at high concentrations of growth regulators.

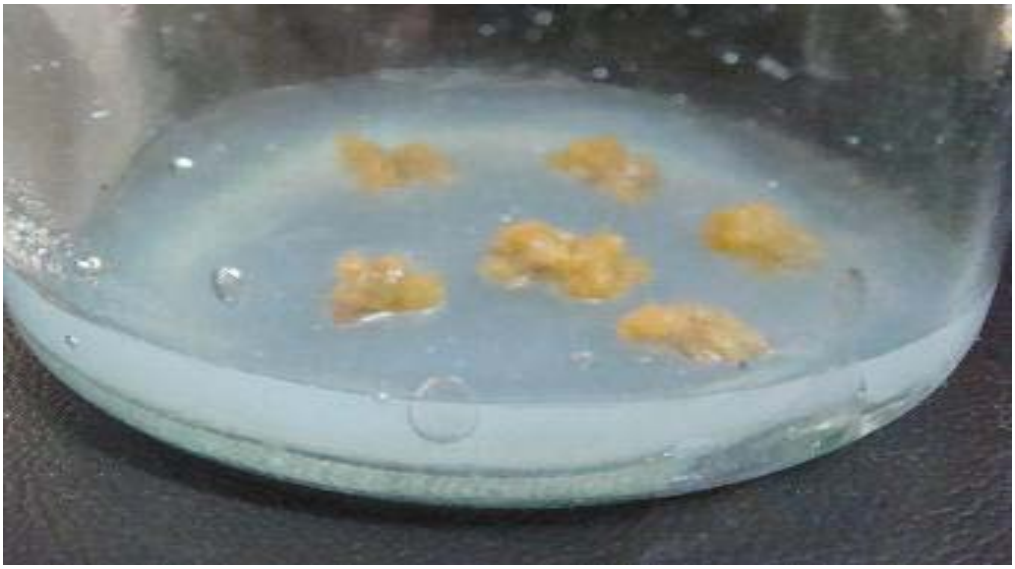


Fig. (3): Callus induction on seed explants of *C. recutita* grown on MS medium containing a combination of 1.0 mg/l BA and 0.1mg/l 2,4-D, 21 days after culture.

Callus induction requires a balanced ratio from auxin(s) and cytokinin(s) as stated by Skoog and Miller, (1957), but in a number of plant species, callus induction favors higher auxins than cytokinins. Seeds of *C. recutita* may contain some levels of endogenous auxins that made a balanced ratio with the exogenous auxin (Ramawat, 2008).

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 upon the physiological status of the explant cells as well as the cultural conditions including the appropriate combination of plant growth regulators.

3.3 Maintenance of callus cultures

All concentrations of BA led to a significant increase in callus fresh weight (431.75, 753 and 432.5mg) at the concentrations (0.5, 1.0 and 2.0mg/l) respectively compared with those not supplemented with BA (93.5mg) (Table 4).

Addition of 2,4-D at (0.1 or 0.5mg/l) caused a significant increase in callus fresh weight (872 and 517.5mg) respectively compared with cultures grown on 2,4-D free medium. Callus fresh weight decreased dramatically when concentration of 2,4-D increased to 1.0mg/l reaching (170)mg.

Table (4): Effect of different concentrations of 2,4-D and BA on callus fresh weight (mg) initiated on seed explants of *C. recutita*.(n=4).

2,4-D (mg/l)	BA (mg/l)				Mean
	0.0	0.5	1.0	2.0	
0.0	**50.0**	**105**	**250**	**200**	**151.25**
0.1	**98**	**800**	**1700**	**890**	**872**
0.5	**99**	**654**	**677**	**640**	**517.5**
1.0	**127**	**168**	**385**	**0.0**	**170**
Mean	**93.5**	**431.75**	**753**	**432.5**	
LSD ≤0.05	BA=51	2,4-D=62		BA×2,4-D=82	

n= number of replicates

The interaction between the two growth regulators resulted in maximum callus production reached (1700mg) at the combination of (1.0 and 0.1mg/l) of BA and 2,4-D respectively (Fig. 4). This combination was significantly higher than all other interactions.

All combinations were significantly higher than those lacking both growth regulators except the combination (2.0 and 1.0mg/l) of BA and 2,4-D respectively where both showed deterioration and death of callus cultures (0.0mg).

Table (5): Effect of different concentrations of 2,4-D and BA on callus dry weight (mg) initiated on seed explants of *C. recutita*. (n=4).

2,4-D (mg/l)	BA (mg/l)				Mean
	0.0	0.5	1.0	2.0	
0.0	**4.1**	**8.75**	**12.5**	**41.6**	**16.75**
0.1	**8.1**	**66.6**	**141.6**	**74.1**	**70.6**
0.5	**8.25**	**54.5**	**56.4**	**53.3**	**43.1**
1.0	**6.75**	**35.9**	**32**	**0.0**	**18.6**
Mean	**6.87**	**41.4**	**60.6**	**42.25**	
LSD ≤0.05	BA=4.7		2,4-D=7.5		BA×2,4-D=9.6

n= number of replicates

The highest dry weights of explants were exhibited in the combination of (1.0mg/l BA and 0.1mg/l 2,4-D) and (2.0mg/l BA and 0.1mg/l 2,4-D). These weights were (141.6 and 74.1mg) respectively which 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 (1.0mg/l BA and 0.1mg/l 2,4-D) 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, 2008).

3.4 Detection of some active compounds in chamomile flowers

Different chemical reagents and solutions were used for detecting various active compounds found in *C. recutita* flowers, which are displayed in table (6). The pH of water and ethanolic extracts was 6.26 and 5.76 respectively. Results obtained by chemical detection indicated the presence of flavonoids, tannins, resins, phenols, alkaloids, coumarins, glycosides and terpenes. Saponins were not detected. These results agree with Al-Azawi, (2007).

Table (6): Detection of some secondary metabolites in *C. recutita* flowers

Secondary Metabolite	Result of detection
Tannins	+
Saponins	-
Flavonoids	+
Glycosides	+
Terpenes	+
Resins	+
Phenols	+
Alkaloids	+
Coumarins	+

+= Presence of secondary metabolite - =Absence of secondary metabolite

3.5 Determination of MIC

Minimum inhibitory concentration (MIC), as defined by Atals *et al.*, (1995), is the least concentration that prevents the growth of bacteria after incubation at 37°C for 24 hrs.

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

3.5.1 MIC of water extracts

Table (7) displays MIC of water extract of chamomile flowers against two types of bacteria (*P. aeruginosa* and *S. aureus*). Results in the table showed that the first two concentrations of chamomile water extract (1:9 and 2:8) had no observed effect against the target bacteria but heavy growth of bacteria was noticed after incubation. Growth decreased at the following concentrations (3:7 and 4:6) for both bacterial types (*P. aeruginosa* and *S. aureus*).

At the concentration of 5:5, no growth for *S. aureus* was observed but *P. aeruginosa* showed light growth. The last four concentrations of water extract (6:4, 7:3, 8:2 and 9:1) were enough to retard any growth of *S. aureus* and *P. aeruginosa*.

Table (7): Minimum inhibitory concentration (MIC) of *C. recutita* flowers' water extract against tested bacteria.

Type of bacteria	Concentration of extract (extract:medium)									
	0:10	**1:9**	**2:8**	**3:7**	**4:6**	**5:5**	**6:4**	**7:3**	**8:2**	**9:1**
P. aeruginosa	+++	+++	+++	+++	++	+	-	-	-	-
S. aureus	+++	+++	+++	++	+	-	-	-	-	-

- = no growth, + = light growth,++ = medium growth, +++ = heavy growth

3.5.2 MIC of ethanolic extracts

Table (8) shows MIC of the ethanolic extract of chamomile flowers against two bacterial types (*P. aeruginosa* and *S. aureus*). Results in the table indicated that the first two concentrations of chamomile ethanolic extract (1:9 and 2:8) had no effect against tested bacteria while heavy growth of these bacteria was noticed after incubation. However, the growth was reduced at the following concentrations (3:7 and 4:6) for both bacteria.

