Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

Study the Effect of Some Biotic and Abiotic Factors on Enhancement of Essential Oils and Rosmarinic Acid in Rosemary *Rosmarinus officinalis* L. *In vitro*

A dissertation

Submitted to the College of Science/Al-Nahrain University as a partial fulfillment of the requirements for the degree of Doctorate of Science in Biotechnology

By

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March 2010

Rabee Al-Awal 1431

Acknowledgements

Praise to **Allah** the first of all causes, the glorious creator of the universe for his mercy and kindness, and blessings upon **Mohammed** the **profit** of **Allah.**

I would like to express my sincere thanks, respect and deep gratefulness to my supervisors Professor Dr. Kadhim M. Ibrahim and Professor Dr. Subhi J. Hamza for their unlimited help, valuable advices, back-up and endurance throughout the preparation of this dissertation.

It is a pleasure to thank all staff members of College of Science and Biotechnology Department at Al-Nahrain University for their help and support.

I express my deep appreciation to Dr. Ali Sadik, College of Science Biotechnology Department at Baghdad University for his help and advice during the course of the study.

My special thanks and grateful are due to Dr. Nazar M. Ali, Dr. Bassam Al-Safadi, Mrs Mervat Nakar and all staff members at Biotechnology and Molecular Biology (AEC-Syria) for their help and support.

I am grateful to my wife and my sister for their love, help and encouragement throughout the time course of study. I also would like to express my deep indebtedness to my son Awus and my daughters Rana and Sara for their support throughout the study.

Finally I would like to thank my parents and to whom that I have not mentioned.

Khaleel

Dedication

Such humble work should be dedicated

to

Almighty Allah who best owed upon my soul with his love

to

My dear father, mother, brothers, sisters

to

My wife, son and daughters who considerably supported me to complete this study

to

My country, Iraq

Khaleel

SUMMARY

The yield of rosemary (*Rosmarinus officinalis* L.) from essential oils and rosmarinic acid were investigated. Yield of callus tissue was compared with the intact plant production. Callus was induced on leaf explants and maintained on Murashige and Skoog medium (MS) supplemented with Dichlorophenoxy acetic acid (2,4-D), Benzyl adenine (BA) and Kinetin (KIN). The highest percentage of callus induction (100%) was recorded on leaf explants when a combination of 2 mg/l 2,4-D and 0.5 mg/l BA was added to the culture medium followed by (90%) at the combination of 2 mg/l 2,4-D and 0.1 mg/l KIN. Maximum callus fresh weight was obtained in the combination of 2 mg/l 2,4-D and 0.5 mg/l BA in cultures grown under 16/8 hrs photoperiod, which reached 1780 mg, while it recorded 1442.5 mg in the combination of 2 mg/l 2,4-D and 0.1 mg/l KIN. Callus cultures were treated with biotic (bacterial, fungal and yeast extract) and abiotic elicitors ($CaCl_2$ and gamma-rays).

The quantitative and qualitative analysis of essential oils was carried out using gas chromatography-mass spectrometry (GC-MS). The most abundant contents of the rosemary leaves essential oils were, camphor (20.54%), cineole (13.40%), verbenone (12.71%), bornyl acetate (12.22%), carene (7.73%), camphene (4.32%), α-pinene (2.28%) and β-pinene (1.77%). The most presented components of leaves ethanol extract were, isocarnosol (5.85%), camphor (3.88%), ferruginol (4.53%), cineole (2.66%), verbenone (2.50%) and borneol (1.56%), while the most presented components of callus ethanol extract were, cineole (4.44%), camphor (2.51%), verbenone (2.30%), borneol (1.91%), bornyl acetate (1.66%),

ferruginol (1.59%), isocarnosol (1.27%), phytol (9.12%) and quinoline (16.78%).

