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Effect of rosemary (*Rosmarinus officinalis* L.) tissue extracts on the growth of some skin infectious microorganisms

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By

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Amaal

Supervisors Certification

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Summary

In an attempt to treat infection of mice skin using ethanolic extract of rosemary (*Rosmarinus officinalis*) callus cultures. Several experiments were carried out. Callus was induced on leaf explants and maintained for 30 days on Murashige and Skoog medium supplemented with 0.5 mg/l of benzyl adenine and 2.0 mg/l of 2,4-dichlorophenoxyacetic acid.

Water and ethanolic extracts of *R. officinalis* leaves and callus cultures were investigated for their antimicrobial activity. Rosemary water leaves extract contained tannins, saponins, flavonoids, terpenes, steroids, alkaloids and phenols, while rosemary ethanolic leaves extract contained tannins, saponins, flavonoids, terpenes, steroids, glycosides and phenols.

Rosemary ethanolic leaf extracts showed inhibitory effect better than water one against *Staphylococcus aureus* and *Candida albicans* and no inhibitory effect against *Pseudomonas aeruginosa.*

Callus extracts showed an inhibitory effect more than leaf extracts on growth of *S. aureus* and *C. albicans* and slight inhibition against *P. aeruginosa*.

It has been noticed that ethanolic callus extract showed better inhibitory activity against *S. aureus* at a concentration of 80 mg/ml, for that this extract applied on laboratory mice skin. Ethanolic callus extract has antibacterial, anti-inflammatory and healing effect at a concentration of 80 mg/ml better than commercial skin ointment (Samacycline) on skin mice infected with *S. aureus*.

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List of Abbreviations

Abbreviation	Full name
2,4-D	2,4-dichlorophenoxy acetic acid
BA	Benzyl adenine
P. aureginosa	*Pseudomonas aeruginosa*
S.aureus	*Staphylococcus aureus*
C. albicans	*Candida albicans*
Spp.	Species
A	alpha
β	beta
°C	Degree Celsius
CRD	Completely randomized design
DDH_2O	Double distilled water
EDTA	Ethylene diamine tetraacetate
GA	Gibberellic acid
Hrs	Hours
LSD	Least significant differences
MS	Murashige and Skoog
n	Number of replicates
NAA	1-naphthalene acetic acid

Abbreviation	Full name
NaOCl	Sodium hypochlorite
min	Minutes
cfu	Colony forming unit
SD	Standard deviation
UV	Ultraviolet (light)
G-ve	Gram negative
G+ve	Gram positive

Chapter One

Introduction and Literatures Review

1. Introduction and Literatures Review

1.1. Introduction

Since ancient times, it has been known that aromatic plants and spices, as well as their essential oils, have varying degrees of antimicrobial activity, for this reason, extracts from these plants can be used to delay or inhibit the growth of pathogenic or spoilage microorganisms (Dorman and Deans, 2000; Rauha *et al.*, 2000). Plant extracts have a wide range of biological activities, such as: antibacterial, antifungal, antiviral, antimalarial, antitumor and their effects on acnes (Jain and Basal, 2003).

It would be well known that the natural activity of plants is due to the presence of secondary metabolites that it would be produced from the plant cell in small amount, in specific parts of plant and in the specific period of plant growth (Hartmann, 1996).

The production of secondary metabolites *in vitro* is possible through plant tissue culture. Plants arising from tissue culture can be a source of useful natural products. The accumulation of secondary products in plant cell cultures depends on many factors including the composition of the culture medium and environmental conditions (Braz and Ellis, 1981; Deus and Zenk, 1982; Stafford and Morris, 1986; Smith, 1996).

Among herbs and spices, (*Rosmarinus officinalis* L.) rosemary is therapeutically significant medicinal plant. Rosemary is a common household plant grown in many parts of the world. It is used to flavor food, in cosmetics and in traditional medicine for its choreretic, hepatoprtective, and antitumorigenic activities (Nemeth and Szekely, 2000; Slamenova *et al*., 2002).

The majority of rosemary extracts is related to its content of important active constituents mainly diterpenes (e.g. carnosic acid); phenolic acids (e.g.

rosmarinic acid) and flavonoids were derived from two common flavones: apigenin and luteolin (Almela *et al.,* 2006).

Rosemary is much appreciated for its aromatic, antioxidant, antimicrobial or antitumoural properties. Rosemary extracts show a strong antioxidant activity that is linked to the presence of substances arising from the secondary metabolism mainly to phenolic compounds (Bicchi *et al.,* 2000; Lo *et al.,* 2002; Lai and Roy, 2004).

Rosemary extracts possesses marked antibacterial, antifungal and antiviral properties and activity against certain bacteria including *Staphylococcus aureus, Staphylococcus albus, Vibrio cholerae, Escherichia coli,* and *Corynebacteria spp.* and yeast including *Candida albicans* (Steinmetz, 1988; Newall *et al*., 1996).

Rosemary extract had been widely used for topical applications such as wound healing, antiaging and disease treatments, and as a stimulant for increased blood supply to the skin (Leung and Foster, 1996; Hsu, 2005).

The aims of the current work were:

1. Preparation of water and ethanolic extracts from rosemary dried leaves and callus cultures.
2. Detection of some active compounds in water and ethanolic rosemary leaf extracts.
3. Examination of water and ethanolic extracts from rosemary dried leaves and callus culture extracts for their antimicrobial activity against some pathogenic microorganisms isolated from skin infections (*S. aureus, P. aeruginosa,* and *C. albicans*).
4. Administration of rosemary ethanolic callus extract on infected mice skin in an attempt to treat the infection.

1.2. Literatures Review

1.2.1. Herbal medicine

Herbal medicine is a growing area of health care that demands attention. It is also an important branch of alternative medicine (Choudhary and Atta-Ur-Rahman, 2004).

Herbs contain different phytochemicals with a biological activity that can provide therapeutic effect. Research interest has focused on herbs that possess hypolipidemic, antimicrobial and antitumor that may be useful in healing and reducing the risk of cardiovascular diseases and cancer (Abuharfeil *et al*., 2000).

The complex composition of herbs varies according to growing conditions (soil, weather, and season), harvest procedure and storage techniques (Humphery *et al.*, 1997).

Medicinal plants have been introduced in drug industries; Arabians scientists have confirmed the therapeutic nature of these plants. They contain active compounds such as alkaloids, glycosides, saponins and volatile oils. Treatment using plant based drug leads to reduce the side effects that often occur with the synthesized medicine (Mussa and Jabber, 1987).

1.2.2. Rosemary classification

Kingdom: Plantae
Subkingdom: Tracheobionta -- Vascular plants
Superdivision: Spermatophyta -- Seed plants
Division: Magnoliophyta -- Flowering plants
Class: Magnoliopsida -- Dicotyledons
Subclass: Asteridae
Order: Lamiales

Family: Lamiaceae

Genus: *Rosmarinus*

Species: *Rosmarinus officinalis* L. – rosemary (I.S.H.S., 1990).

1.2.3. The Labiatae family (Lamiaceae)

Is one of the largest and most distinctive families of flowering plants, with about 220 genera and almost 4000 species worldwide. This family has an almost cosmopolitan distribution. Some genera like *Nepeta*, *Phlomis*, *Eremostachys*, *Salvia* and *Rosmarinus* have a great diversity in the Mediterranean (Hedge, 1986; Hedge, 1992; Jamzad *et al.*, 2003).

Labiates are known for their essential oils common to many members of the family. Many biologically active essential oils have been isolated from various members of this family. The family is also famous for the presence of diterpenoids in its members. These plants have been used by humans since prehistoric times. Evidence from archaeological excavations showed that some species of this family, which are now known only as wild plants, had been cultivated at local scales in the past. This family is one of the major sources of culinary, vegetable and medicinal plants all over the world. Species of *Mentha*, *Thymus*, *Salvia*, *Origanum*, *Coleus* and *Rosmarinus* are used as food flavorings, vegetables and in industry. Also several species of family are used in traditional and modern medicine (Nunez and Obon de Gastro, 1992).

1.2.4. Rosemary nomenclature

Rosemary (*Rosmarinus officinalis* L.) is a common household plant grown in many parts of the world (Al-Sereitia *et al.*, 1999). The genus name *Rosmarinus* is a Latin word which means Dew of the Sea, (Ros=Dew; Marinus=Sea), a reference to its pale blue dew-like flowers and the fact that it

is often grown near the sea. It is a symbol of remembrance and friendship, and is often carried by wedding couples as a sign of love and fidelity (Leth, 1976).

The species name *officinalis* is a Latin word which means medicinal indicates that the plant has been used for medicinal purposes (Amr and Dordevic, 2000).

1.2.5. Rosemary common names

R. officinalis can be named also Rosemary, Old man, Compass Weed, Dew of the Sea, Poplar Plant, Compass Plant, Incensor (Fahim *et al.*, 1999). Also its name in Arabic language is Iklil Al Jabal, Rosemary in English, Rosmarin in German, Romarin in France, Romero and Rosmario in Spanish, Rosmarino in Italian and Hasalban in Turkish (Michel, 1995).

1.2.6. Rosemary description

Rosemary is an evergreen branched bushy shrub (Fig. 1), attaining a height of about one meter with upright stems. Pale-blue, labiates flowers appear in winter and spring. Dark green needle-like leaves are small with edges turned over backward. Underneath these rolled edges are little glands containing aromatic oils (Tyler *et al.,* 1976; Kotb, 1985).

1.2.7. Rosemary distribution

Rosemary grows wildly along the north and south coasts of the Mediterranean Sea, particularly on the dry rocky hills of the Mediterranean region and also in the sub-Himalayan areas (Tyler *et al.*, 1976; Kotb, 1985). The main producers are Italy, Spain, Greece, Turkey, Egypt, France, Portugal and North Africa (Svoboda and Deans, 1992).

Fig. (1): Rosemary (*R. officinalis*) plant grown in (a local garden) in Baghdad used as a source for plant material in this experimental work.

1.2.8. Rosemary history and popular uses

Rosemary has a rich history, holding a special position among herbs for the symbolism connected with it. In Europe rosemary has a very old reputation for improving memory, and has been used as a symbol of friendship, loyalty and for remembrance during weddings, war commemorations and funerals (Moss, 2003). In ancient Greece, students would place rosemary sprigs in their hair to improve memory and concentration when studying for examination (Calucci *et al.*, 2003).

Rosemary leaves are burnt as an incense, fumigant and disinfectant, in times past, the herb was burned in sick chambers to purify the air and was placed in law courts as a protection from jail fever (typhus), and during the Plague of 1665, and rosemary was carried and sniffed in suspicious areas to protect against plague. Reinforcing those antiseptic uses, a mixture of

rosemary and juniper was burned during World War II in French hospitals to kill germs (Usher, 1974; Phillips and Foy, 1990).

In ancient China, rosemary was used for headaches, insomnia, and mental fatigue and topically for baldness (Leung and Foster, 1996). In India, rosemary enjoys a history of use as a stimulant and as a carminative to expel gas from the stomach and intestines and as antimigraine (Nadkarni, 1994).

The Egyptians used the plant for incense in ritual cleansing and healing. Anthropologists and archaeologists have found evidence that rosemary herbs were used as medicinal, culinary and cosmetics virtues in the ancient Egypt, Mesopotamia, China and India (Stefanovits-Banyai *et al.*, 2003).

Rosemary oil was first extracted in the 14th century, after which it was used to make Queen of Hungary water, a very popular cosmetic used at that time. In the 16th and 17th centuries, rosemary became popular as a digestive aid in apothecaries (Ensminger *et al*., 1983). Throughout history, rosemary was traditionally used as food preservative, and before the invention of refrigeration, used to preserve meat (Castleman, 1991).

Rosemary herb and oil are commonly used as a culinary spice, especially in Mediterranean dishes for flavoring food as soup, beverages, and stews and in meat preparation and preservation for its desirable flavor and high antioxidant activity (Ho *et al.,* 1994).

Rosemary is famous as a rejuvenating tonic and is used to slow the aging process; the essential oil is widely applied in cosmetic industry producing various cologne waters, bathing essences, hair lotions and an infusion of the dried plant (both leaves and flowers) is used in shampoos (Allardice, 1993; Stefanovits-Banyai *et al.*, 2003). The oil is used widely in high quality perfumes, soaps and in certain medicinal preparations (Polunin and Huxley, 1987).

The powdered leaves are used as an effective natural flea repellent and branches of the leaves are placed in books and in clothes cupboards to protect them from moths and to produce a pleasant odor (Niebuhr, 1970; Holtom and Hylton, 1979). Rosemary has been in folk use to treat headaches, epilepsy, poor circulation and many aliments (Aruoma *et al.,* 1996).

1.2.9. Rosemary secondary metabolites

Plants produce large, diverse array of organic compounds that appear to have no direct function in plant growth and development. These substances are known as secondary metabolites, secondary products, or natural products Hartmann (1996), while primary metabolites (proteins, carbohydrates and fats) are important in plant physiological process such as growth and development (Mann, 1987). Secondary products have a variety of functions in plants. It is likely that their ecological function may have some potential medicinal effects for human. For example, secondary products involved in plant defense through cytotoxicity toward microbial pathogens could prove useful as antimicrobial medicines in human, if not too toxic (Briskin, 2000).

1.2.9.1. Phenolic compounds

The term phenolic compounds includes a wide range of plant substances which posses in common an aromatic ring bearing one or more hydroxyl substituent. Some phenolic substances tend to be water-soluble, some are soluble only in organic solvents and others are large insoluble. Phenolic compounds are formed via the shikimic acid pathway or malonic acid pathway (Taiz and Zeiger 2002).

These phenolic metabolites function to protect the plants against biological and environmental stresses and therefore are synthesized in

response to pathogenic attack such as fungal or bacterial infection or high energy radiation exposure such as prolonged UV exposure (Shetty, 1997 and Briskin, 2000).