At the concentrations (5:5) no bacterial growth was observed. The last four concentrations (6:4, 7:3, 8:2 and 9:1) were sufficient to cease the growth of *P. aeruginosa* and *S. aureus*.

Table (8): Minimum inhibitory concentration (MIC) of *C. recutita* flowers' ethanolic extract against tested bacteria.

Type of bacteria	Concentration of extract (extract:medium)									
	0:10	**1:9**	**2:8**	**3:7**	**4:6**	**5:5**	**6:4**	**7:3**	**8:2**	**9:1**
P. aeruginosa	+++	+++	+++	++	+	-	-	-	-	-
S. aureus	+++	+++	+++	++	+	-	-	-	-	-

- = no growth, + = light growth, ++ = medium growth, +++ = heavy growth

3.6 Determination of the antibacterial activity of dried chamomile flowers and callus *in vitro*

Water and ethanolic extracts of dried chamomile flowers and dried callus were tested for their antibacterial activity against (*P. aeruginosa* and *S. aureus*).

3.6.1 Flower extracts

3.6.1.1 Water extract

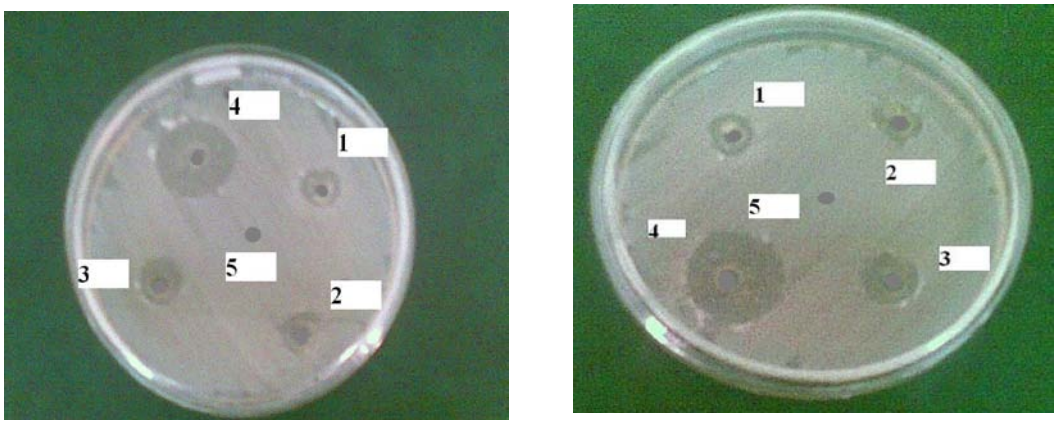
Results displayed in table (9) indicate that high concentrations of dried flowers' water extract (60 and 80mg/ml) had an inhibitory effect against Gram positive bacteria (*S. aureus*) with (14 and 15)mm inhibition zones diameter respectively, while 9mm was recorded at the concentration (40mg/ml). Very slight inhibition was noticed at the concentration (20mg/ml) with 8mm (Fig. 4-A).

The inhibitory activity against Gram negative bacteria (*P. aeruginosa*) was less. The highest concentrations of the water extract (60 and 80mg/ml) had an inhibitory effect with (12 and 14)mm inhibition zones diameter respectively, 7mm was recorded at the concentration (40mg/ml), while 5mm was recorded at (20mg/ml) (Fig. 4-B).

Table (9): Diameter of inhibition zones caused by *C. recutita* flowers' water extracts at various concentrations on Gram positive and negative bacteria.

Diameter of inhibition zone (mm) of bacterial isolates ±S.E.	Concentration (mg /ml)				
	20	40	60	80	Control
P. aeruginosa	**5±0.4**	**7±0.5**	**12±1**	**14±0.2**	**0.0**
S. aureus	**8±0.6**	**9±1**	**14±0.5**	**15±0.9**	**0.0**

Values= mean of 3 replicates ± S.E.



A **B**

Fig. (4): Effect of *C. recutita* flowers' water extract on the growth of A- *S. aureus* and B- *P. aeruginosa*.

1= 20mg/ml, 2=40mg/ml, 3=60mg/ml, 4=80mg/ml, 5= control (PBS).

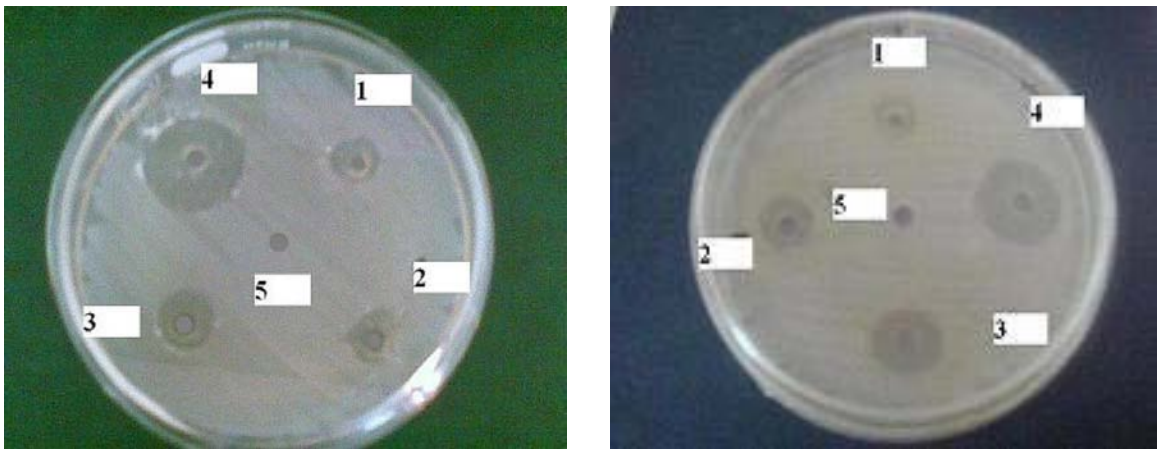
3.6.1.2 Ethanolic extract

Chamomile ethanolic extract exhibited an antibacterial activity against tested bacteria (*P. aeruginosa* and *S. aureus*) as shown in table (10). The diameter of the inhibition zone against *S. aureus* was (18mm) at the concentration (80mg/ml), whereas it decreased to (14.5mm) at the concentration (60mg/ml). Lower concentrations of the extracts (20 and 40)mg/ml against *S. aureus* showed (10 and 12)mm respectively (Fig. 5-A). *P. aeruginosa* displayed inhibition zone of (16mm) in diameter at the concentration (80mg/ml) and (13.5mm) at the concentration (60mg/ml), whereas the diameter of the inhibition zones decreased to (9.5 and 11)mm at the concentrations (20 and 40)mg/ml respectively (Fig 5-B).

Table (10): Diameter of inhibition zones caused by *C. recutita* flowers' ethanolic extracts at various concentrations on Gram positive and negative bacteria.

Diameter of inhibition zone (mm) of bacterial isolates ±S.E.	Concentration (mg/ml)				
	20	40	60	80	Control
P. aeruginosa	**9.5±1**	**11±0.4**	**13.5±0.5**	**16±0.5**	**0.0**
S. aureus	**10±0.4**	**12±0.5**	**14.5±0.5**	**18±0.4**	**0.0**

Values= mean of 3 replicates ± S.E.



A **B**

Fig. (5): Effect of *C. recutita* flowers' ethanolic extracts on the growth of A- *S. aureus* and B- *P. aeruginosa*.