Among the biotic and abiotic elicitors fungal elicitor (*Fusarium oxysporum*) at the concentration of 2 ml/l ($2x10^4$ CFU/ml) was the most effective in stimulating secondary products in rosemary callus followed by the bacterial elicitor (*Pseudomonas aeruginosa*) at the concentration of 1 m/l ($1.5x10^5$ CFU/ml). Addition of sucrose at 40 g/l in acombination with 0/1 mg/l of Phe and Tyr as precursors to the callus culture medium showed increase in cineole and camphor concentrations.

High performance liquid chromatography (HPLC) analysis of phenolic acids; rosmarinic acid (RA), caffeic acid (CAF), and phenolic diterpenes; carnosic acid (CA), carnosol (CAR) and rosmanol (ROL) in rosemary leaf and callus extracts revealed that RA, CAF, CA and CAR production reached 4.5, 2.7, 3.3 and 2.8 µg/ml respectively when *F. oxysporum* was added at 2 ml/l, except ROL which was found at high levels (4.3, 4.2 and 4.6 µg/ml) only from leaf extracts, untreated callus and when callus treated with 0.4 g/l of $CaCl_2$ respectively. Biological activity of leaf ethanol extracts was examined against three bacterial isolates (*Escherichia coli, P. aeruginosa, Bacillus cereus*) and two fungal isolates (*Aspergillus nigar, F. oxysporum*). The MIC values were 320 µg/ml for *E. coli*, 640 µg/ml for *P. aeruginosa* and 160 µg/ml for *B. cereus*. However, *P. aeruginosa* was found the most resistant bacteria. The MIC values of ethanol extracts against *F. oxysporum* and *A. nigar* were recorded as 160, 320 µg/ml respectively. *F. oxysporum* seems to be more sensitive than *A. nigar*.

List of abbreviations

2,4-D	2,4-Dichlorophenoxy Acetic Acid
AEC	Atomic Energy Commission
BA	Benzyl Adenine
CA	Carnosic Acid
CAF	Caffeic Acid
CAR	Carnosol
CFU	Cell Forming Unit
C. L. S. I	Clinical and Laboratory Standards Institute
DAD	Diode Array Detector
DDH_2O	Double Distilled water
DMSO	Dimethyl sulphoxide
EID	Electron Ionization Detector
EOs	Essential Oils
ESI	Electro-Spray Ionization
eV	Energy of Fragmentation
FID	Flame Ionization Detector
GC-MS	Gas Chromatography-Mass Spectrometry
Gy	Gray (Gamma-rays measurement unit)
HPLC	High Performance Liquid Chromatography
I.S.H.S	International Society for Horticultural Science
KIN	Kinetin
LB	Luria-Bertani
LC	Lethal Concentration
LDL	Low Density Lipoprotein
LSD	Least Significant Differences
MH	Mueller-Hinton medium
MeJ	Methyl Jasmonate
MIC	Minimum Inhibitory Concentration
MS	Murashige and Skoog 1962 medium
MWD	Multi-Wave Detector
NAA	Naphthalene Acetic acid
OD	Optical Density
PBS	Phosphate Buffer Saline
PDA	Potato Dextrose Agar

PE	Penicillium expansum
Phe	Phenylalanine (amino acid)
Pi	Inorganic Phosphate
RA	Rosmarinic Acid
ROL	Rosmanol
RT	Retention Time
SA	Salicylic Acid
SAS	Statistical Analysis System
TDZ	Thidiazuron
Tyr	Tyrosine (amino acid)
µg/ml	Microgram/Milliliter
µl/ml	Microliter/Milliliter
UV	Ultraviolet
WHO	World Health Organization
YE	Yeast Extract

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Chapter One: 1- Introduction and Literature Review 1

1-1: Introduction

Plants have been an important source of medicine for thousands of years. Even today, the world health organization estimates that up to 80 percent of people still rely mainly on traditional remedies such as herbs which contain plant extracts with active ingredients. Biotechnological tools are important for multiplication and genetic manipulation of the medicinal plants through callus induction, cell suspension in bioreactors, *in vitro* regeneration of plantlets and genetic transformations (Katzung, 1995).