1.2.9.1.1. Phenolic acids

Rosemary leaf contains (2-3)% phenolic acids such as caffeic, chlorogenic, labiatic, neoclorogenic and rosmarinic acid (Newall *et al.,* 1996).

Rosmarinic acid (Fig. 2) the diphenolic compound is an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid. It is commonly found in species of Lamiaceae family. The biosynthesis of rosmarinic acid starts with the amino acids L-phenylalanine and L-tyrosine (Petersen and Simmonds, 2003). Rosmarinic acid is regarded as a potential pharmaceutical plant product and is noted for its potent antioxidant properties (Exarchou *et al.,* 2000; Malencic *et al.,* 2000; Ly *et al*., 2006).

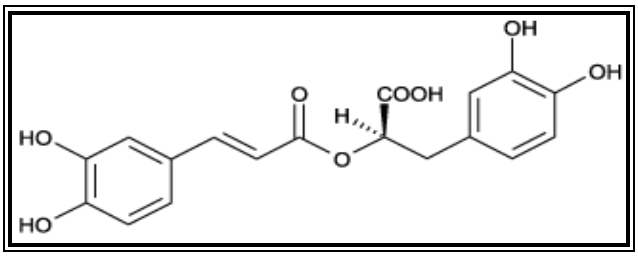

Fig. (2): Chemical structure of rosmarinic acid (Almela *et al.,* 2006).

1.2.9.1.2. Flavonoids

Flavinoids in rosemary include diosmetin, diosmin, genkwanin, eriocitrin, hesperidin and hispidulin and two common flavones apigenin and luteolin (Fig.3) (Del Bano *et al.,* 2004; Almela *et al.,* 2006). Three flavonoids including glucuronides, luteolin 3'-O-beta-D-glucuronide and hesperidin, were isolated from 50% methanol extract of the leaves of rosemary.

The structures were established by chemical and spectroscopic methods. Their antioxidant activities were evaluated showing the greatest activity (Okamura *et al.,* 1994b).

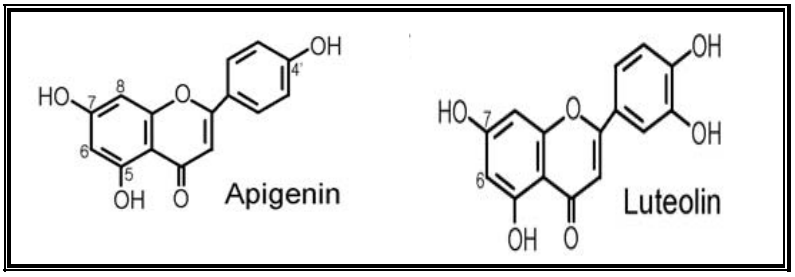

Fig. (3): Chemical structure of apigenin and luteolin (Almela *et al.*, 2006).

1.2.9.2. Terpenoid compounds

Terpenoids are aromatic chemical compounds which are dissolved in fats; it's found in the cytoplasm of plant cells or in special glands like the volatile oil, or it could be found in green plastids (Harborne, 1993). Terpenoids are based on the isoprene molecule and built from the union of two or more of (C5) units. Terpenoids are classified according to whether they contain two (C10) monoterpenes, three (C15) sesquiterpenes, four (C20) diterpenes, six (C30) triterpenes or eight (C40) carotenoids units (Trease and Evans, 1989).

1.2.9.2.1. Diterpenes

The most effective antioxidants in rosemary is the tricyclic diterpene carnosic acid in concentrations higher than 4% (Fig. 4). Some other less effective diterpenes in this plant are degradation products of carnosic acid, which are mostly converted to carnosol, carnosic acid methylester, epirosmanol, rosmanol and 7-methylrosmanol (Schwarz and Ternes, 1992; Okamura *et al.,* 1994a; Cuvelier *et al.,* 1996).

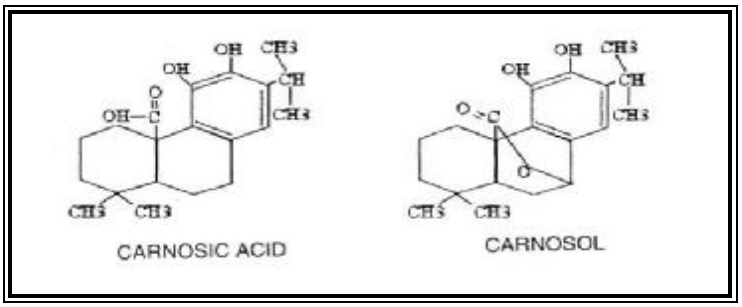

Fig. (4): Chemical structure of carnosic acid and carnosol (Almela *et al.*, 2006).

Diterpenes are not found in all rosemary tissues. The leaf was the tissue showing the highest concentrations of diterpenes. Diterpenes are also present in the flowers of rosemary, although the concentrations found in the sepals are not comparable with those found in the petals. Sepals contained approximately 30% fewer diterpenes than leaves, but 3.2 times more than petals. Diterpenes were also found at low concentrations in seeds and trace amounts were detected in stems. Roots do not contain diterpenes (Munne-Bosch and Alegre, 2001).

1.2.9.2.2. Triterpenes

Other terpenoid constituents in rosemary include triterpenes oleanolic and ursolic acids at (2-4)% (Newall *et al.*, 1996).

1.2.9.3. Essential oils

The leaves of rosemary contain between (1.0-2.5)% essential oil, such composition may markedly vary according to the chemo type and the development stage at which the plant has been harvested. It is an almost colorless to pale yellow liquid with a characteristic, refreshing and pleasant odor (Bauer *et al.,* 1997). Twenty components were identified in rosemary oils.

The main constituents are α-pinene (40.55-45.55)%, 1,8-cineole (17.40-19.35)%, camphene (4.73-6.06)%, verbenone (2.32-3.86)%, borneol (2.24-3.10)%, bornyl acetate, α-terpineol camphor, limonene, β-pinene and geraniol <2% (Atti-Santos *et al.,* 2005).

1.2.9.4. Alkaloids

Alkaloids are a large group of secondary products offer protection against predators, act as growth regulators, maintain ionic balance, act as a nitrogen reserve and possibly serve as nitrogen excretion products and exhibit important pharmacological properties. They are substances containing one or more nitrogen atoms in combination as part of a cyclic system. The most common precursors of alkaloids are amino acids (Goodwin and Mercer, 1983).The nitrogen-containing secondary products are biosynthesized primarily from amino acids (Taiz and Zeiger, 2002). Water extract of rosemary contain the alkaloid rosmaricine7 (Hoefler *et al.,* 1987).

1.2.10. Rosemary biological activity

1.2.10.1. Antimicrobial activity

Different extracts of rosemary have demonstrated inhibitory activity on cultures of *S. aureus, S. albus, E. coli, Corynebacterium spp., Bacillus subtilis, Micrococcus luteus, Salmonella spp., Listeria monocytogens* and *Vibrio cholerae*. Two of its components, carnosol and ursolic acid, are responsible for this antimicrobial effect and fungi of the *Aspergillus sp, Penicillium spp., Alternaria spp.* genera and other food fermenting microorganisms such as *Lactobacillus brevis*, *Pseudomonas fluorescens, Rhodotorularia glutinis* and *Kluyveromyces bulgaricus* (Alonso, 2004).

Methanol extract is containing 30% of carnosic acid, 16% of carnosol and 5% of rosemarinic acid. It is an effective antimicrobial against Gram-positive bacteria, Gram-negative bacteria and yeast (Moreno *et al*., 2006). Rosemary essential oil, like many essential oils, has antimicrobial properties when it comes in direct contact with bacteria and other microorganisms (Oluwatuyi *et al.*, 2004; Santoyo *et al.*, 2005).

Alpha-Pinene, beta-Pinene, 1,8-cineole, camphor, verbenone, and borneol standards showed antimicrobial activity against all the microorganisms tested, borneol being the most effective followed by camphor and verbenone Gram-positive bacteria (*S. aureus* and *B. subtilis*), Gram-negative bacteria (*E. coli* and *P. aeruginosa*), a yeast (*C. albicans*), and a fungus (*Aspergillus niger*), therefore, the active ingredients with bactericidal activity from rosemary extract are ideal when formulating purifying and antiseptic cosmetic products (Santoyo *et al.*, 2005).

One of the most recent studies to look at the effect of rosemary essential oil on drug-resistant infections found rosemary effective against several of the most common pathogens affecting humans, including the fungi that cause nail infections and the pathogen responsible for most vaginal yeast infections (Luqman *et al.*, 2007).

1.2.10.2. Antioxidant activity

As one of the most potent antioxidants known, rosemary prevents free-radical damage, protects cells from deterioration and aids in the prevention of cancer. Research into the free-radical quenching effects of rosemary has found it to be a potent antioxidant, possessing greater activity than the common food additives. The discovery of the antioxidant activity of rosemary in biological systems supports the historical use of rosemary as a preservative for meats and foods (Ho *et al.*, 1994).

1.2.10.3. Anti-inflammatory activity

Rosmarinic acid is a naturally occurring non-steroidal anti-inflammatory agent. It inhibits the complement factor C_3, a mediator in the inflammatory process (Al-Sereitia *et al.*, 1999; Alonso, 2004). Rosmarinic acid has a scavenging effect on the active oxygen free radical (Zhao *et al.*, 2001).

Manez *et al.* (1997) verified that ursolic acid reduces the chronic inflammation and neutrophil infiltration. This activity is closely linked to the structure of this compound. Rosemary extract is highly recommended when formulating cosmetic products for sensitive or irritated skin.

1.2.10.4. Antiseptic activity

As an antiseptic, rosemary cleanses the blood and helps to control many pathogenic organisms. It is potent enough to kill bacterial infection but not so potent, however, to completely wipe out the natural bacterial populations of the digestive tract that keep the intestines in healthy balance. Rosemary's antiseptic quality heals wounds (Williamson *et al.*, 1988; ESCOP, 2003). Distilled water from the flowers is used as eyewash (Chiej, 1984). It can be used as a disinfectant, as a mouth wash and to treat fever or rheumatism (Calabrese *et al.*, 2002).

1.2.11. Rosemary medicinal uses

One of the potential therapeutic effects of rosemary is its role in preventing cancer. Researchers have demonstrated that natural polyphenols found in rosemary have potent anticarcinogenic properties. To date, rosemary extract, or its active components, carnosol, carnosic acid, and rosmarinic acid, have been shown to prevent cancer (Singletary *et al.*, 1996; Zhu *et al.*, 1998).

Scientists have found that rosemary extract can significantly help to protect DNA against free radical damage (Slamenova *et al.*, 2002).

Rosemary is an excellent memory and brain stimulant that is said to improve brain function by feeding it with oxygen-rich blood and it play a role in memory loss associated with Alzheimer (Duke, 1997). It helps with headaches, migraines, neuralgia, mental fatigue and nervous disorders (Amini, 1997). Also rosemary is a central nervous system stimulant (Kovar *et al.*, 1987). Carnosic acid and carnosol are able to promote markedly enhanced synthesis of nerve growth factor (Kosaka and Yokoi, 2003).

Rosemary infusion used as a mouthwash alleviates halitosis (Chevallier, 1996). It is used internally for flatulence, stimulating the digestion, dyspepsia, carminative and as appetizer (Amini, 1997; Blumenthal *et al.*, 2000; ESCOP 2003; Mills and Bone 2005; Bradley, 2006; Barnes *et al*., 2007). Rosemary is used as an antispasmodic in renal colic, relaxes smooth muscles of intestine and its constituents have a therapeutic potential in treatment or prevention of liver damage and peptic ulcer (Al-Sereitia *et al.*, 1999; Masuda *et al.*, 2002; Sotelo-Fleix *et al.*, 2002; Osakabe *et al.*, 2004). It improve hepatic and biliary function and increases bile flow (Hoefler *et al*., 1987; ESCOP, 1997).

Rosemary improves blood circulation and decreases the permeability and fragility of the capillaries due to the flavinoid (diosmin) (Bown, 1995). The plant has significant antithrombotic activity (blood thinning) and lowers blood pressure in hypertension patients (Yamanoto *et al.*, 2005).

Rosemary has therapeutic properties as a mild diuretic making it effective in reducing swollen ankles and bloating. Its diuretic action increases the flow of urine that flushes bacteria from the body before they have chance to cause infection (Haloui *et al.*, 2000).

Rosemary and its constituents have a therapeutic potential in treatment or prevention of bronchial asthma and in relieving respiratory disorders (Al-Sereitia *et al.,* 1999; Masuda *et al.,* 2002; Sotelo-Fleix *et al.,* 2002; Osakabe *et al.,* 2004). It is effective for asthma, bronchitis, catarrh, sinus, whooping cough, colds, cough and influenza (Small, 1997). Constituents of this herb include eucalyptol relaxes smooth muscles of trachea and lungs (Aqel, 1991).

Rosemary oleanolic acid extract decreased pain in patients suffering from rheumatic conditions and arthritis (Lukaczer *et al.,* 2005). Rosemary facilitates menstruation and regulates its flow, treating low or excessive bleeding and its constituents have a therapeutic potential in treatment of poor sperm motility, rosemary and its constituents have a therapeutic potential in treatment or prevention of cataract (Al-Sereitia *et al.,* 1999; Masuda *et al*., 2002; Sotelo-Fleix *et al*., 2002; Osakabe *et al.,* 2004).

Rosemary enhances immunity because it stimulates sweating and toxin removal (Babu *et al.,* 1999). It alleviates allergies due to the ursolic acid content of rosemary inhibiting mast cell degranulation and histamine release from mast cells in the presence of antigens (Tsuruga *et al.,* 1991). It exhibited strong inhibitory effects against human immunodeficiency virus (HIV) (Paris *et al.,* 1993).