1= 20mg/ml, 2= 40mg/ml, 3= 60mg/ml, 4= 80mg/ml, 5= control (PBS).

3.6.2 Callus extracts

3.6.2.1 Water extract

Water extract of chamomile callus (Table 11) showed antibacterial action against tested bacteria (*P. aeruginosa* and *S. aureus*) at the concentrations (60 and 80)mg/ml. The largest inhibition zones were observed against *S. aureus* recording (18.5mm) in diameter at (80mg/ml) while decreased to (15mm) at the concentration (60mg/ml). Lower concentrations of extracts (20 and 40)mg/ml against *S. aureus* displayed inhibition zones of (11 and 12.5)mm in diameter respectively (Fig. 6-A). Inhibition zones at the concentrations (60 and 80)mg/ml against *P. aeruginosa* were (13.5 and 16.5)mm in diameter, while at the concentrations (20 and 40)mg/ml the inhibition zones recorded were (10 and 11)mm in diameter (Fig. 6-B).

Table (11): Diameter of inhibition zones caused by *C. recutita* water extract of callus cultures at various concentrations on Gram positive and negative bacteria.

Diameter of inhibition zone (mm) of bacterial isolates ±S.E.	Concentration (mg/ml)				
	20	40	60	80	Control
P. aeruginosa	**10±0.4**	**11±0.5**	**13.5±0.8**	**16.5±0.4**	**0.0**
S. aureus	**11±0.5**	**12.5±0.3**	**15±0.2**	**18.5±0.5**	**0.0**

Values= mean of 3 replicates ± S.E.

A **B**

Fig. (6): Effect of *C. recutita* callus water extract on the growth of A- *S. aureus* and B- *P. aeruginosa*.

1= 20mg/ml, 2= 40mg/ml, 3= 60mg/ml, 4= 80mg/ml, 5= control (PBS).

These results agree with Korting and Schafer-Corting, (1993) who regarded Gram positive bacteria especially *S. aureus* sensitive to *C. recutita* water extract, and Gram negative bacteria especially *P. aeruginosa* 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 100µg/ml. α-bisabolol and its spiro-ether derivatives were effective antibacterial agents (Smith, 2006).

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

The resistance of Gram negative bacteria could be due to the permeability barrier provided by cell wall (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*.

3.6.2.2 Ethanolic extract

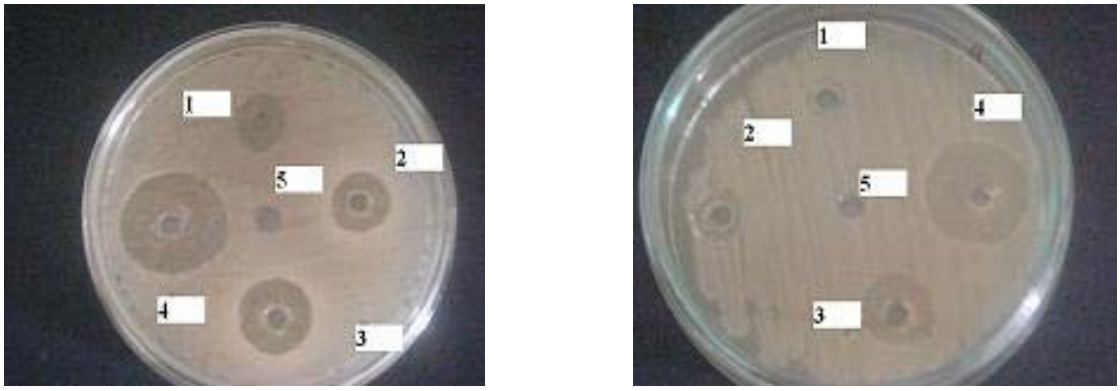
Results displayed in (Table 12) confirmed the activity of chamomile ethanolic extract of callus cultures against tested bacteria. The callus ethanolic extracts (60 and 80)mg/ml have inhibitory effects against *S. aureus* with (16 and 25)mm inhibition zones diameter respectively, and showed (11 and 12.5)mm inhibition zones diameter at concentrations (20 and 40)mg/ml respectively (Fig. 7-A). While *P. aeruginosa* showed (14 and 21)mm inhibition zones diameter at the concentrations (60 and 80)mg/ml respectively, and showed (10 and 11.5)mm inhibition zones diameter at the concentrations (20 and 40)mg/ml (Fig. 7-B).

Table (12): Diameter of inhibition zones caused by *C. recutita* ethanolic callus extracts at various concentrations on Gram positive and negative bacteria.

Diameter of inhibition zone (mm) of bacterial isolates ±S.E.	Concentration (mg/ml)				
	20	40	60	80	Control
P. aeruginosa	**10±0.5**	**11.5±1.5**	**14±1**	**21±0.5**	**0.0**
S. aureus	**11±0.4**	**12.5±0.2**	**16±0.6**	**25±0.5**	**0.0**

Values= mean of 3 replicates ± S.E.

It appears that ethanolic extracts are more efficient than water extracts. The reason may be due to the compounds extracted by ethanol particularly flavonoids and terpenes. These results agree with Al-Naymi, (2005) who reported that ethanolic extracts of *C. recutita* flowers have higher activity than water ones.



A **B**

Fig. (7): Effect of *C. recutita* ethanolic extracts on the growth of A- *S. aureus* and B- *P. aeruginosa*.
1= 20mg/ml, 2= 40mg/ml, 3= 60mg/ml, 4= 80mg/ml, 5= control (PBS).

It is clear from the data presented in tables (9, 10, 11 and 12) that *S. aureus* was more susceptible than *P. aeruginosa*. Furthermore, our results are in agreement with Mann and Staba, (1986) who showed that *C. recutita* water and ethanolic extracts are widely used as anti-inflammatory and antibacterial activity.

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

Although *C. recutita* contains many active compounds, most studies attributed the antimicrobial activity in chamomile to terpene compounds (Mann

and Staba, 1986; Der Mardersian and Liberti, 1988; Tyler, 1999; Gyllenhaal, 2000).

The results from (3.6.1 and 3.6.2) were compared with the positive control (chloramphenicol solution 0.05mg/ml) which had an antibacterial activity against the tested becteria (*P. aeruginosa* and *S. aureus*) represented by inhibition zones of (6 and 77mm) in diameter respectively (Fig. 8-A, B) indicating that chamomile callus ethanolic extract is more effective against *P. aeruginosa* while the antibiotic solution is far more effective than callus ethanolic extract against *S. aureus*. Al-Naymi, (2005) suggested that the activity of *C. recutita* could be attributed to the existence of chamazulen, α-bisabolol (sesquiterpenes) that showed high inhibitory activity against *S. aureus, S. epidermidis, St. pyogenes, St. pneumoniae* and *Candida albicans*.

A **B**

Fig. (8): The effect of chloramphenicol solution (0.05mg/ml) on the growth of A- *S. aureus* B- *P. aeruginosa*.

Essawi and Srour (2000) mentioned that well diffusion method is preferred over disk diffusion one; therefore, this method was used for this purpose.

3.7 Isolation of *Lactobacillus spp.*

From a total of 25 vaginal swabs collected from healthy premenopausal women from Kamal Al-Samarai hospital, Baghdad, 20 of them gave pale, round shape, soft, mucoid colonies on MRS agar as the first isolation step. Such result coincides with De Man *et al.*, (1960) who suggested that MRS medium is the most selective medium for *Lactobacillus spp.* In the second step, they had the ability to form clear zones around the colonies when cultured on MRS agar containing 1% $CaCO_3$, due to the acid produced by the isolates which dissolved the $CaCO_3$. To isolate *L. acidophilus* from other species, third step was applied by growing on MRS agar containing raffinose. Results showed that only *L. acidophilus could* grow due to its ability to utilize raffinose in this medium (Holt and Krieg, 1986).