There is a series of distinct advantages to produce a valuable secondary product in plant cell culture, rather than *in vivo* in the whole crop plant. These include the following:

- Production can be more reliable, simpler, and more predictable.
- Isolation of the phytochemicals can be rapid and efficient, as compared to extraction from complex whole plants.
- Compounds produced *in vitro* can directly parallel compounds in the whole plant.
- Interfering compounds that occur in the field-grown plant can be avoided in cell cultures.
- Cell cultures can yield a source of defined standard phytochemicals in large volumes.
- Cell cultures are a super model to test elicitation.
- Cell cultures can be radio labeled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically (Mary, 2005).

Rosemary (*Rosmarinus officinalis* L.) which has long been known as a spice and medicinal herb belongs to the Lamiaceae family and receives increasing attention due to its antimicrobial, anti-inflammatory, and antioxidative constituents (Eva *et al*., 2003).

Plant material from rosemary is of commercial interest for its essential oil content and its antioxidant compounds. Carnosic acid and carnosol were shown to be the major phenolic diterpenes in leaves of rosemary (Sergi *et al*., 2000). Natural compounds such as phenolic diterpenes and triterpenes, present in various plants, have been the subject of intense research due to their potential benefits for human health (Masa *et al*., 2007).

Rosemary leaf extracts show a very high antioxidant activity and are increasingly used as food additives, proposed as important human dietary factors, and investigated as inhibitors of skin tumorgenesis (Sergi and Leonor, 2001). The Lamiaceae family seems to be a rich source of plant species containing large amounts of phenolic acids, which have already been analyzed using HPLC. The later was used for the determination of rosmarinic acid and other phenolic acids (Alica and Eva, 2003).

Plant cell suspension and callus cultures produce all the primary and secondary metabolites that are produced by the parent plant from which cultures are derived (Arouma *et al*., 1992; Mehrabani *et al*., 2005).

Using special techniques such as elicitation and hairy root cultures provide methods to enhance the production of secondary metabolites.

Various biotic and abiotic factors influence their production by activating genes for *de novo* synthesis or by stimulating the physiological processes leading to enhanced accumulation of such products.

Elicitations are considered to be an important strategy towards improved *in vitro* production of secondary metabolites. In cell cultures, biotic and abiotic elicitors have effectively stimulated the production of plant secondary metabolite (Shilpa and Jayabaskarn, 2007; Ramawat, 2008).

Biotic and abiotic elicitors are often applied in the examination of secondary metabolism and the responses of cultured plant cells to UV, MeJ, and YE have been characterized at various levels of detail in several species (Namdeo, 2007).

The aims of this study are:

1- To investigate the effect of biotic and abiotic elicitors in increasing the amount of essential oils and rosmarinic acid in rosemary leaf and callus tissues.

2- Determination of phenolic and terpenic compounds in rosemary extracts using GC-MS and HPLC techniques.

3- To determine the antimicrobial activities of rosemary extracts.

1-2: Literature Review 4

1-2-1: Medicinal plants and herbs

Medicinal plants are rich in secondary plant products, these secondary metabolites or products exert a profound physiological effect on the mammalian system. This effect of the active principles is used for curing ailments and therefore, these are drugs of plant origin or natural drugs.

The use of plant compounds for pharmaceutical purpose has gradually increased (Robert, 1988; Alsereitia and Abu-Amer, 1996). The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. *In vitro* regeneration holds tremendous potential for the production of high-quality plant-based medicine. *In vitro* production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants (Goldstein, 1988; Leena and Jaindra, 2003).

Micropropagation of various plant species including medicinal plants has been reported. Numerous factors influence the success of *in vitro* propagation of different medicinal plants (Withers and Anderson, 1986).

According to WHO (1978) medicinal plants would be the best source for obtaining a variety of drugs since about 80% of world population in developed countries use traditional medicines, derived from medicinal plants.

Therefore, such plants should be investigated thoroughly to determine their structural and functional properties, as well as the constitutes of various parts (Ellof, 1998).