Ursolic acid and oleanolic acid, have been used in tonics to enhance hair growth and prevent scalp irritation, they encourage hair growth by stimulating the peripheral blood flow in the scalp and activating the hair mother cells. They also furnish alopecia-preventing and dandruff-preventing effects (Okazaki *et al.,* 1989; Kikuko *et al.,* 1993).

1.2.12. Plant cell culture

The history of plant cell culture dates back to the beginning of the twentieth century, and since the 1930. The concept of culturing plant cells includes the culture of plant organs, tissue, cells and protoplast. The application of plant cell culture has three main aspects: the production of secondary metabolites, micropropagation and the study of plant cell genetics, physiology, biochemistry and pathology (Zhong, 2001).

The useful aspects of the plant tissue culture in the field of secondary metabolites are:

- Used for the large scale culture of plant cells from which secondary metabolites can be extracted.
- The advantage of this method is that it can ultimately provide continuous, reliable source of natural products.
- Compounds from tissue culture are more easily purified because of the absence of significant amounts of pigments, thus reducing production costs (Barnum, 2003).

1.2.13. *In vitro* production of secondary metabolites

The production of secondary metabolites *in vitro* is possible through plant tissue culture (Barnum, 2003). Secondary products such as vivid pigments, aromatic compounds, flavors, and bioactive phytochemicals can now be successfully accumulated in many plants *in vitro* cultures. *In vitro* production of valuable plant secondary products (phytochemicals) has become an industrially promising alternative to synthetic compounds (Smith *et al.,* 1987).

Large scale production of phytochemicals using plant cell and tissue culture technologies has the following advantages: controlled environmental

factors (climate, pests), no geographical and seasonal constraints, defined production systems and when required, more consistent product quality and yield, continuous and homogenous supply of plant material in a uniform physiological state, use of recombinant DNA technologies for yield improvement, production of novel compounds *in vitro*, which are absent in the parent plant material and the ability for large-scale cultivation of cells and organs in bioreactors for easier and higher product recoveries (Zafar and Datta, 1992).

1.2.14. Callus cultures

Callus cultures are undifferentiated, proliferating mass of cells usually arising on wounds of differentiated tissues. Generally, plants which accumulate relatively high yields of specific secondary metabolites, give rise to tissue cultures producing high levels of secondary metabolites (Lindsey and Yeoman, 1985).

The degree of callus formation depends on the type of explants even when taken from the same plant. Juvenile plants (with physiologically active tissues) give better callus formation. Exogenous plant growth regulators (type, concentration, auxin to cytokinin ratio) which required for callus formation depends upon the endogenous hormone content of the tissues under investigation (Pierik, 1987).

1.2.15. Applied strategies for increasing secondary metabolites

Various factors affecting the production of secondary metabolites in tissue culture. Initially growth and production of secondary metabolites are optimized by manipulating the physicochemical factors followed by selection of high-productive cells (Ramawat, 2008).

Different strategies have been employed to increase secondary metabolite production:

1.2.15.1. Culture medium

Media components play a vital role in stimulating the secondary metabolite production, e.g. many plant cells that grown in limited amount of nitrogen or phosphate gave enhanced yields of secondary metabolites (Zenk *et al.*, 1977). The general consensus is that sucrose is better than all other carbohydrate sources for growth and concentrations above 3% often enhance the biosynthesis of phytochemicals (Sass *et al.*, 1982; DiCosmo and Towers, 1984).

Phosphate level in the growth media may affect the biosynthesis of phytochemicals by cultured plant cells. Many studies have shown that cultured cells of *Catharanthus roseus* and *Nicotina tabacum* grown in media depleted of phosphate showed increased synthesis of indolic alkaloids and coumarins respectively (Knoblocu and Berlin, 1980; Okazaki *et al.*, 1982). In contrast increased phosphate concentrations caused increased anthraquinone production in *Morinda citrifolia* (DiCosmo and Towers, 1984).

1.2.15.2. Plant growth regulators

Another important component of the culture media is the growth regulator (phytohormone). Auxins and cytokinins have shown the most remarkable effects on growth and productivity of plant metabolites (Kuang and Cheng, 1981). There are numerous reports indicate that the type and concentration of auxin or cytokinin or auxin:cytokinin ratio or gibberellins in the growth medium alter dramatically both the growth and secondary products accumulation of cultured plant cells (Mackenzie and Street, 1970; DiCosmo and Towers, 1984).

Kinetin is one of the most popular cytokinins, it was reported to stimulate production of an anti-tumor compound like tripdiolide (Misawa, 1985). The suspension cultures of *Catharanthus roseus* initiated from stem and leaf explants on a medium containing NAA and kinetin has been established to increase secondary products formation by Zhao *et al*. (2001).

Xu *et al.* (2008) reported *in vitro* production of rosmarinic acid from callus and cell suspension culture system of *Agastache rugosa* and it was found that a combination of high levels of 2,4-D (2.0 mg/l) and low levels of BA (0.5 mg/l) are suitable for cell growth and rosmarinic acid production from cell cultures of *Agastache rugosa.* While Lui and Staba (1982) reported that GA_3 was also effective on plant cell cultures, the growth of callus of digoxin-producing plant, *Digitalis lanata*, was promoted by the addition of GA_3 into the media.

1.2.15.3. Physical factors

In vitro production of secondary metabolites by plant cell cultures is largely dependent on environmental factors: light, pH, temperature, oxygen and other factors.

Ramawat (2008) reported that blue light induced maximum anthocyanin formation in *Haplopappus gracilis* cell suspension cultures, whereas white light induced anthocyanin synthesis in *Catharanthus roseus* and *Populus spp.* Cells of *Ruta graveolens* produce several coumarins and alkaloids when grown in continuous white light, while it produces 2-nonanone, 2-nonanyl acetate and 2-nonanol preferentially in dark (DiCosmo and Towers, 1984).

A temperature of 17-25 °C is normally used for induction of callus tissues and growth of cultured cells. But, each plant species may favor a

different temperature. It was found that lowering the cultivation temperature to 10°C causes increased phospholipids in *Rauwolfia serpentine* (Yamada *et al*., 1980).

Plants are usually cultured in media having pH range of 5 to 6. The pH of the growth medium can influence the production of phytochemicals in cultured cells. Cultures of *Daucus carota* produced less anthocyanin when grown at pH 5.5 than those grown at pH 4.5, since anthocyanin content decreased by 90% at pH 5.5 compared to tissues grown at 4.5 (Ramawat, 2008).

1.2.15.4. Addition of precursors

A precursor is a compound which is transformed to a class of functional molecules within short steps (Arita *et al.*, 2000). Sometimes, addition of appropriate precursors to the culture media or related compounds stimulates secondary metabolites production. This approach is advantageous if the precursors are inexpensive. For example, phenylalanine is one of the biosynthetic precursors of rosmarinic acid (Zenk *et al*., 1977). Addition of this amino acid to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid. Addition of phenylalanine to the callus cultures of *Taxus cupsidata* cells stimulated the biosynthesis of the anticancer compound, taxol (Fett *et al.*, 1995).

1.2.16. Skin infections

Skin infections are common and may be caused by bacteria, fungi or viruses. Breaks in the skin integrity, particularly those that inoculate pathogens into the dermis, frequently cause or exacerbate skin infections (Michael and Odell, 1998).

1.2.16.1. Bacterial skin infections

Skin infections are often the result of a break in the integrity of the skin. Some infections are self-limiting and resolve on their own, but many others require medical attention. Bacterial skin infections technically have specific names based on their origin and extent, but even in the medical community we often lump these technical terms into the common term boil or abscess (Daniel *et al.*, 2002).

Common bacterial skin infections include boils, cellulitis, erysipelas, impetigo, folliculitis, furuncles, carbuncles and hot tub folliculitis. Boils is a skin infection caused by *S. aureus* are quite common. Cellulitis is an infection of the deeper layers of the skin and the connective tissues below the skin's surface that has poorly demarcated borders. People with cellulitis usually have an area of red, swollen, tender, warm and is usually caused by *Streptococcus spp.* or *Staphylococcus spp.* (Bjornsdottir *et al.*, 2005; Roberts and Chambers, 2005).

Erysipelas is a superficial form of cellulitis with sharply demarcated borders and is caused almost exclusively by *Streptococcus spp*. Impetigo is a skin infection caused by *Streptococcus spp.* or *Staphylococcus spp.* (Krasagakis *et al.*, 2006).

In impetigo, fluid-filled blisters develop and often burst and form yellowish crusts. Impetigo is a contagious infection that can spread if a person scratches the blisters and then scratches or touches another area of the body (Stulberg *et al.*, 2002).

Carbuncles and furuncles also known as boils, are *Staphylococcal* infections that produce a red, swollen bump filled with pus in the skin surrounding a hair follicle. With boils pus forms in a single hair follicle, whereas carbuncles form from grouped furuncles and have several small

chambers, like a series of connected boils. Folliculitis is an inflammation of the hair follicles caused by *Staphylococcus spp*. If the infection of the follicle is deeper and involves more follicles, it moves into the furuncle and carbuncle stages and usually requires incision and drainage. A special case of folliculitis that is not caused by *S. aureus* is hot tub folliculitis. This self-limited infection is caused by *P. aeruginosa* contracted from hot tubs, whirlpools, and pools with inadequate chlorine levels (Daniel *et al.,* 2002).

1.2.16.2. Fungal and yeast skin infections

Fungi and yeast are capable of causing many different forms of skin infections, broadly referred to as dermatomycoses. *C. albicans*, *Trichophyton*, *Epidermophyton* and *Microsporum spp.* are the most common infecting organisms. Many of the lesions caused by fungi are distinctive and diagnosis is primarily based on the presenting pattern rather than on culture or other test results (Drake *et al.,* 1996).

The dermatophytoses are a group of common infections generally caused by *Trichophyton spp*. These infections include tinea capitis, tinea corporis and tinea pedis (Kovacs and Hruza, 1995). Infection of the skin by *C. albicans* occurs principally in moist, warm parts of the body, such as the axilla, intergluteal folds, and groin or inframammary folds.

It is most common in obese and diabetic individuals. These areas become red and weeping and may develop vesicles (Jawetz *et al.,* 1982).

1.2.17. Microorganisms cause skin infection

Many types of microorganisms infect skin such as *S. aureus, Streptococcus pyogenes,* Overgrowth of *Corynebacterium spp., P. aeruginosa, Mycobacterium spp., Clostridium perfringens, Klebsiella*

rhinoscleromatis and others, in addition to fungi such as *C. albicans* and *Trichophyton rubrum* (Michael and Odell, 1998).

The major related microorganisms to skin infection are discussed in some detail below:

1.2.17.1. *Staphylococcus aureus*

The Staphyllococci is Gram positive, spherical usually arranged in grape-like irregular clusters. Some are members of the normal flora of the skin and mucous membranes of human. The pathogenic Staphylococci often cause hymolyse blood, coagulate plasma and produce variety of extra-cellular enzyme and toxins. The most common type of food poisoning is caused by a heat stable Staphylococcal Entero-toxin. Staphylococci rapidly develop resistance to antimicrobial agents and present difficult therapeutic problems (Jawetz, 1998).

S. aureus is a major pathogen for humans. Almost every person will have some types of *S. aureus* infection during poisoning or minor skin infections to sever life-threatening infections. *S. aureus* infection can also result from direct contamination and a wound like the post operative Staphylococcal wound infection or infection following trauma (Chronic osteomyelitis subsequent to an open fracture, meningitis following skull fracture) (Baily and Scott, 1974).

1.2.17.2. *Pseudomonas aeruginosa*

The *Pseudomonas* is a large genus of Gram negative aerobic rods of the family pseudomonadacea (Jawetz *et al.,* 1995). The *Pseudomonas spp.* is widely distributed in soil, water, plants and animals. *P. aeruginsa* is the major

pathogen of the group. Other *Pseudomonas spp.* infrequently causes diseases (Cruickshank *et al.,* 1975; Nester *et al.,* 1988).

P. aeruginsa invasive and toxigenic produces infection in patients with abnormal host defenses and considered as important nosocomial pathogen. *P. aeruginsa* is pathogenic only when introduced into areas devoid of normal defenses, like the mucous membranes and skin when they are disrupted by direct tissue damage. The bacterium attaches to and colonizes the mucous membranes or skin, invades locally and produces systemic disease (Bodey, 1983 and Jawetz, 1995).

1.2.17.3. *Candida albicans*

C. albicans appears as unicellular, yeast like fungi, reproduced by budding and it is polymorphic, able to change reversibly between round budding and elongated hyphen or filamentous growth. This morphological flexibility appears to be a key contributor to virulence (El-Barkani *et al*., 2000).

Yeasts live as normal microorganisms in and on the human body. Many species are without any clinical significance, however, some of them develop pathological changes in debilitated persons, e.g. following administration of anticancer drugs or immunosuppressive agents such as corticosteroids and by overuse of broad-spectrum antibiotics. *C. albicans* causes systemic and topical infections in children (Murray *et al.,* 1995).

C. albicans is a component of the normal skin floral and also the chief cause of mucocutaneous fungal disease in humans (Hay, 1993). *C. albicans* can also infect fingernails, producing onychomycosis and paronychia and is more common with advanced human immunodeficiency virus (HIV) disease (Conant, 1994).

1.2.18. Skin treatment with rosemary

Rosemary has developed a reputation for antibacterial and antifungal action, and herbalists recommend that the leaves be used externally for skin infections (Leung and Foster, 1996). External use of rosemary preparations for the promotion of wound healing and easing congestion, puffiness and swelling and can also be used for acne and boils (Hussain, 1979; Ashoor, 1985; El-Gadi and Bshina, 1989; ESCOP, 1997).