According to the results above, three isolates were obtained which used in further experimental work represented 80% of the total number of isolates. These results agree with Velraeds *et al.*, (1996) since they found that lactic acid bacteria comprise (50-90)% of the total vaginal isolates in healthy women and this was confirmed by Lawson and Reid, (2001) who proved that *Lactobacillus spp.* are dominant over other bacterial types that comprise normal vaginal flora in women capable of giving birth.

3.8 Identification of *Lactobacillus* species

3.8.1 Cultural characteristics

When grown on MRS agar containing 1% $CaCO_3$, suspected *Lactobacillus* isolates produced colonies surrounded by clear zones.

Colonies were white to pale in color, round, soft, mucoid, convex and having smooth edges. Such cultural characteristics are concerned with those of *Lactobacillus* species (Kandler and Weiss, 1986).

3.8.2 Morphological characteristics

Microscopical examination after Gram staining demonstrated that suspected *Lactobacillus* isolates were Gram positive, short or long bacilli, grouped in long and short chain containing (3-8) cells but sometimes are single, non-spore former and non-motile. So they are related to the *Lactobacillus spp*. (Atlas *et al.*, 1995).

3.8.3 Biochemical tests

Biochemical tests shown in table (13) indicated that suspected isolates were able to produce clot when grown on litmus milk medium and change its color leading to decrease the pH from 6.5 to 4.5. Furthermore, all suspected isolates gave negative results for the catalase test when no bubbles were observed after addition of hydrogen peroxide to the colonies. The isolates also gave negative results for both oxidase and gelatinase tests and all of the isolates had the ability to hydrolyze starch when grown on starch agar medium. Moreover, all isolates were unable to grow on nutrient agar. Some isolates were able to grow in 45°C, while others were able to grow at 15°C (Holt and Kreig, 1986).

Table (13): Biochemical tests carried out for the identification of *L. acidophilus*.

Isolates / Tests	*L.* 1	*L.* 2	*L.* 3	*L.* 4	*L.* 5	*L.* 6	*L.* 7	*L.* 8	*L.* 9	*L.* 10	*L.* 11	*L.* 12	*L.* 13	*L.* 14	*L.* 15	*L.* 16	*L.* 17	*L.* 18	*L.* 19	*L.* 20
Growth in litmus milk	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 15	-	-	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-	+	-
Growth at 45° C	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+
Growth nutrient medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrates Fermentation																				
Fructose	+2	+1	+1	+1	+1	+2	+1	+1	+2	+1	+2	+1	+2	+1	+1	+2	+1	+2	+1	+1
Sucrose	+1	+1	+1	+2	+1	+1	+1	+1	+1	+2	+1	+1	+1	+1	+2	+1	+1	+1	+1	+1
Lactose	+4	+1	+2	+1	+1	+1	+2	+2	+4	+1	+1	+1	+4	+2	+1	+1	+1	+1	+1	+1
Maltose	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1
Mannitol	-	+1	+1	-	-	+1	+1	+1	-	+1	+1	+1	-	+1	+1	-	+1	+1	+1	-
Raffinose	+1	-	-	-	-	-	-	-	+1	-	-	-	+1	-	-	-	-	-	-	-
Xylose	-	+2	+1	+1	+1	-	+2	+1	-	-	-	+1	-	+2	+1	+1	-	-	+1	+1

+ = Positive result, - = Negative result, numbers= no. of days to change the color.

In order to differentiate the twenty isolates of *Lactobacillus* species, carbohydrates fermentation test was performed. The isolates were different in their ability to ferment the carbohydrate sources used. Isolates which were able to ferment all sugars but xylose and mannitol were identified as *L. acidophilus* (Hammes and Vogel, 1995).

These results revealed that only 3 isolates from the 20 suspected isolates were identified as *L. acidophilus* representing 15% of the LAB isolates. While other bacterial types (*E. coli*, *S. spp.* and *St. spp.*) represented 20% of the total number of bacterial isolates (Fig. 9).

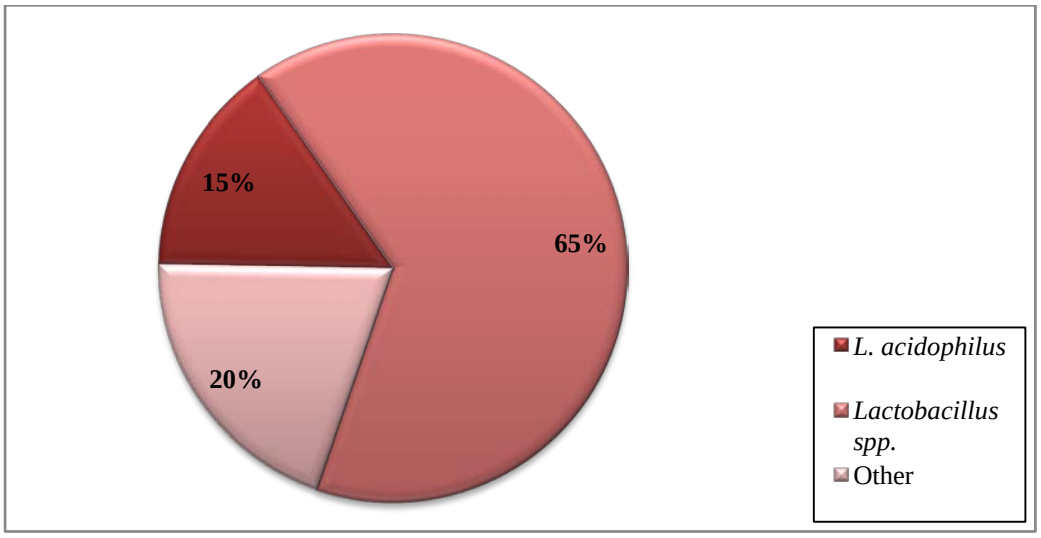


Fig. (9): The percentages of bacterial types in vaginal isolates.

The difference in LAB existence depends on several factors such as age, taking antibiotics and antipregnency drugs. Hormones concentrations especially estrogen has an important role in the existence of lactic acid bacteria as the increase in its concentration leads to the increase of glycogen layer in the vagina which encourages LAB to use as a carbon source leading to increased production of lactic acid and decreasing the vaginal pH which

results in the inhibition of many bacterial types which do not grow in acidic environment (Hawes *et al.*, 1999; Reid *et al.*, 2001).

3.9 Detecting the ability of *Lactobacillus acidophilus* to produce surlactin

The ability of *L. acidophilus* isolates (3 isolates) to produce surlactin was detected by inhibiting the adhesion of biofilm produced by the target bacteria (*P. aeruginosa*) in test tubes as in (2.2.16.1).

All of the three isolates were able to produce surlactin and inhibit the adhesion of *P. aeruginosa* biofilm but in different degrees. Results showed that the isolates (1 and 13) showed high capability in inhibiting the adhesion of biofilm of *P. aeruginosa* to the walls of test tubes, while the isolate 9 was less active.

These results agree with Borise *et al.*, (1998) as they mentioned that the biosurfactant produced by *L. acidophilus* is the most effective in inhibiting the adhesion of pathogenic bacteria in comparison to other types.

According to these results, the isolates (1 and 13) were chosen to be used in the subsequent experiments of this study.

3.10 Extraction and purification of surlactin produced by *Lactobacillus acidophilus*

Surlactin was extracted from the isolates (1 and 13) using a simple method because it is produced during the stationary phase of bacterial growth, this phase was considered as a start point for the extraction of surlactin by precipitating the cells of the two isolates after 18hrs of growth in MRS broth and washing them with PBS to get rid of logarithmic phase

products represented by bacteriocins, hydrogen peroxide and others. Then it was filtrated by Millipore filter papers (0.2µm) to purify it.