1-2-2: Rosemary (*Rosmarinus officinalis* L.) **5**

Rosemary is a very important medicinal plant (figure 1-1), which has been cultivated for a long time (Eva *et al*., 2003).

Figure 1-1: Rosemary plant (*Rosmarinus officinalis* L.) grown in the garden (AEC-Syria, 2008)

Rosemary's name is derived from rosmarinus, which means " dew of the sea " it is cultivated world-wide, but France, Spain and Tunisia are the main essential oil producers from this plant. Bushy and branched evergreen grows six feet high. Leaves are stiff, leathery, like pine needles, and opposite. Flowers are light blue and bloom from March to May.

There are silver and gold-striped varieties, but the green-leaved variety is the kind used medicinally. Various varieties such as Corsican blue; growing about 90 cm tall, which is a highly aromatic plant with porcelain-blue flowers (Genders, 1994). Miss Jessopp's Upright; an erect and very robust form that tolerates temperature about -10ºC, it is suitable for hedging (Brickell, 1990).

Postractus, it can be used as a ground cover in a position, it is the least cold-hardy form, it is very tolerant of the winds (Phillips and Rix, 1998).

Rosemary is propagated by seeds, cuttings, layering and division of roots. It succeeds in light, rather dry soil, and in a sheltered situation, such as the base of a low wall with a south aspect. Rosemary is used as a ground cover along roads and as embankments because of it's deep root system, which helps stabilize the soil, withstands hot and dry periods (Misra and Chaturvedi, 1984).

1-2-3: Rosemary classification

Kingdom: Plantae

Subkingdom: Tracheobionta--Vascular plants

Superdivision: Spermatophyta--Seed plants

Division: Magnoliophyta--Flowering plants

Class: Magnoliopsida--Dicotyledons

Subcalss: Asteridae

Order: Lamiales

Family: Lamiaceae (Labiatae)

Genus: *Rosmarinus*

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

The Lamiaceae (Labiatae) family is one of the largest and most distinctive families of flowering plants, with about 220 genera and almost 4000 species worldwide. Some genera like *Nepeta, Phlomis, Eremostachys, Salvia* and *Rosmarinus* have a great diversity in the Mediterranean (Jamzad *et al.*, 2003).

1-2-4: Rosemary popular uses **7**

Rosemary is widely used as a culinary spice, and is also used for its fragrance in soaps and therapeutic potential (table 1-1). Traditionally, rosemary has been used medicinally to improve memory, relieve muscle pain and spasm, stimulate hair growth, and support the circulatory and nervous systems (Foster and Tyler, 1999).

It's action include, analgesic, antidepressive, anti-inflammatory, antispasmodic, astringent, carminative, diaphoretic, digestive, diuretic, fungicidal, insecticide, relaxant and reputed cardiac tonic (Svoboda and Hampson, 1999). Dried rosemary leaves, whole or ground, are used as seasoning for soups, stews, sausages, meat, fish and poultry. The essential oil is used in food products, perfumes and cosmetics, such as creams, deodorants, hair tonics and shampoos. As a medicinal plant, rosemary wine has been used as an external stimulant and as a relaxant for nervousness, muscle spasms and headaches. Rosemary is specially indicated in depressive states accompanied with general debility and indication of cardiovascular weakness and is of value as a tonic for elderly people with weak circulation. The camphor compound from rosemary extract has a general tonic effect on the circulation and nervous system, especially in the vascular nerves, making it an excellent drug for all states of chronic circulatory weakness including hypotension (Al-Sereitia *et al.*, 1999). It is of particular value as a tonic in conditions of the stomach. Several species of the family Lamiaceae are used in traditional and modern medicine (Nunez and Obon de Gastro, 1992; Maganga, 2004). The antiseptic oil of rosemary is also used in the treatment of arthritis, colic depression, memory loss, migraine, coughs, influenza, and diabetes (Offord *et al*., 1995).