Its supreme skin regenerative and wound healing properties make rosemary verbenon especially useful for treating chronic skin conditions, dermatitis, eczema and psoriasis. Acne prone skin may respond favorably to the renewing effects of rosemary verbenon, as well as to its action to fight infection and promote glandular balance and function. Its skin nourishing action makes it ideal for dry and mature skin. Rosemary verbenon is excellent for the treatment of oily skin, scalp and dandruff (Stiles, 2007).

Rosemary is known to enhance the skin's natural metabolism, improve the nutrient flow into skin cells and speed the elimination of metabolic wastes and toxins (Cech, 2007).

Rosemary has been used in antiaging creams for several years (Jellin *et al.,* 2000). It helps minimize the effects of ageing on skin because ursolic acid has been used to treat photoaged skin because it prevents and improves the appearance of wrinkles and age spots by restoring the skin’s collagen bundle structures and its elasticity (Katsuo *et al.*, 1997).

Because rosemary stimulates and improves circulation throughout the body, it increases the blood supply to the skin, which is thought to help restore a youthful glow (Masuda *et al.*, 2002).

It was shown to safeguard a protective protein called HSP70. The role of this protein is to reduce damage caused by stress, free radicals and other toxins on the skin (Calabrese *et al.,* 2001).

Application of rosemary extract has been shown to prevent chemically induced skin tumors in a mouse model of human skin cancer. Depending on the concentrations of the extract, tumors were inhibited by up to 99% (Huang *et al.,* 1994).

Ursolic acid, derived from rosemary, significantly inhibited the proliferation of melanoma cells (Harmand *et al.*, 2005). In addition, research has demonstrated that when specially formulated with lipids, ursolic acid enhances the dermal collagen and ceramide content of normal human epidermal keratinocytes. Collagen provides the skeleton that gives shape and structure to the skin, while ceramide is a lipid that helps maintain proper immune function, as well as youthful moisture content, in the skin. Keratinocytes make up as much as 95% of epidermal tissues and are responsible for producing keratin, the tough protein that contributes to healthy hair, nails, and skin. (Yarosh *et al.*, 2000; Both *et al.*, 2002).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Apparatus and equipments

The following equipments and apparatus were used in this study:

Apparatus	Company / Origin
Autoclave	Karl / Germany
Distillator	GFL / Germany
Electric balance	Mettler / Switzerland
Hot plate with magnetic stirrer	Gallenkamp / England
Incubator	Sanyo / Japan
Laminar air flow cabinet	ESCO / Singapore
Micropipettes	Brand / Germany
Millipore filters	Millipore and Whattman / England
Oven	Gallenkamp / England
pH-meter	Metter Gmbh-Teledo / England
Refrigerator	Ishtar / Iraq
Rotary evaporator	Buchi / Switzerland
Sensitive balance	Delta Range / Switzerland
Shaker incubator	Sanyo / Japan
Soxhlet	Electrothermal / England
Vortex	Buchi / Switzerland
Water bath	Gallenkamp / England

2.1.2. Chemicals

Chemicals used in this study and their suppliers:

Chemical	Company / Origin
2,4-dichlorophenoxy acetic acid	BDH / England
Acetic anhydride	BDH / England
Agar	Oxoid / England
Agar-Agar	Sleeze / England
Ammonium nitrate	Fluka / Switzerland
Benzyl adenine	BDH / England
Boric acid	Fluka / Switzerland
Calcium chloride.$2H_2O$	Fluka / Switzerland
Chloroform	BDH / England
Cobalt chloride.$6H_2O$	BDH / England
Cupric sulphate.$5H_2O$	BDH / England
Ethanol	BDH / England
Ferric chloride	BDH / England
Ferrous sulfate.$7H_2O$	BDH / England
Glycine	BDH / England
Hydrochloric acid (HCl)	BDH / England
Lead-acetate	BDH / England
Magnesium sulphate.$4H_2O$	Fluka / Switzerland
Manganese sulphate.$4H_2O$	BDH / England
Molybdic acid (sodium salt).$2H_2O$	BDH / England
Myoinositol	BDH / England
Nicotinic acid (B3)	BDH / England
Potassium hydroxide (KOH)	Fluka / Switzerland

Chemical	Company / Origin
Potassium iodide	BDH / England
Potassium iron cyanide	BDH / England
Potassium nitrate	BDH / England
Potassium phosphate monobasic	Fluka / Switzerland
Pyridoxine.HCl (B6)	BDH / England
Sodium ethylene diamine tetraacetate	Fluka / Switzerland
Sodium hydroxide (NaOH)	BDH / England
Sulphuric acid	BDH / England
Thiamine.HCl (B1)	BDH / England
Zinc sulphate.$7H_2O$	BDH / England

2.1.3. Culture media

Medium	Company / Origin
Nutrient broth	Oxoid / England

2.1.4. Plant material

Rosemary plants, *R. officinalis* (Lamiaceae) were purchased from local nurseries in pots. Plants were identified by Prof. Dr. Ali H. AL-Moosawi, Dept. of Biology, College of Science, University of Baghdad. Some plants were grown directly in the soil for continuous supply of explants for tissue culture work and as a source for vegetative parts for intact plant studies.

2.1.5. Skin ointment (Samacycline)

In this study skin ointment (Samacycline) considered as antibacterial ointment was used to treat the infected area, this ointment used for comparing with the effect of ethanolic callus extract. It's manufactures by the state company for drug industries and medical appliances, Samarra, Iraq. Samacyclin ointment contains Tetracycline HCl 3%.

2.1.6. Microorganisms isolates

The following microorganisms were used in this study. They were isolated from patients suffering from skin infections and obtained from the Dept. of Biotechnology, Al-Nahrain University: *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans.*

2.1.7. Laboratory animals

Healthy albino adult female mice were obtained from the animal house of Biotechnology Research Center, AL-Nahrain University.

Twelve female mice were used in this experiment weighted 20-22 g and their age at the start of the experiment was 4-6 weeks. The animals were kept in suitable environmental conditions at 20-25 °C and a photoperiod of 12 hrs. daily.

2.2. Methods

2.2.1. Sterilization of explants

Mature leaf explants were excised, rinsed with running tap water for 1 hr., then transferred to laminar air flow-cabinet where submerged in 70% ethanol for less than one minute, washed with sterilized DDH_2O, then rinsed

with sodium hypochlorite at the different concentrations 0, 0.5, 1.0 or 3.0 % for 5 or 15 min. Commercial bleach was purchased from the local market at a concentration 5.25% of NaOCl. To prepare the following concentrations the volumes of 9.5, 19 or 57 ml of sodium hypochlorite were taken and completed with the volumes 90.5, 81 or 43 ml of DDH_2O respectively, in order to prepare the following concentrations 0.5, 1.0 or 3.0 %.

Explants then rinsed with sterilized DDH_2O for three times. For each concentration 12 explants were used and both ends of each explant were cut to remove tissues affected by sterilization solution. The final length of leaves was 1 cm (Pierik, 1987).

2.2.2. Preparation of culture medium

Murashige and Skoog (1962) (MS) medium was prepared and used (Table 1). Sucrose 20 g/l (Tawfik *et al.*, 1997), Myoinositol 0.1 g/l and the plant growth regulators (2,4-D and BA) at different concentrations were added. The pH was adjusted to 5.8 using NaOH or HCl (1N), then 8 g/l of the agar type (Agar-Agar) was added to the medium, placed on a hot plate magnetic stirrer till boiling, then aliquots of 20 ml were dispensed into (10×5 cm) culture vessels.

Culture media were sterilized by autoclaving at 121°C under (15 Ib/in^2) pressure, for 15 min. Glassware and other instruments either autoclaved or placed in electric oven at 200°C for 2 hrs. (Cappuecino and Sherman, 1987).The medium was left at room temperature (25°C) to cool and became ready to culture explants.

Table (1): Composition of Murashige and Skoog medium (1962)

Components	Chemical formula	Weight (mg/l)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride.$2H_2O$	$CaCl_2.2H_2O$	440
Magnesium sulphate.$4H_2O$	$MgSO_4.7H_2O$	370
Potassium phosphate monobasic	KH_2PO_4	170
Micronutrients		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Manganese sulphate.$4H_2O$	$MnSO_4.4H_2O$	22.30
Zinc sulphate.$7H_2O$	$ZnSO_4.7H_2O$	8.60
Molybdic acid (sodium salt).$2H_2O$	$Na_2MoO_4.2H_2O$	0.25
Cupric sulphate.$5H_2O$	$CuSO_4.5H_2O$	0.025
Cobalt chloride.$6H_2O$	$CoCl_2.6H_2O$	0.025
Chelated Iron		
Sodium ethylene diamine tetraacetate	Na_2-EDTA	37.3
Ferrous sulfate.$7H_2O$	$FeSO_4.7H_2O$	27.8
Vitamins and organics		
Thiamine.HCl (B_1)	$C_{l2}H_{17}ClN_4OS.HCl$	0.1
Nicotinic acid (free acid) (B_3)	$C_8H_{11}NO_3.HCl$	0.5
Pyridoxine.HCl (B_6)	$C_6H_5NO_2$	0.5
Glycine (free base)	$C_2H_5NO_2$	2.0
Myoinositol	$C_6H_6(OH)_6$	100
Sucrose	$C_{12}H_{22}O_{11}$	20000

2.2.3. Plant growth regulators

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

2.2.4. Incubation of cultures

Surface sterilized leaf explants 1 cm long were inoculated into the culture vessels with their abaxial surface down under aseptic conditions, placed in the incubator at 22-25 °C in dark (Caruso *et al.*, 2000).

2.2.5. Initiation of callus cultures

Different combinations of plant growth regulators were examined to determine the most effective one for callus induction. Leaves were placed onto MS medium containing 2,4-D and BA as in 2.2.3. Cultures were placed in the incubator at 25°C. The response of these leaves to auxin and cytokinin combinations was evaluated after 30 days in culture to determine the proper combination for callus induction.

2.2.6. Maintenance of callus cultures

The initiated callus was removed from the explants using forceps and scalpel, then pieces weighting 50 mg were subcultured onto fresh medium supplemented with the same combinations of 2,4-D and BA as in 2.2.3. Callus fresh weight was determined using sensitive balance, and then oven dried at 40°C for 24 hrs. for callus dry weight measurements (Bos, 1997).

2.2.7. Preparation of water and ethanolic extracts of *R. officinalis* leaves for antimicrobial activity

Leaves of rosemary (*R. officinalis*) were air-dried in shade and ground into fine powder using grinding machine (Abu-Shanab *et al.*, 2004).

2.2.7.1. Water extract

A quantity of 50 g of the leaves powder was mixed with 250 ml DDH_2O. The mixture was left in a shaker incubator at 37°C for 24 hrs., then filtered through a filter paper (Whatman no. 1). The filtrate was concentrated using rotary evaporator at 40°C until dryness and the extract residue was weighted and kept until used (Swanston *et al.,* 1990).

2.2.7.2. Ethanolic extract

A quantity of 50 g of leaves powder was extracted with 250 ml of 75% ethanol by soxhlet apparatus for 6 hrs. at 40-60 °C. The solution then evaporated to dryness using a rotary evaporator at 40°C. and the extract residue was weighted and kept until used (Harborne, 1973).

2.2.8. Preparation of water and ethanolic extracts of *R. officinalis* callus for antimicrobial activity

The water and ethanolic extracts of callus that originally initiated on explants was prepared for antimicrobial activity as below:

2.2.8.1. Water extract

A quantity of 10 g of callus powder was extracted with 50 ml DDH_2O. The mixture was left in a shaker incubator at 37°C for 24 hrs., then filtered through a filter paper (Whatman no. 1). The filtrate was concentrated using rotary evaporator at 40°C until dryness and the extract residue was weighted and kept until used (Swanston *et al.,* 1990).

2.2.8.2. Ethanolic extract

A quantity of 10 g of callus powder was extracted with 50 ml of 75% ethanol by soxhlet apparatus for 6 hrs. at 40-60 °C. The solution then evaporated to dryness using a rotary evaporator at 40°C. and the extract residue was weighted and kept until used (Harborne, 1973).

2.2.9. Detection of some active compounds of rosemary water and ethanolic leaf extracts

2.2.9.1. Detection of alkaloids

A liquot of 0.2 ml of the water or ethanolic extract was thoroughly mixed with 5 ml of 1% HCl in a steam bath, and then 1 ml was treated with Mayer's reagent. Appearance of white precipitate indicates a positive result (Stahl, 1969).

2.2.9.2. Detection of flavonoids

A) A quantity of 10 g of the powdered explants were macerated in 95% ethanol then filtered.

B) A liquot of 10 ml of 50% ethanol was added to 10 ml of 50% aqueous KOH. A solution was mixed with B solution. Appearance of yellow color indicates a positive result (Harborne, 1973).

2.2.9.3. Detection of phenols

Equal quantity of aqueous ferric chloride (1%) was mixed with potassium iron cyanide (1%). Equal quantity of the reagent and water or alcoholic plant extract was mixed. Appearance of blue-green color indicates a positive result (Harborne, 1984).

2.2.9.4. Detection of saponins

A) Water or ethanolic extract of the powdered plant material was shacked vigorously in a test tube. Formation of foam standing for a time indicates a positive result.

B) A liquots of 5 ml plant extract was added to 1-3 ml of (1%) ferric chloride solution. Appearance of white precipitate indicates a positive result (Stahl, 1969).

2.2.9.5. Detection of glycosides

Equal amounts of the water or ethanolic extract was mixed with Fehling's reagent in a test tube, and then boiled in a water bath for 10 min. Formation of red precipitate indicates a positive result (Shihata, 1951).

2.2.9.6. Detection of terpenes and steroids

One gram of dried water or ethanolic extract was suspended with a few drops of chloroform, and then a drop of (1%) acetic anhydride and a drop of concentrated sulphuric acid were added. Appearance of brown color indicates a positive result. Appearance of dark blue color after few minutes indicates the presence of steroids (Al-Abid, 1985).