Simple and short methods for surlactin purification were used in this study, many complicated steps were avoided which were used in previous studies because of the interaction between the products of stationary and logarithmic phases after proving that surlactin is produced during the stationary phase of bacterial growth and disclaiming its production before this stage (Van Hoogmoed *et al.*, 2000; Rojas *et al.*, 2002).

3.11 Biological and medical application of surlactin

3.11.1 Testing the antibacterial activity of surlactin

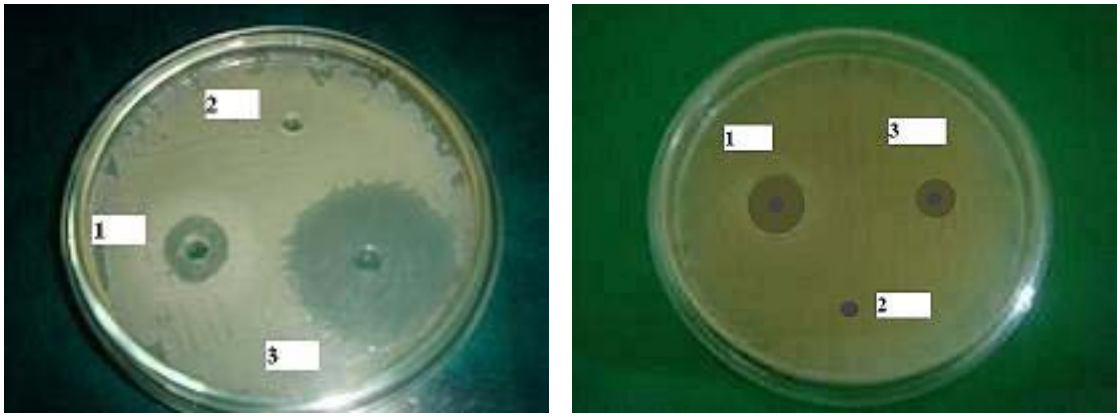
The results of this test are shown in (Fig. 10) where it is noticed that the primary filtrate of *L. acidophilus* cultivated in MRS broth for 18hrs had an antibacterial activity against the tested bacteria (*S. aureus* and *P. aeruginosa*) as the filtrate contains the products of logarithmic phase (bacteriocins, hydrogen peroxide and others) while surlactin extract of the isolates (1 and 13) had no effect against them.

These results (Table 14) indicate that surlactin lack the ability to inhibit the growth of pathogenic bacteria, and this agrees with Velraeds *et al.*, (1998) as they noticed the absence of any antibacterial activity of surlactin against pathogenic bacteria and *C. albicans* even when its concentration reached 1000µg/ml.

Table (14): Diameter of inhibition zones caused by primary filtrate of *L. acidophilus*, surlactin and chloramphenicol on Gram positive and negative bacteria.

Diameter of inhibition zone (mm) of bacterial isolates ±S.E.	Primary Filtrate of *L. acidophilus*	Surlactin	Chloramphenicol
P. aeruginosa	**11±0.3**	**0.0**	**6±0.5**
S. aureus	**13±0.4**	**0.0**	**77±0.6**

Values= mean of 3 replicates ± S.E.



A **B**

Fig. (10): Antibacterial activity of *L. acidophilus* extracts (isolate no. 1) against the growth of A- *S. aureus* and B- *P. aeruginosa*.

1= primary filtrate (after 18hrs of growth in MRS broth

2= surlactin, 3= positive control (chloramphenicol).

3.11.2 Inhibiton of pathogenic bacterial adhesion to the industrial contact lenses

The results of this test showed inhibition in the ability of *P. aeruginosa* to adhere to the contact lenses when treated with purified surlactin for both isolates (1 and 13), difference in crystal violet intensity was noticed in lenses treated with surlactin (less intensity) in comparison to negative control (lenses not treated with surlactin) (Fig. 11).

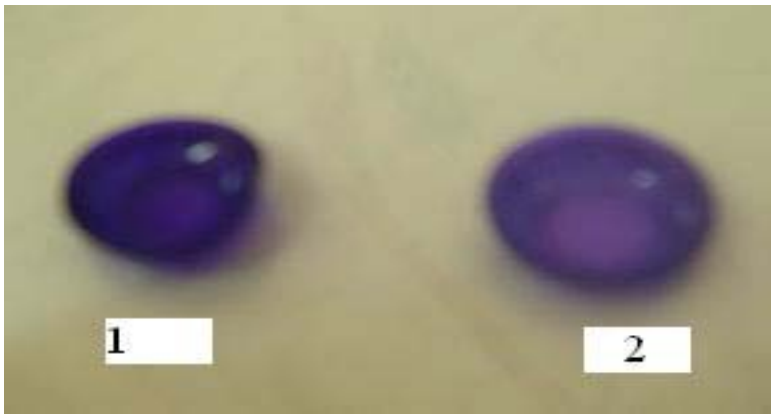


Fig. (11): Inhibition of *P. aeruginosa* adhesion to synthetic contact lenses using surlactin extracted from isolate (1).

1= contact lens treated with *P. aeruginosa* only (control).

2= contact lens treated with *P. aeruginosa* and surlactin.

Growth intensity of *P. aeruginosa* was reduced to 60% when treated with surlactin extracted from isolate (1) and 55% when treated with surlactin extracted from isolate (13). These results agree with Kamil, (2005) who stated that surlactin extracted from *L. acidophilus* had a good activity in removing biofilm formed by *St. epidermidis* from contact lenses.

The difference in surlactin activity to inhibit the adhesion of *P. aeruginosa* in contact lenses and glass tubes was due to the chemical

composition of those substances affecting the ability of bacterial cells to adhere to their surfaces.

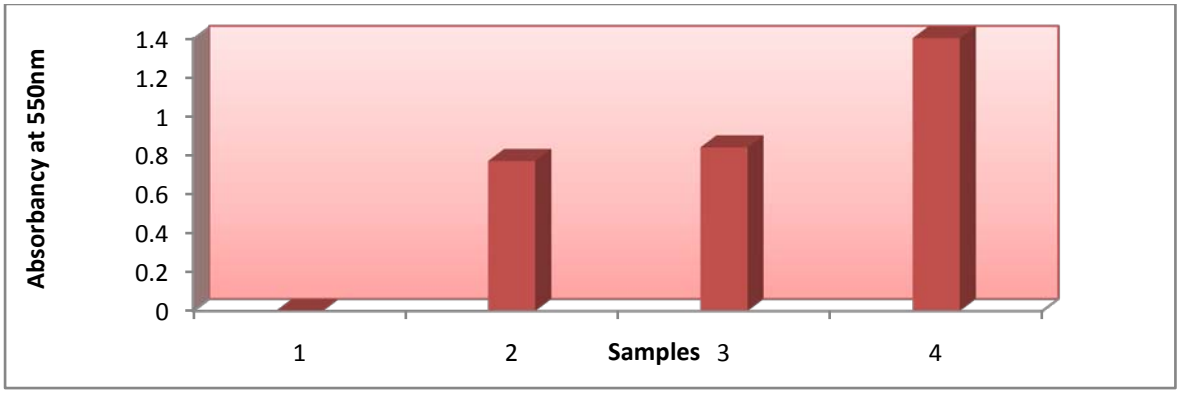


Fig. (12): The effect of surlactin samples extracted from isolates (1 and 13) on inhibiting the adhesion of *P. aeruginosa* in nutrient broth containing contact lenses.

1= Negative control (nutrient broth without bacteria).

2= Growth intensity in nutrient broth (in tubes containing surlactin from isolate (13) and bacterial culture of *P. aeruginosa*) after removing the lenses.

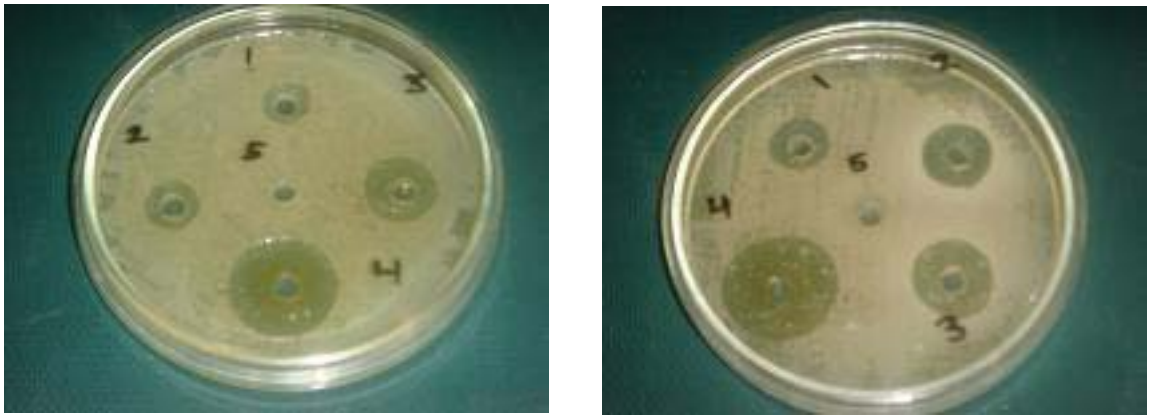
3= Growth intensity in nutrient broth (in tubes containing surlactin from isolate (1) and bacterial culture of *P. aeruginosa*) after removing the lenses.

4= Growth intensity in nutrient broth (in tubes containing contact lenses and bacterial culture of *P.* aeruginosa without surlactin) after removing the lenses (control).

3.12 The antibacterial activity of chamomile extract and surlactin (*in vitro*)

The synergetic effect of *C. recutita* callus ethanolic extract and surlactin against *P. aeruginosa* and *S. aureus* was tested; results displayed in (Fig. 13-A, B) indicate that diameters of inhibition zones were almost the same like those represented in fig. (8) revealing that surlactin had no effect against tested bacteria and the results obtained were only the effect of chamomile callus ethanolic extract.

The concentrations (60 and 80)mg/ml of chamomile extract and surlactin showed inhibition zones of (14 and 21mm) in diameter respectively against *P. aeruginosa* (Fig. 13-A) and (16 and 25mm) in diameter against *S. aureus* (Fig. 13-B), while the concentrations (20 and 40)mg/ml of the extract and surlactin showed inhibition zones of (10 and 12mm) in diameter respectively against *P. aeruginosa* (Fig. 13-A) and (11.5 and 13mm) in diameter against *S. aureus* (Fig 13-B).



A **B**

Fig. (13): Synergetic effect of chamomile callus ethanolic extract at different concentrations and surlactin on the growth of:

A- *P. aeruginosa* and B- *S. aureus*.

1= 20mg/ml, 2=40mg/ml, 3=60mg/ml, 4=80mg/ml, 5= control (PBS).

3.13 Synergetic effect of callus ethanolic extracts and Surlactin on bacteria causing eye infection in rabbits' eyes (*in vivo*)

The groups (1, 2, 3 and 4) inoculated with control samples showed swallowing, semi closed eyes with red lid filled with pus after 24hrs of injection (Fig. 14).

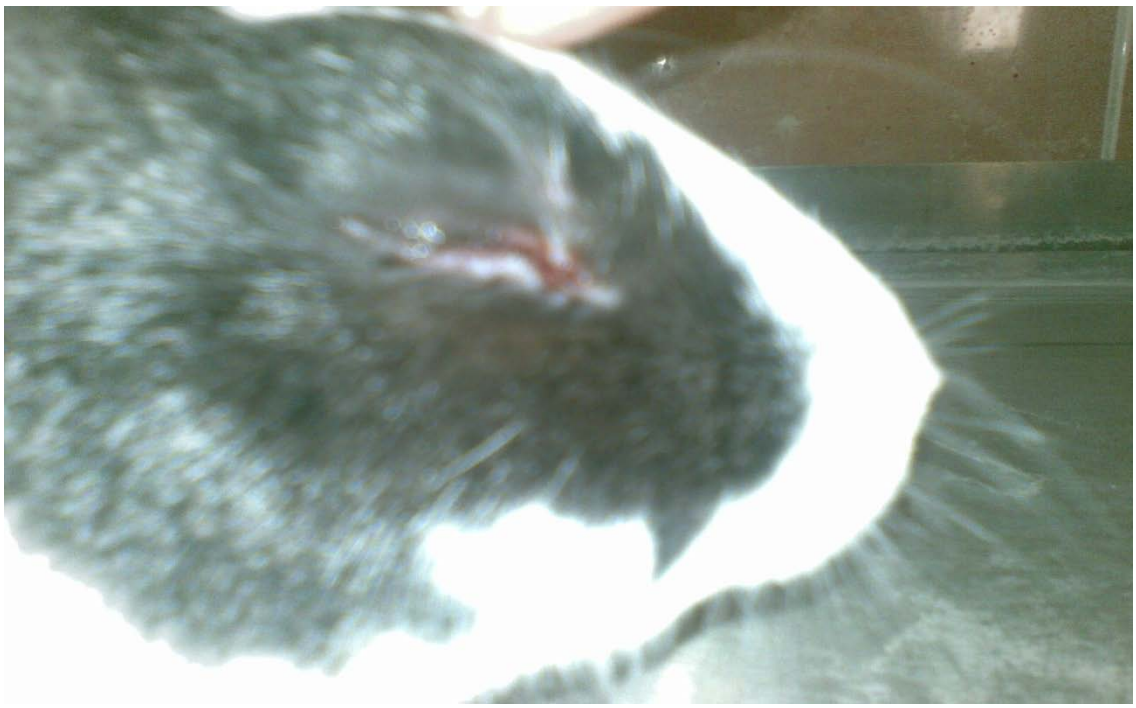


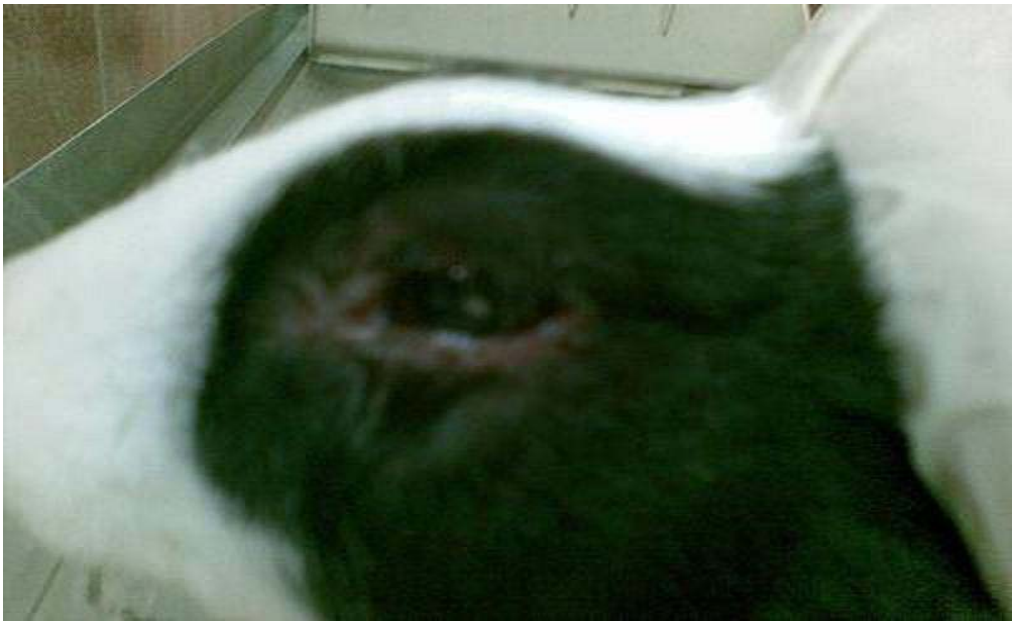
Fig. (14): Infection of rabbit eye (after inoculation with *P. aeruginosa* and PBS (as control).