2.2.9.7. Detection of tannins

A quantity of 10 g of the plant powder was mixed with 50 ml DDH_2O using a magnetic stirrer. The mixture was left boiling in a water bath for few minutes, and then filtered, and the filtrate was treated with a few drops of 1% lead-acetate solution. Development of greenish-blue precipitate indicates a positive result (Shihata, 1951).

2.2.10. Study of the antimicrobial activity

2.2.10.1. Preparation of microorganism culture media

The microorganisms (*S. aureus, P. aeruginosa* and *C. albicans*) were cultured on nutrient agar by mixing 20 g of agar powder with 8 g of nutrient broth powder in a conical flask, then the volume was completed to 1 liter with DDH_2O (Tilton *et al.,* 1992). Nutrient broth medium was prepared as recommended by manufacturing company. Culture media were sterilized by autoclaving at 121°C under (15 Ib/in^2) pressure, for 15 min (Cappuecino and Sherman, 1987).

These microorganisms were reidentified by using a set of biochemical tests as follow:

a. Identification of *S. aureus*

- Mannitol salt agar, coagulases test (Baird, 1996).

b. Identification of *P. aeruginosa*

- Oxidase test (Atlas, 1995), indole test (Collee *et al*., 1996).

c. Identification of *C. albicans*

- Germ tube formation test (Evans and Richardson, 1989), sugar fermentation test (Forbes *et al*., 1998).

2.2.10.2. Determination of the antimicrobial activity of water and ethanolic extracts (*in vitro*)

The activities of extracts were determined against target cells (bacterial and yeast isolates). For water and ethanolic extract, the stock solutions were prepared by dissolving 1 g of plant extract residue with 10 ml sterile DDH_2O. The stock solutions were sterilized by filtration using Millipore filter 0.45 μm under aseptic conditions (Ibrahim, 2003).

The extracts were prepared at different concentrations (20, 40, 60 and 80 mg/ml) and the antimicrobial effect of each concentration was measured. The nutrient agar medium was mixed well and 20 ml was poured in Petri-dishes. The medium was swabbed with 0.1 ml of a suspension containing 1.5×10^5 cfu/ml of the pathogenic bacteria (*S. aureus* or *P. aeruginosa*) and 1.5×10^3 cfu/ml of the pathogenic yeast (*C. albicans*) using sterile cotton swab. The hole-plate diffusion technique was used. Five plugs were removed from each agar plate using a sterile cork borer to produce 5mm-diameter hole. To each hole, 100 µl from different concentrations of each extract was added and allowed to diffuse at room temperature for 20 min (Clark *et al.,* 1981; Shihchih *et al.,* 1990; El-Astal *et al.,* 2005).

To identify the intrinsic extracts activity (water and ethanolic extracts for leaf and callus cultures), one control well was filled with 100 µl sterile DDH_2O. The plates were incubated at 37°C for 24 hrs. for bacteria and 48 hrs. for yeast. Each extract was tested against each microorganism in triplicate. The antimicrobial activity of the plant extracts were recorded as the mean diameter of the resulting inhibition zones of growth measured in millimeters (Santoyo *et al.,* 2005).

2.2.10.3. The effect of ethanolic callus extract on mice skin (*in vivo*)

To evaluate the antimicrobial effect of rosemary ethanolic callus extract on mice skin infected with pathogenic *S. aureus* isolated from infected skin.

The mice were divided into 4 groups (A, B, C and D), each consisted of 3 mice. The back of the mice skin was cleaned with distilled water and then shaved with paste. After removal of the hair from the back and cleaned with distilled water and cotton sterilized with 75% ethanol and scratched using

sterilized scalpel; the skin became visually damaged and was characterized by reddening but no regular bleeding (Kugelberg *et al.,* 2005).

Animal groups:

Group A: after scratching of the skin, a bacterial infection was initiated by placing on the skin 50µl droplets containing 1.5×10^5 cfu/ml *S. aureus* concentrated from an overnight *S. aureus* culture. The Infected animals treated topically with callus ethanolic extract of 80 mg/ml.

Group B: the scratched skin infected with *S. aureus* by placing on the skin 50µl droplets containing 1.5×10^5 cfu/ml concentrated from an overnight *S. aureus* culture. The infected animals treated topically with commercial skin ointment Samacycline.

Group C: the scratched skin infected with *S. aureus* by placing on the skin 50µl droplets containing 1.5×10^5 cfu/ml concentrated from an overnight *S. aureus* culture. The infected animals treated topically with DDH_2O.

Group D: the scratched skin infected with sample which prepared as follow: a liquot of 0.5 ml of ethanolic callus extract 80 mg/ml mixed with 0.5 ml of nutrient broth medium, and then 0.1 ml of 1.5×10^5 cfu/ml *S. aureus* suspensions was inoculated and incubated at 37°C for 24 hrs., then 50µl droplets of this sample were placed on the scratched skin.

2.2.11. Statistical analysis

A completely randomized design (CRD) was used with 12 replicates. Least significant differences (LSD) were calculated. Means were compared at probability of 0.05. For antimicrobial activity, means were calculated and standard deviations were computed for three sample replicates (Gomes and Gomes, 1984).

Chapter Three

Results and Discussion

3. Result and Discussion

3.1. Sterilization of explants

NaOCl was used for explants sterilization (leaves) of *R. officinalis.* (Fig. 5) shows that the most effective concentration of NaOCl was 1% for 15 min. that gave the highest percent 100% of survival, increasing the concentration to 3% caused damage to plant tissues, whereas lowering the concentration of NaOCl led to high rate of contamination.

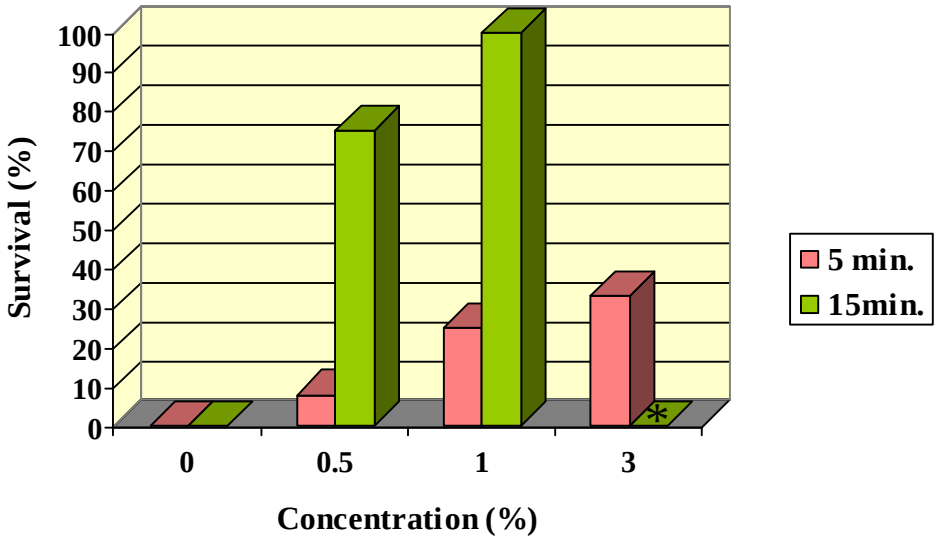

Fig. (5): Effect of different concentrations of NaOCl on explants survival at sterilization periods of 5 or 15 min. (n= 12). *Explants were seriously damaged by NaOCl.

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

Pierik, (1987) referred to the importance of NaOCl in explants sterilization and found that increasing the surface sterilization period and concentration often leads to serious reduction in survival rate. Therefore, optimization experiment is 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 (%) to callus induction on leaf explants is shown in (Table 2).

All concentrations of 2,4-D led to a significant increase in the percentage of explants showed callus induction 50.0, 72.2 and 80.4% at the concentrations 0.5, 1.0 or 2.0 mg/l respectively compared with the control one 22.2%.

Treatment with 1.0 mg/l of BA resulted in a mean value of 54.0 mg/l which decreased significantly than those treated with 0.5 mg/l of BA which gave 72.9 mg/l.

The interaction between the two growth regulators achieved 100% response (Fig. 6) in a combination of 0.5 mg/l BA and 2.0 mg/l 2,4-D. However, it was found that the presence of 2,4-D only was less effective than a combination of 2,4-D and BA for callus induction.

These results are in agreement with Ramawat (2008) who stated that callus induction in a number of plant species favors higher auxins than cytokinins.

Table (2): Effect of different concentrations of 2,4-D and BA and their interactions on the response (%) of callus induction on *R. officinalis* leaf explants in dark (n=12).

2,4-D (mg/l)	BA (mg/l)			Mean
	0.0	0.5	1.0	
0.0	16.7	25.0	25.0	22.2
0.5	41.7	75.0	33.3	50.0
1.0	50.0	91.7	75.0	72.2
2.0	58.3	100	83.0	80.4
Mean	41.6	72.9	54.0	
LSD 0.05	**BA**= 4.3	**2,4-D**= 6.7	**BA ×2,4-D**= 12.4	

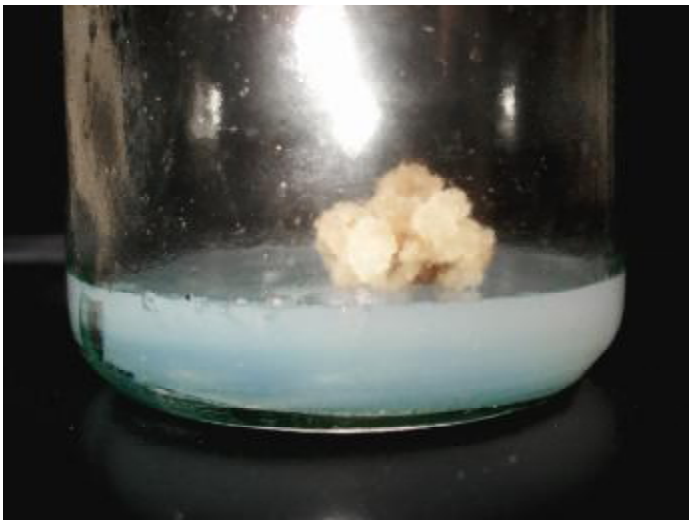

Fig. (6): Callus induction on leaf explants grown on MS medium containing a combination of 0.5 mg/l BA and 2.0 mg/l 2,4-D, 30 days cultured in dark.

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

3.3. Maintenance of callus cultures

A quantity of 50 mg of callus cultures induced on leaf explants from the best combination of BA and 2,4-D 0.5 and 2.0 mg/l respectively, were inoculated into the same combinations of plant growth regulators used for callus induction to determine the appropriate concentration for callus maintenance.

Inclusion of 2,4-D at the concentration 2.0 mg/l gave significantly higher callus fresh weight (1042.89mg) than other concentrations, while the lowest fresh weight (105.26mg) obtained in 2,4-D free culture medium (Table 3).

The result agree with Caruso *et al.* (2000) who proved that rosemary callus grown on medium containing 2.0 mg/l 2,4-D in the dark increased in size. The highest callus fresh weight obtained in BA treated callus cultures 992.24 mg was at the concentration 0.5 mg/l.

The interaction between the two growth regulators at the levels 0.5 mg/l BA and 2.0 mg/l 2,4-D and resulted in a maximum callus fresh weight reached 1856.330 mg, and all the treatments were significantly higher than the control one (30.90mg) as shown in (Fig. 7).

Table (3): Effect of different concentrations of 2,4-D and BA and their interactions on callus fresh weight (mg) grown on a maintenance medium in dark. Initial weight was 50 mg (n= 12).

2,4-D (mg/l)	BA (mg/l)			Mean
	0.0	0.5	1.0	
0.0	30.90	136.618	148.267	105.26
0.5	350.21	877.167	165.717	464.36
1.0	498.00	1098.850	680.230	759.026
2.0	551.983	1856.330	720.383	1042.89
Mean	357.77	992.24	428.64	
LSD 0.05	**BA=** 59.13	**2,4-D=**175.61	**BA×2,4-D=** 265.63	

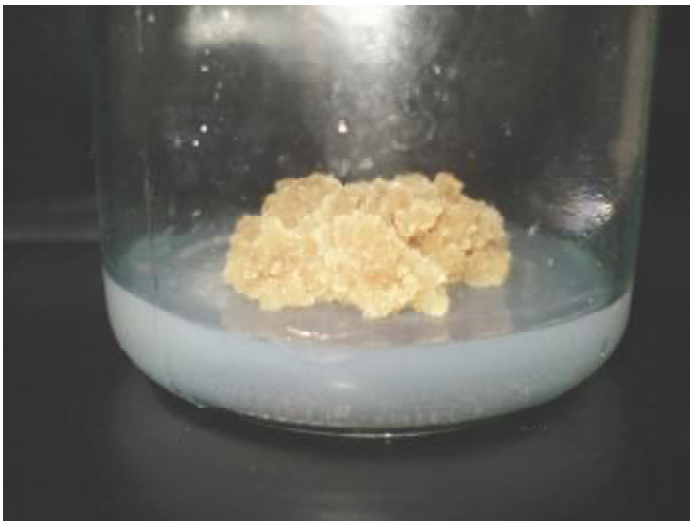

Fig. (7): Callus cultures originated from leaf explants grown on maintenance medium containing 0.5 mg/l BA and 2.0 mg/l 2,4-D. Cultures were continuously cultured on fresh medium at 30 days intervals in dark.

Dry weights of callus cultures initiated from leaf explants are shown in (Table 4).

Treatment with 2,4-D at the concentration of 2.0 mg/l gave significantly higher callus dry weight (187.150 mg) than control treatment where no 2,4-D was added to the culture medium.

The highest callus dry weight obtained in BA treated callus cultures (175.09 mg) was at the concentration 0.5 mg/l.

The highest callus dry weights were exhibited at the combination of 0.5 mg/l BA and 2.0 mg/l 2,4-D. The weight was 278.707 mg which was significantly higher than all other treatments except between those treated with the combination of 1.0 mg/l 2,4-D and 0.5 mg/l BA.

Table (4): Effect of different concentrations of 2,4-D and BA and their interaction on callus dry weight (mg) initiated on leaf explants of *R. officinalis* and grown on maintenance medium in dark (n= 12).

2,4-D (mg/l)	**BA (mg/l)**			**Mean**
	0.0	0.5	1.0	
0.0	7.73	52.64	66.471	42.28
0.5	93.798	163.218	80.307	112.407
1.0	110.079	205.802	144.82	153.567
2.0	130.286	278.707	152.459	187.150
Mean	85.44	175.09	111.01	
LSD 0.05	**BA=** 27.80	**2,4-D=** 49.96	**BA×2,4-D=** 114.28	

It was found that the presence of 2,4-D only was less effective than a combination of 2,4-D and BA for callus growth. Xu *et al*. (2008) published similar result with callus growth of *Agastache rugosa.*

According to the results stated above, callus was induced on leaf explants then maintained for many subcultures on MS medium containing 0.5 mg/l of BA and 2.0 mg/l of 2,4-D for subsequent experiments.

Induction and maintenance of callus cultures in *R. officinalis* seem to favor high levels of 2,4-D and low level of BA. Callus induction requires a balanced ratio from auxin(s) and cytokinin(s) as stated by Skoog and Miller (1957). It would be convenient from the practical point of view to induce and maintain callus on the same growth nutrients and plant growth regulators requirements.

The increase of callus mass is important for the production of secondary metabolites since they are proportionally related (Ramawat, 2008).

Callus induction and differentiation are influenced by many factors: medium component, type and concentration of plant growth regulators, plant physiology, source of explants and environmental conditions (Torbert *et al.*, 1998).

3.4. Plant extracts

3.4.1. Water extracts

Weight of the dried residue from leaves extract resulted after evaporation of water was 8 g representing 16% of the dried weight of leaves. The extract appeared with dark brown color with a specific odor. Weight of the dried residue from callus extract resulted after evaporation of water was 1.2 g representing 12% of the dried weight of callus. The extract appeared with dark brown color (Table 5).

Table (5): Weight and percentages of dried leaf and callus tissues used for water extracts.

Powder weight (g)		Water extract	Percentage
Leaves	50	8	16
Callus	10	1.2	12

3.4.2. Ethanolic extracts

Weight of the dried residue from leaves extract resulted after evaporation of ethanol was 3.5 g representing 7% of the dried weight of leaves. The extract appeared with greenish black color with a specific odor. Weight of the dried residue from callus extract resulted after evaporation of ethanol was 0.56 g representing 5.6% of the dried weight of callus. The extract appeared with brown color (Table 6).

Table (6): Weight and percentages of dried leaf and callus tissues used for ethanolic extracts.

Powder weight (g)		Ethanolic extract	Percentage
Leaves	50	3.5	7
Callus	10	0.56	5.6

The relative proportion between the amount of plant used for extraction and crude product was variable depending on several factors, such as the method of extraction and solvent used in extraction process as well on plant species (Henning *et al*., 2003).

The specific odor of plant extracts is caused by the high percentage of phenol compounds and terpens (Cherevaty *et al.*, 1980).

3.5. Detection of some active compounds in rosemary leaves

The pH of water and ethanolic extracts was 6.5 and 5.3 respectively indicating the acidity of water and ethanolic extracts.

Results obtained using chemical detection indicated the presence of alkaloids, flavonoids, phenols, saponins, terpenes, steropids and tannins in rosemary water extract, while flavonoids, phenols, saponins, glycosides, terpenes, steroids and tannins was detected in rosemary ethanolic extract (Table 7).

Table (7): Secondary metabolites detected in water and ethanolic extracts of *R. officinalis* leaves.

Type of secondary metabolite	Result of detection (leaves)	
	Water extract	Ethanolic extract
Alkaloids	+ve	-ve
Flavonoids	+ve	+ve
Phenols	+ve	+ve
Saponins	+ve	+ve
Glycosides	-ve	+ve
Terpenes	+ve	+ve
Steroids	+ve	+ve
Tannins	+ve	+ve

+ve indicates the presence of secondary metabolites.
-ve indicates the absence of secondary metabolites.

Hossnzadeh and Nourbakhsh, (2003) detected the presence of flavonoids, tannins and saponins in *R. officinalis* water and ethanolic extracts, while alkaloids was detected only in the water extract. Phenols and terpenes were isolated and identified from *R. officinalis* by Almela *et al.* (2006).

3.6. Identification of microorganisms

a. Identification of *Staphylococcus aureus*

S.aureus gave positive results for mannitol fermenting ability and coagulase.

b. Identification of *Pseudomonas aeruginosa*

P. aeruginosa gave positive results for oxidase, while it gave negative results for indol production.

c. Identification of *Candida albicans*

C. albicans gave positive results for germ tube formation and ability to ferment glucose and maltose.

3.7. Antimicrobial activity of water and ethanolic extracts (*in vitro*)

3.7.1. Effect of leaves and callus water extracts

Results displayed in (Table 8) indicate that the high concentrations of rosemary leaves water extract 80 or 60 mg/ml have inhibitory effects against *S. aureus* with 13.01 and 12.13 mm inhibition zone diameter respectively, while 11.06 mm was recorded at the concentration 40 mg/ml and slight inhibition at the concentration of 20 mg/ml (Fig. 8a).

While *P. aeruginosa* had no response against *R. officinalis* water extract (Fig. 8b).

In *C. albicans*, the high concentrations of water extract 80 or 60 mg/ml showed the diameter of 11 and 10.13 mm inhibition zone respectively and 9.67 mm at the concentration of 40 mg/ml. No inhibition was observed at the concentration of 20 mg/ml (Fig. 8c).

Table (8): Diameter of inhibition zone caused by *R. officinalis* leaves water extract at various concentrations against G+ve, G-ve bacteria and yeast.

Conc. mg/ml	Diameter of inhibition zone (mm)		
	S. aureus	***P. aeruginosa***	***C. albicans***
Control (DDH_2O)	0.00	0.00	0.00
20	Slight inhibition	0.00	0.00
40	11.06±0.63	0.00	9.67±0.32
60	12.13±0.47	0.00	10.13±0.13
80	13.01±0.46	0.00	11.00±0.57

Values = are means of 3 sample readings± SD.

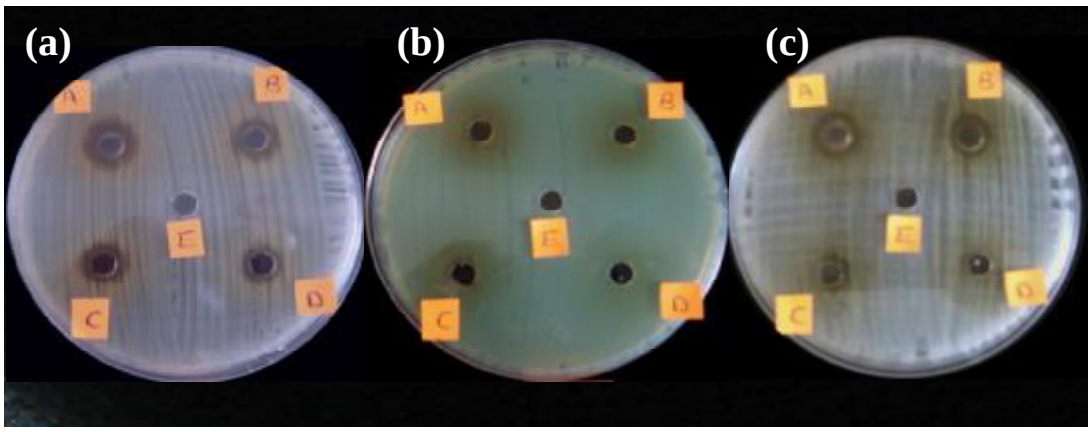

Fig (8): Effect of *R. officinalis* leaves water extract on the growth of a- *S. aureus*, b- *P. aeruginosa*, c- *C. albicans.*

A= 80 mg/ml, B= 60 mg/ml, C= 40 mg/ml, D= 20 mg/ml, E= control (DDH_2O).

Water extract of rosemary callus (Table 9) showed the high concentrations of rosemary callus water extract 80 or 60 mg/ml have inhibitory effects against *S. aureus* with 16.00 and 15.01 mm inhibition zone diameter respectively, while 12.03 mm was recorded at the concentration of 40 mg/ml and 11.00 mm at the concentration of 20 mg/ml (Fig. 9a).

While *P. aeruginosa* had no response against *R. officinalis* callus water extract (Fig. 9b).

In *C. albicans*, the high concentrations of water extract 80 or 60 mg/ml showed the diameter of 14.00 and 12.13 mm inhibition zone subsequently and 11.09 mm at the concentration of 40 mg/ml. Slight inhibition was observed at the concentration of 20 mg/ml (Fig. 9c).

Table (9): Diameter of inhibition zone caused by *R. officinalis* callus water extract at various concentrations against G+ve, G-ve bacteria and yeast.

Conc. mg/ml	Diameter of inhibition zone (mm)		
	S. aureus	***P. aeruginosa***	***C. albicans***
Control (DDH_2O)	0.00	0.00	0.00
20	11.00±0.81	0.00	Slight inhibition
40	12.03±0.92	0.00	11.09±0.32
60	15.01±0. 45	0.00	12.13±0.17
80	16.00±0. 47	0.00	14.00±0.61

Values = are means of 3 sample readings± SD.

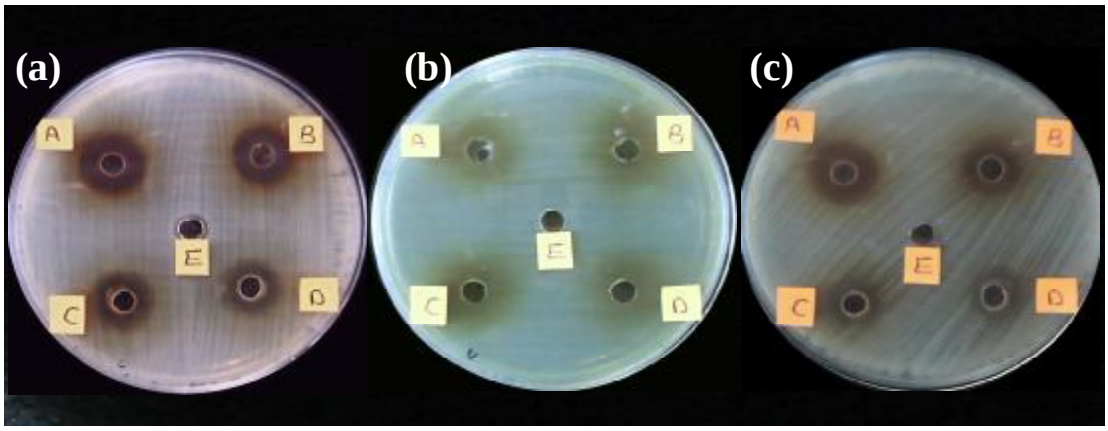

Fig (9): Effect of *R. officinalis* callus water extract on the growth of a- *S. aureus*, b- *P. aeruginosa*, c- *C. albicans.*
A= 80 mg/ml, B= 60 mg/ml, C= 40 mg/ml, D= 20 mg/ml, E= control (DDH $_2$O).

It appears that callus water extract is more efficient than leaves water extract. The reason for this may be due to the compounds produced in callus cultures.

Rosmarinic acid is readily accumulated in undifferentiated plant cell cultures; in some cases its concentrations are much higher than in the plant itself (Razzaque and Ellis, 1997). Plants arising from tissue culture can be a source of useful natural products (Smith, 1996).

The inhibitory ability was more pronounced against *S. aureus*, whereas it showed no activity against Gram-negative bacteria presented in *P. aeruginosa*.

These results are in agreement with these of Abu-Shanab *et al.* (2004) who proved that rosemary water leaves extract at 100 mg/ml showed inhibitory effects against *S. aureus* and no inhibitory effects against *P. aeruginosa*.

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 Duke (1985) who proved that *R. officinalis* contains some of the most powerful *C. albicans* killing substances like eucalyptol.

The antimicrobial action of the water extract of rosemary could be ascribed to the water soluble component of rosemary. Water extract contains 15% of rosmarinic acid (Moreno *et al*., 2006). While rosmarinic acid exhibits antimicrobial activity as stated by Szabo *et al.* (1999) and Hras *et al.* (2000).

3.7.2. Effect of leaves and callus ethanolic extracts

Results displayed in (Table 10) showed that rosemary ethanolic leaves extract had antibacterial action against *S. aureus* at the high concentrations of the extract 80 and 60 mg/ml.

The largest inhibition zones were observed against *S. aureus* recording 15.11 mm in diameter at the concentration of 80 mg/ml whereas, reached 13.07 mm at the concentration of 60 mg/ml. At low concentrations of the extract 40 or 20 mg/ml, *S. aureus* showed the inhibition zone reached 10 and 9.97 mm respectively (Fig. 10a).

This followed by *P. aeruginosa* which showed no inhibition zone at all the tested concentrations (Fig. 10b).

C. albicans growth was inhibited at the extract concentrations 20, 40, 60 or 80 mg/ml. It showed 9.03, 10.13, 11.07 and 12.39 mm inhibition zones diameter respectively (Fig. 10c).

Table (10): Diameter of inhibition zone caused by *R. officinalis* leaves ethanolic extract at various concentrations against G+ve, G-ve bacteria and yeast.