When administrated to group (2), chamomile callus ethanolic extract (80mg/ml) showed an obvious effect on destroying *S. aureus* cells in the rabbit eye after 24hrs of administration. Complete cure from *S. aureus* was achieved after 36hrs of administration (Fig. 15) while it took 48hrs to fully recover from *P. aeruginosa* after administration.

Fig. (15): Appearance of rabbit's eye after administration of chamomile callus ethanolic extract at a concentration of (80mg/ml) after 36 hrs of administration.

When surlactin was administrated to group (3), rabbit's eye infected with *P. aeruginosa* showed a noticed recovery, and full cure occurred after 72hrs of administration (Fig. 16-A), while eyes infected with *S. aureus* did not show any recovery and infection persisted even after one week of surlactin administration and increasing the dose. These results agree with Velraeds *et al.*, (1998) who stated that surlactin does not have any antibacterial activity while it has the ability to inhibit the adhesion of biofilm forming bacteria to moist surfaces (Fig. 16-B).

A



B

Fig. (16): Appearance of rabbit's eye after administration of surlactin extract

A- Full cure from *P. aeruginosa* after 36hrs of administration

B- Persistence of infection with *S. aureus* after 7 days of administration.

When chamomile callus ethanolic extract (80mg/ml) and surlactin were administrated to group (4), results showed faster recovery in eyes infected with *P. aeruginosa* and it took only 36hrs for full cure to occur.

While eyes infected with *S. aureus* showed slow recovery compared to those infected with *P. aeruginosa*, full cure occurred after 48hrs as the callus ethanolic extract only had antibacterial activity against *S. aureus* while surlactin did not change the effect of chamomile extract (Fig. 17).

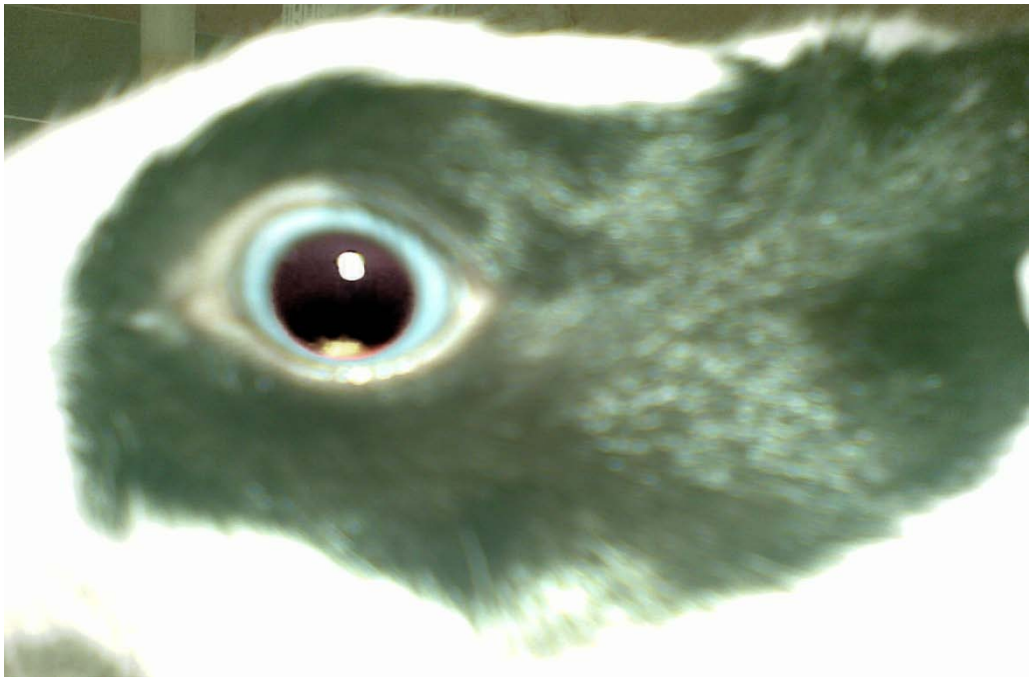


Fig. (17): Appearance of rabbit's eye after administration of chamomile callus ethanolic extract at a concentration of (80mg/ml) and surlactin extract after 2 days.

The groups (5, 6 and 7) when administrated with samples (A, B and C) respectively did not show any infection or eye redness after 24hrs of administration as shown in fig. (18).

Fig. (18): Appearance of rabbit's eye after administration of sample (A) after 24hrs.

These results agree with Smith, (2006) who showed that chamomile tea helps to relieve eyes redness and swollen eyes. Chamomile contains azulene, an anti-inflammatory compound, α-bisabolol which also has been shown to shorten the healing time of skin burns in laboratory animals (Der Mardersian and Liberti, 1988).

Biosurfactants might contain signaling factors that interact with the host and/or bacterial cells, leading to the inhibition of infections. Moreover, they support the assertion of a possible role in preventing microbial adhesion

(Millsap *et al.*, 1996, Rodrigues *et al.*, 2006) and their potential in developing anti-adhesion biological coatings for implant materials (Rodrigues *et al.*, 2006).

3.14 Effect of chamomile ethanolic extract and surlactin on inhibiting the adhesion of pathogenic bacteria to industrial contact lenses

The results of this test showed inhibition in the ability of *P. aeruginosa* to adhere to the contact lenses when treated with purified surlactin for both isolates (1 and 13) and chamomile callus ethanolic extract. Difference in crystal violet intensity was noticed in lenses treated with surlactin and chamomile extract (less intensity) in comparison to negative control (lenses not treated with surlactin and chamomile extract) (Fig. 19 A-B).

Fig. (19): Inhibition of *P. aeruginosa* adhesion to synthetic contact lenses using surlactin extracted from isolate (1) and chamomile extract.
A= contact lens treated with *P. aeruginosa* only (control).
B= contact lens treated with *P. aeruginosa*, surlactin and chamomile ethanolic extract (80mg/ml).

When measured by spectrophotometer at 550nm, growth intensity of *P. aeruginosa* decreased to 50% when treated with chamomile and surlactin extracted from isolate (1) and 45% when treated with chamomile extract and surlactin extracted from isolate (13).

4.1 Conclusions

1. Callus cultures of *C. recutita* can be induced and maintained on MS medium supplemented with 1.0mg/l BA and 0.1mg/l 2,4-D using seeds as a source of explants.

2. Ethanolic extracts can be used against all tested microorganisms at concentrations (80 and 60)mg/ml. The largest inhibition zone was observed against *S. aureus*. Callus ethanolic extract showed a better inhibition activity than flower extract.

3. High concentrations of chamomile water extract (60 and 80)mg/ml have inhibitory effects against tested bacteria. Callus water extract has a better activity than flower extract.

4. LAB formed the largest percentage of the vaginal normal flora in healthy women (65%), while *L. acidophilus* represented (20%) of the total number of bacterial types isolated from the vagina.

5. All *L. acidophilus* isolates (3 isolates) showed the ability to produce surlactin and its biological activity was determined by its ability to remove biofilm produced by *P. aeruginosa* using test tubes method. Isolates (1 and 13) were the most effective. Purified surlactin extracted from isolates 1 and 13 showed good activity in removing the biofilm formed by *P. aeruginosa* from contact lenses but it did not show any antibacterial activity against tested bacteria.