Conc. mg/ml	Diameter of inhibition zone (mm)		
	S. aureus	***P. aeruginosa***	***C. albicans***
Control (DDH_2O)	0.00	0.00	0.00
20	9.97±0.06	0.00	9.03±0.49
40	10.00±0.06	0.00	10.13±0.10
60	13.07±0.18	0.00	11.07±0.12
80	15.11±0.34	0.00	12.39±0.68

Values = are means of 3 sample readings± SD.

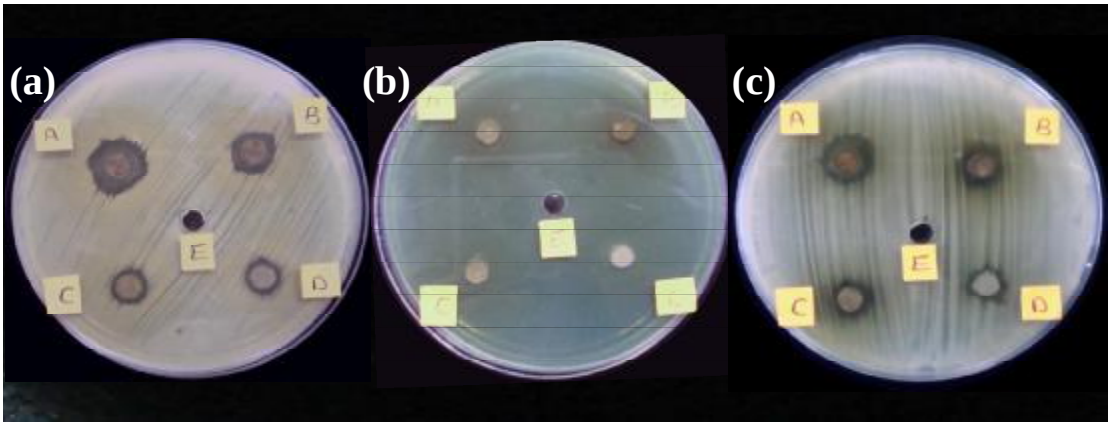

Fig (10): Effect of *R. officinalis* leaves ethanolic extract on the growth of a- *S. aureus,* b- *P. aeruginosa,* c- *C. albicans.*

A= 80 mg/ml, B= 60 mg/ml, C= 40 mg/ml, D= 20 mg/ml, E= control (DDH_2O).

Ethanolic extract of rosemary callus (Table 11) showed antimicrobial action against all the tested microorganisms at the concentration of 80 mg/ml. The largest inhibition zone was observed against *S. aureus* recording 19.03 mm in diameter at 80 mg/ml whereas, decreased to 15.19 mm at the concentration of 60 mg/ml. The lower concentrations of the extract 40 or 20 mg/ml, *S. aureus* showed 13.13 and 11.34 mm respectively (Fig. 11a).

P. aeruginos showed a slight inhibition zone at the concentration of 80 mg/ml (Fig. 11b).

While *C. albicans* was inhibited at the extract concentrations 20, 40, 60 or 80 mg/ml. It showed 10.79, 11.09, 13.30 and 15.11 mm inhibition zones diameter (Fig. 11c).

Table (11): Diameter of inhibition zone caused by *R. officinalis* callus ethanolic extract at various concentrations against G+ve, G-ve bacteria and yeast.

Conc. mg/ml	Diameter of inhibition zone (mm)		
	S. aureus	***P. aeruginosa***	***C. albicans***
Control (DDH_2O)	0.00	0.00	0.00
20	11.34±0.41	0.00	10.79±0.36
40	13.13±0.13	0.00	11.09±0.18
60	15.19±0.54	0.00	13.30±0.79
80	19.03±0.26	slight inhibition	15.11±0.34

Values = are means of 3 sample readings± SD.

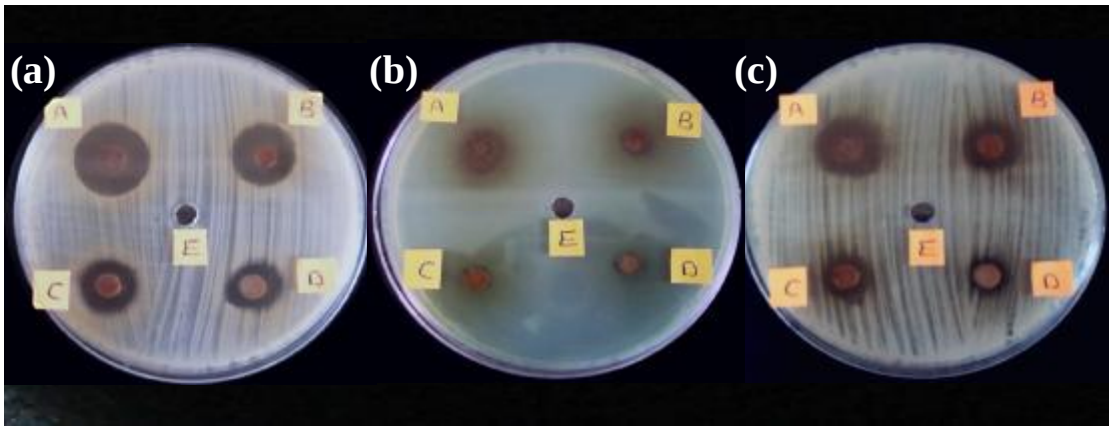

Fig (11): Effect of *R. officinalis* callus ethanolic extract on the growth of a- *S. aureus*, b- *P. aeruginosa*, c- *C. albicans*.
A= 80 mg/ml, B= 60 mg/ml, C= 40 mg/ml, D= 20 mg/ml, E= control (DDH $_2$O).

It appears that ethanolic callus extract is more efficient than ethanolic leaves extract. The reason for this may be due to the accumulation of secondary products in plant cell cultures as stated by (Stafford and Morris, 1986). Additionally, Tawfik *et al.* (1997) who found that β-pinene levels in rosemary callus cultures reached highest when 20 g /l sucrose was added to rosemary culture medium. β-pinene was inhibitory to bacterial and yeast cells, the effect can be ascribed to a localization of β-pinene on membranes caused an increase in the fluidity of the membrane (Uribe *et al.*, 1985; Scortichini and Rossi 1991).

These results agree with Abu-Shanab *et al.*, (2004) who showed that Gram-positive bacteria *S. aureus* is sensitive to *R. officinalis* ethanolic leaves extract at the concentration of 100 mg/ml, while *P. aeruginosa* was resistant to the ethanolic leaves extract at the same concentration.

In addition Nascimento *et al.,* (2000) referred that rosemary ethanolic leaves extract has antimicrobial activity against *C. albicans* and no activity against *P. aeruginosa*.

Data presented in this study confirm that ethanolic extracts is more efficient than water extracts. The reason for this may be due to the compounds already extracted by ethanol particularly terpenoids and polyphenols as stated by (Cowan, 1999).

Although *R. officinalis* contains many active compounds, most studies attributed the antimicrobial activity in rosemary to diterpens and triterpens, carnosol and ursolic acid respectively (Alonso, 2004).

Additionally, Moreno *et al*. (2006) suggested that the antimicrobial of rosemary leaves extracts efficacy is associated with their specific phenolic composition.

Santoyo *et al.* (2005) proved that the activity of *R. officinalis* attributed to the existence of borneol that showed high antimicrobial activity.

It has been hypothesized that the microorganisms' inhibition involves phenolic compounds, because these compounds sensitize the phospholipids bilayer of the microbial cytoplasm membrane causing increased permeability, unavailability of vital intracellular constituents and/or impairment of bacterial enzymes systems (Farag *et al.,* 1989; Juven *et al.,* 1994; Kim *et al.,* 1995; Wendakoon and Skaguchi, 1995).

In addition Manohar *et al.* (2001) referred that rosemary has thymol which is considered active ingredient as fungicide, disinfectant, bactericide and virucide. Thymol inhibits the formation of the germinative tube of *C. albicans,* besides inhibiting the growth of the yeast.

Generally, Gram-negative bacteria have been reported to be more resistant than Gram-positive bacteria to rosemary extracts because of their cell wall lipopolysaccharide (Russel, 1991). Cell wall lipopolysacaride may prevent extracts active compounds in reaching the cytoplasmic membrane of Gram-negative bacteria (Chanegriha *et al.,* 1994).

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

It is clear from the data presented in (Table 8, 9, 10 and 11) that among the three tested microorganisms, *S. aureus* was the most susceptible microbe to the four extracts. The finding that *S. aureus* is susceptible to a variety of extracts was recorded by several researchers such as Okemo *et al*. (2001) and Madamombe and Afolayan (2003). Generally, the antimicrobial activity of each extract was dependant on the type of the extract, method of extraction, concentration and the type of microorganism.

3.8. Antimicrobial activity of ethanolic callus extract (*in vivo*)

After scratching the back of the mice skin and infected with *S. aureus*, the infection was appeared at the 8^{th} day and the skin of the infected animals appeared with congestion and lesions. Red granulated tissue, pus and separation of small sheets of epidermis were clear for the infected area of group A (Fig. 12) and group B (Fig. 15).

These changes may due to many virulence factors caused by *S. aureus* such as exfoliative toxin which is an exotoxin produced by *S. aureus*, causing blisters in human and animal skin, detachment within the epidermal layer and the loss of keratinocyte cell–cell adhesion in the superficial epidermis (Ladhani *et al.,* 1999; Yamaguchi *et al.,* 2002; Nishifuji *et al.,* 2008).

For group D the scratched skin of the animals infected with the sample which consist of (*S. aureus* and ethanolic callus extract of 80 mg/ml incubated at 37°C for 24 hrs.) appeared without infection at the 8th day of the infection (Fig. 19), this indicate that rosemary ethanolic callus extract beside inhibition the growth of *S. aureus* and develop infection it facilitate the healing of the damaged skin, the result agree with Germany's Commission E, it approves the external use of rosemary preparation for promotion of wound healing and as antiseptic (ESCOP, 1997).

3.9. Treatment of the infected animals

Group A (ethanolic callus extract treatment): animals infected with *S. aureus* and then treated with *R. officinalis* ethanolic callus extract have shown certain regenerative changes. The redness disappeared at the 7th day of treatment (Fig. 13). The initial depression of the healing incision line disappeared at the 12th day of the treatment. This incision was completely recovered newly epithelium and hair as shown in (Fig. 14).

This extract was considered to have antibacterial activity. This activity is due to the phenolic and terpenoid compounds extracted by ethanol (Cowan, 1999).

Group B (commercial skin ointment treatment): the same regenerative changes but with remaining skin swelling were seen with the animals infected with *S. aureus* and treated with commercial skin ointment (Samacycline). The redness disappeared at the 7th day of the treatment (Fig. 16), but the initial depression of the healing incision lines disappeared at the 17th day of the treatment. This incision was completely recovered newly epithelium and hair as shown in (Fig. 17).

Group C (DDH_2O treatment): showing epidermal splitting and skin peeling at the 7^{th} day of the treatment (Fig. 18).

According to these results, the healing of animal skin infected with *S. aureus* and treated with ethanolic extract (extracted from rosemary callus) took 12 days, while it took 17 days for infected area to heal using commercial skin ointment for treatment. This indicates that ethanolic callus extract was more efficient than commercial skin ointment in treatment of skin infected with *S. aureus*.

The results agree with Hsu, (2005) who showed that rosemary leaves extract used topically for wound healing and disease treatments. In addition rosemary has high degree of skin penetration, anti-microbial and anti-inflammatory activity (Al-Sereitia *et al.,* 1999).

Calabrese *et al.* (2002) reported evidence that an alcoholic extract of rosemary leaves, is endowed with strong antioxidant activity and, as evaluated by both *in vitro* and *in vivo* systems, is capable of inhibiting oxidative alterations to skin surface lipids.

Morphological changes and repair of group A

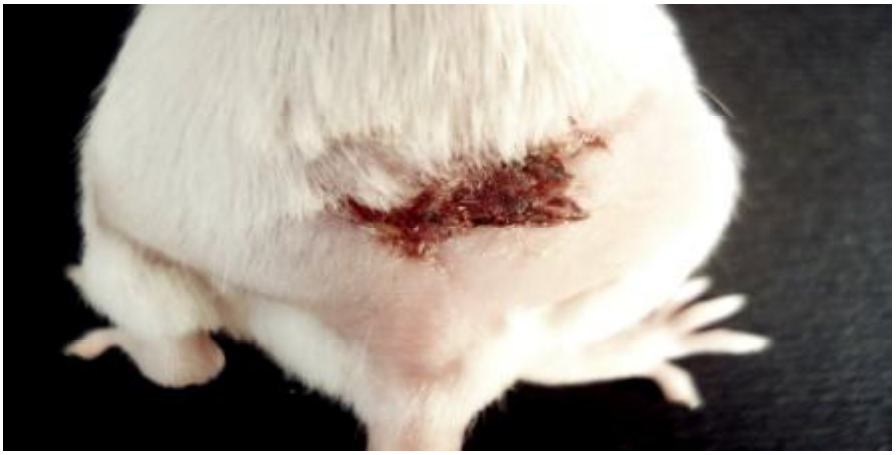

Fig. (12): Morphological changes caused by *S. aureus* at 8th day of the infection.