6. Ethanolic extract is a potential cure for eyes infection and inflammation in rabbits. When surlactin and ethanolic extract were used together to treat eye infection in rabbits, it was found that they cured the infection caused by *P. aeruginosa* faster than the ethanolic extract did when it was used separately, while the effect did not differ when they were used against *S. aureus.*

7. Synergetic effect of surlactin and ethanolic extract decreased the adhesion of *P. aeruginosa* to contact lenses up to 50 and 45%.

4.2 Recommendations

1. Investigation for other medicinal plants as sources for phytochemicals using tissue culture techniques.
2. Examination of *C. recutita* extracts on other microorganisms and other kinds of infections.
3. Comparing the activities of different biosurfactants produced by other microorganisms.
4. Studying the applications of surlactin in different immune responses *in vivo* and *in vitro*, and the possibility of using surlactin as antitumor agent using cancer tissue cultures and laboratory animals.
5. Utilization of molecular tools to produce biosurfactants from microorganisms.

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الخـــلاصـــــــــــــــة

تضمنت هذه الدراسة معرفة تأثير كل من مستخلصات نبات البابونج *Chamomilla recutita* ومادة surlactin المنتجة من بكتريا *Lactobacillus acidophilus* فضلاً عن التأثير التآزري لهما معاً في بعض الانواع البكتيرية المسببة لالتهابات العيون وكالآتي:

استحث الكالس من بذور نبات البابونج وأديم على وسط Murashige and Skoog, 1962 (MS) المجهز بـ 1.0 ملغم/لتر من BA 0.1 ملغم/لتر من 2,4-D.

اظهرت نتائج دراسة مستخلصات ازهار وكالس النبات فعاليتها المضادة لكل من بكتريا *Staphylococcus aureus* و *Pseudomonas aeruginosa* المسببة في التهاب العيون.

كما تم الكشف عن مكوناتها الكيميائية ووجد ان المستخلصات قد احتوت على التانينات، الفينولات، الكومارينات، الفلافونات، الراتنجات والكلايكوسيدات.

اوضحت النتائج ايضاً بأن المستخلص الكحولي للازهار اعطى فعالية تثبيطية اعلى من المستخلص المائي في نمو بكتريا *S. aureus* و *P. aeruginosa*، في حين اظهرت مستخلصات الكالس فعالية تثبيطية اعلى تجاه العزلات اعلاه.

تم في هذه الدراسة ايضاً تحديد قيمة التركيز المثبط الادنى (MIC) للمستخلصات النباتية على الانواع البكتيرية المدروسة واظهرت النتائج بأن فعالية المستخلص الكحولي اعلى من المائي للازهار ونسيج الكالس، وتباينت النتائج تبعاً لاختلاف نوع المستخلص ونوع البكتريا.

جمعت 25 عزلة مهبلية من نساء صحيحات من مستشفى كمال السامرائي/بغداد للفترة من نيسان 2007 ولغاية كانون الاول 2007 بهدف عزل وتشخيص بكتريا *L. acidophilus*. شخصت 3 عزلات من هذه المسحات كونها *L. acidophilus* والتي شكلت 15% من مجموع العزلات البكتيرية التي تم الحصول عليها، وكانت نسبة الانواع الاخرى لبكتريا *Lactobacillus* 65% (20 عزلة)، في حين كانت نسبة الانواع الجرثومية الاخرى20%.

اجري اختبار كفاءة عزلات *L. acidophilus* لانتاج الـsurlactin عن طريق قياس فعاليته الحيوية بمنع التصاق الغشاء الحيوي للبكتريا المنتجة له *P. aeruginosa* بالسطوح الملساء بطريقة انابيب الاختبار. ووجد ان العزلات الثلاثة كانت منتجة للـsurlactin ولكن اختلفت الفعالية بين العزلات، اذ كان الـsurlactin المنتج من العزلتين 1 و13 الاكفأ من حيث الفعالية.

درست التطبيقات الحيوية للـ surlactin عن طريق تثبيط التصاق الخلايا الممرضة (*P. aeruginosa*) المنتجة للغشاء الحيوي في عدسات العين اللاصقة الصناعية، اذ وجد أن له القابلية في تثبيط الالتصاق بنسبة60% و55% للعزلتين 1و13 على التوالي. كما اختبرت قابلية الـ surlactin في تثبيط النمو الجرثومي ووجد عدم امتلاكه لهذه القابلية.

لوحظ ان المستخلص الكحولي لنسيج الكالس له القدرة في معالجة الالتهاب في عيون الارانب عند اعطائه بجرعة 80ملغم/مل. اظهر ايضاً فعلاً مضاداً للبكتريا مما ادى الى عدم اصابة الارانب التي حقنت عيونها بالمستخلص بتركيز 80ملغم/مل والبكتريا المسببة للالتهاب.

اظهرت مادة surlactin القدرة على معالجة الالتهابات في عيون الارانب المصابة ببكتريا *P. aeruginosa* ولم تكن لها القدرة لمعالجة الارانب المصابة ببكتريا *S. aureus* في عيون الارانب. كما منعت حدوث الاصابة في عيون الارانب عند حقنها مع بكتريا *P. aeruginosa* بينما لم يكن لها اي تاثير في الحد من الالتهاب عند حقنها مع بكتريا *S. aureus* في عيون الارانب.

عند اضافة المستخلص الكحولي لنسيج الكالس لنبات البابونج بتركيز 80ملغم\مل بوجود مادة الـ surlactin، لوحظ ان لهما تاثير تآزري في معالجة الالتهابات في عيون الارانب التي سببتها بكتريا *P. aeruginosa* في وقت اسرع مقارنةً مع استخدامهما كلٌ على حدة بينما كان التأثير اقل في الالتهابات التي سببتها بكتريا *S. aureus*.

اخيراً تم دراسة تأثير المستخلص الكحولي لنسيج الكالس ومادة الـ surlactin في تثبيط التصاق بكتريا *P. aeruginosa* في عدسات العين الصناعية، واظهرت النتائج ان نسبة تثبيط الالتصاق كانت 50% لمستخلص الكالس الكحولي ومادة الـ surlactin المستخلصة من العزلة رقم 1 وانخفضت الى 45% لمستخلص الكالس الكحولي ومادة الـ surlactin المستخلصة من العزلة رقم 13.

الإهـ ـ ـداء

الى من حملتني واحتملتني

الى معينِ حنان لا ينضب .... أمي الغالية

الى من لم يبخل بشئ في سبيل اسعادي

الى أطيبِ قلبٍ في الوجود .... أبي الحبيب

الى أخي وصديقي وسندي .... أخي أحـمـد

الى صغيرتي وحبيبتي الجميلة .... أختي آيـة

اهدي لكم ثمرة عملي المتواضع ....

رنـ ـ ـ ـ ـ ـ ـد

بِسمِ الله الرحمٰنِ الرحيمِ

اقْرأْ بِاسمِ ربك الذي خلَق ۝ خلَق الإِنسانَ

من علَقٍ ۝ اقْرأْ وربك الأَكْرم ۝ الذي علم

بِالْقَلَمِ ۝ علم الإِنسانَ ما لَم يعلَم ۝

صدق اللهُ العظيم

سورة العلق

الآية(1-5)

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

التأثير التآزري لمستخلصي الكالس وازهار نبات البابونج *Chamomilla recutita* ومادة الـ Surlactin المنتجة من بكتريا *Lactobacillus acidophilus* على البكتريا المسببة لالتهابات العيون في الارانب

رسالة

مقدمة الى كلية العلوم – جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

رنـــــد طـلال حمـــــيد

بكالوريوس تقانة احيائية جامعة النهرين 2005

باشراف

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