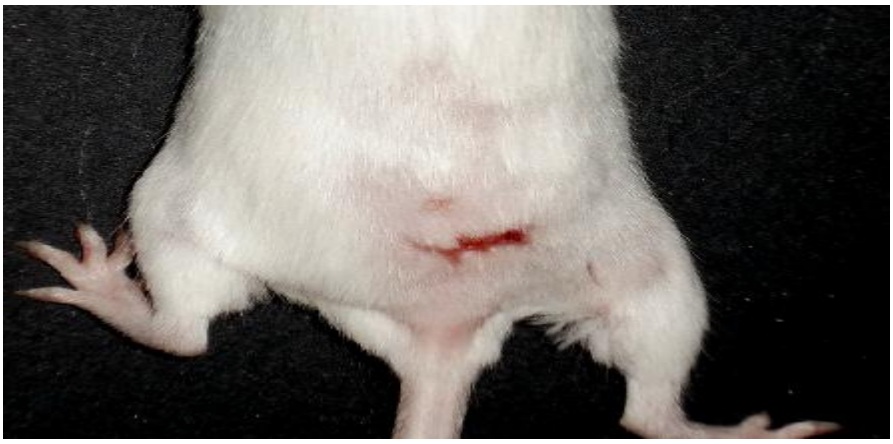

Fig. (13): Morphological repair of an animal infected with *S. aureus* treated with rosemary ethanolic callus extract (80 mg/ml) at the 7 th day of treatment.

Fig. (14): Morphological repair of an animal infected with *S. aureus* treated with rosemary ethanolic callus extract (80 mg/ml) at the 12 th day of treatment.

Morphological changes and repair of group B

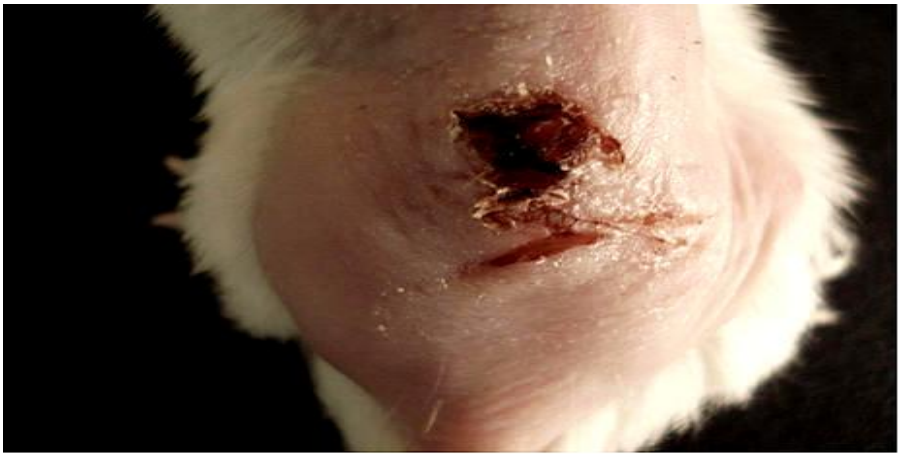

Fig. (15): Morphological changes caused by *S. aureus* at the 8th day of the infection.

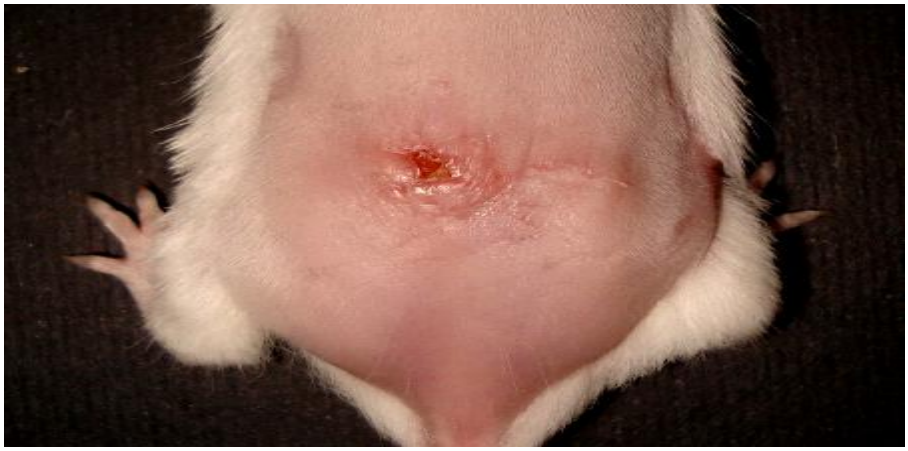

Fig. (16): Morphological repair of an animal infected with *S. aureus* treated with skin ointment (Samacycline) at the 7th day of treatment.

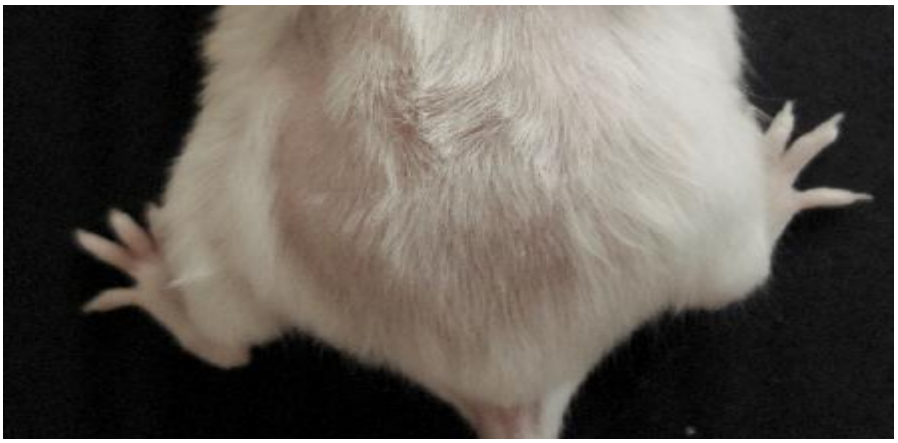

Fig. (17): Morphological repair of an animal infected with *S. aureus* treated with skin ointment (Samacycline) at the 17th day of treatment.

Morphological changes of group C

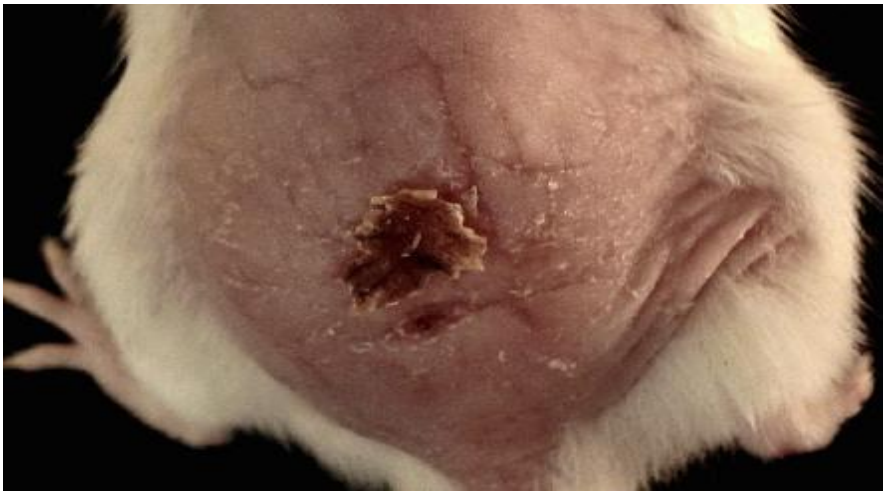

Fig. (18): Morphological changes caused by *S. aureus* treated with DDH_2O at the 7th day showing epidermal splitting and skin peeling.

Morphological changes of group D

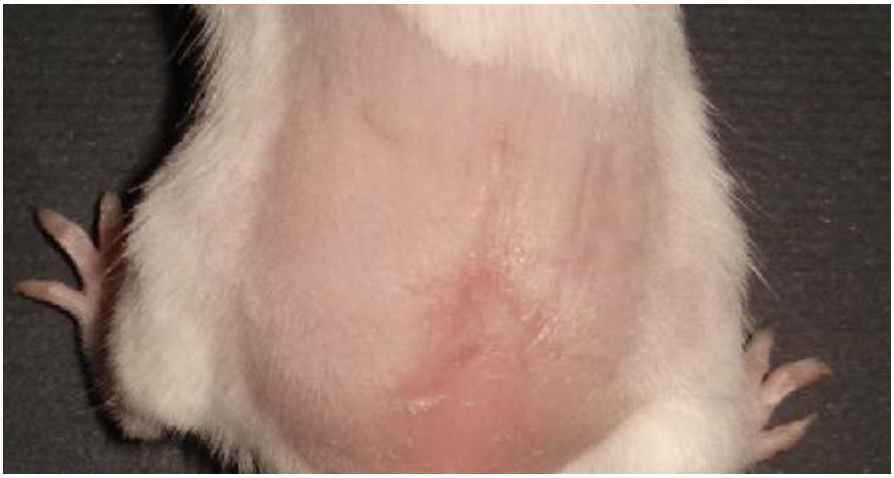

Fig. (19): Morphological changes caused by the sample (*S. aureus* and ethanlic callus extract of 80 mg/ml incubated at 37°C for 24 hrs.) at the 8th day of the infection.

4. Conclusions and Recommendations

4.1. Conclusions

1. Ethanolic extracts are more efficient than water extracts, while callus extracts are more efficient than leaf extracts as inhibitory effect on growth of *S. aureus* and *C. albicans*.
2. High concentrations of rosemary water callus extract 60 and 80 mg/ml have inhibitory effects against Gram-positive bacteria *S. aureus* and yeast *C. albicans*, whereas it showed no activity against *P. aeruginosa.*
3. Ethanolic callus extract can be used at the concentrations 60 and 80 mg/ml against the growth of *S. aureus* and *C. albicans*.
4. Ethanolic callus extract superior in healing wound in tested animals compared to the commercial skin ointment (Samacycline).

Chapter Four

Conclusions and Recommendations

4.2. Recommendations

1. Investigation of other medicinal plants as a source for phytochemicals using tissue culture techniques.
2. Investigating physical elicitors such as high or low pH, different light intensities, different light qualities and different temperature regimes for increasing the production of secondary metabolites in tissue cultures of rosemary.
3. Using genetic engineering approaches for the manipulation of genes responsible for key enzymes for higher production of secondary metabolites such as gene transfer techniques and hairy root production using *Agrobacterium rizogenes* as a vector.
4. Purification of the target secondary metabolite then examination for antimicrobial activity to find out which compound is the most effective.
5. Examination of the synergistic effect of antibiotics and plant extracts.
6. Examination of extracts on cell lines to be used as antitumor.

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الخلاصـــة

في محاولة لمعالجة اصابة جلد الفئران باستخدام المستخلص الكحولي لنسيج كالس نبات اكليل الجبل تم تنفيذ عدد من التجارب لهذا الغرض. استحث الكالس واديم على وسط Murashige and Skoog لمدة ثلاثين يوما المجهز بـ 2.0 ملغم/لتر من 2,4-dichlorophenoxyacetic acid و 0.5 ملغم/لتر من benzyl adenine باستعمال الاوراق النباتية لاكليل الجبل كمصادر لنشوء الكالس.

اشارت النتائج الى ان المستخلصين المائي والكحولي لاوراق اكليل الجبل والكالس له فعالية مضادة للبكتريا و الخميرة. تم الكشف الكيميائي لمكونات المستخلص المائي لاوراق النبات وقد احتوى على شبه القلويدات، الفلافينويدات، الفينولات، الصابونيات، التيربينات، الستيرويدات والتانينات، بينما احتوى المستخلص الكحولي لاوراق النبات على الفلافينويدات، الفينولات، الصابونيات، الكلايكوسيدات، التيربينات، الستيرويدات والتانينات.

كما واوضحت النتائج بان المستخلص الكحولي للاوراق قد اعطى اعلى فعالية تثبيطية من المائي في نمو *Staphylococcus aureus* و *Candida albicans* ولم يعطي اي فعالية تثبيطية في نمو*Pseudomonas aeruginosa* . في حين اظهرت مستخلصات الكالس فعالية تثبيطية اعلى من مستخلصات الاوراق ولنفس العزلات المايكروبية.

لوحظ ان المستخلص الكحولي لانسجة الكالس له اعلى فعالية تثبيطية في نموعزلة *S. aureus* المسببة لالتهاب الجلد بتركيز 80 ملغم/مل، لذالك استعمل هذا المستخلص على الفئران مختبريا. لقد اظهرالمستخلص الكحولي لانسجة الكالس بان له تاثيرات مضادة للبكتريا، للالتهابات وتاثير معالج اسرع من مرهم الجلد التجاري بتركيز 80 ملغم/مل على جلد الفئران المصابة بـ *S. aureus* .

الاهـــداء

الى واحتي الخضراء ومن كان دعاؤها نوراً اضاء لي الارجاء ....

والدتي الحبيبة

الى بحر العطاء وقدوة المربين والاباء ....

والدي الغالي

الى نجوم لي في السماء ....

اخواتي

الى سندي الدائم ....

زوجي العزيز

اهدي لهم ثمرة جهدي المتواضع ... كبعض من الوفاء

آمـــال

بِسْمِ ٱللَّهِ ٱلرحمَنِ ٱلرَّحِيمِ

قُل كُلٌ يَعمَلُ عَلى شَاكِلَتِهِ فَرَبُّكُم اعلَمُ
وَيَسئَلُونَك عَنِ ۞ بِمَن هُوَ اهدَى سَبِيلاً
الرُّوحِ قُلِ الرُّوحُ مِن أمرِ رَبِّي وَمَا
۞ أُوتِيتُم مِن العِلمِ اِلاَّ قَلِيلاً

صَدَقَ ٱللَّهُ ٱلعَظِيمِ

سورة الاسراء
الاية (٨٤-٨٥)

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

تأثير مستخلصات أنسجة نبات اكليل الجبل (*Rosmarinus officinalis* L.) في نمو بعض الاحياء المجهرية المسببة لالتهاب الجلد

رسالة

مقدمة الى كلية العلوم - جامعة النهرين و هي جزء من متطلبات
نيل درجة الماجستير في علوم التقانة الاحيائية

من قبل

آمال سليم عبد الصاحب المظفر

بكالوريوس تقانة احيائية -٢٠٠٥
جامعة النهرين

محرم
١٤٣٠

كانون الثاني
٢٠٠٩