It stimulates the smooth muscle of the digestive tract and gallbladder. A salve made from the oil can be applied to bruises and wounds. The anti-inflammatory action of the herb is thought to be due to rosmarinic acid, ursolic acid and apigenin (Arouma *et al*., 1998).

Table 1-1: Therapeutic potential of rosemary plant (Al-Sereitia *et al*., 1999)

	Pharmacological action	**Therapeutic potential**
1	Relaxation of bronchial smooth muscle	Bronchial asthma
2	Relaxant of smooth muscle	Antispasmodic
3	Reduction of leukotrienes	Bronchial asthema, Peptic ulcer, inflammatory diseases
4	Inhibition of lipid peroxidation	Hepatoxicity, Atherosclerosis and ischaemic heart diseases, inflammatory diseases, Asthenozoospermia
5	Inhibition of the complement	Inflammatory diseases
6	Prevention of the carcinogen	Cancer (Protection)

1-2-5: Rosemary secondary metabolites

The family Lamiaceae contains an extremely wide variety of aromatic plants, among this rich array of plants yielding essential oils (Chalchate *et al*., 1993). Labiates are known for their essential oils common to many members of the family. Many of the active essential oils have been isolated from various members of this family. The family is famous for the presence of diterpenoids in its members. Rosemary is a common household plant grown in many parts of the world.

One of the most important constituents of rosemary is caffeic acid and its derivatives such as rosmarinic acid. These compounds have antioxidant effect (Al-Sereitia *et al*., 1999).

Rosemary leaves contain about 1-2.5% essential oil; cineol (30%), Camphor (15-25%), Borneol (16-20%), Bornyl acetate (Max. 7%), alpha-Pinene (Max. 25%) and others contribute to the complex taste (Haas *et al*., 1993). The chemical composition of rosemary aqueous extract revealed the presence of many substances which anti oxidant and anti-lipoperoxidant activities have been demonstrated, namely rosmarinic acid, caffeic acid, chlorogenic acid, carnosic acid, rosmanol, carnosol and different diterpenes.

The essential oil contains esters mainly boneol acetate and 10-18% free alcohol including, borneol and linalool, camphor, camphene, and cineol; diterpenes such as, carnosilic acid, carnosol (picrosalvin), and rosmariquinone; triterpenic (Angioni *et al*., 2004).

Table 1-2: List of some plants belong to Lamiaceae family containing rosmarinic acid (RA) and caffeic acid (CAF) (Al-Sereitia *et al*., 1999)

Plant scientific name	RA or CAF
Calendula officinalis	RA
Anchusa officinalis	CAF
Lavandula officinalis	RA
Lithospermum officinalis L.	CAF
Melissa officinalis	CAF, RA
Salvia officinalis L.	RA
Rosmarinus officinalis L.	RA, CAF

1-2-6: *In vitro* production of secondary metabolites 10

Plant cells and callus cultures have been extensively used to explore the possibility of producing useful secondary metabolites through biotechnological methods. Plants are the traditional source for many chemicals used as pharmaceuticals. Medicinal plants are used in crude or purified form in the preparation of drugs in different systems (Robins, 1999). Secondary metabolites are synthesized in specialized cells at particular developmental stages making their extraction and purification difficult (Dean and Svoboda, 1993; Dornenburg and Knorr, 1995). There are numerous reports describing the production of diverse secondary metabolites, viz..,anthocyanins, carotenoids, flavones, coumarins, saponins, sesquiterpenes, steroidal alkaloids, sterols, tannins, terpenoids and several others (Smita and Ashok, 2007). Primary metabolism provides critical substrates for secondary metabolic pathways. Co-A is an essential component in both primary and secondary metabolic reactions, and the regulation of enzymes utilizing Co-A or its thioesters is often affected by the induction of secondary metabolism (Alex *et al.*, 2000). Several products, were found to be accumulated in cultured cells at high level than those in native plants through optimization of cultured conditions. For example, RA by *R. officinalis* and *Colleus blumei*, ginsenosides by *Panax ginseing*, shikonin by *Lithospermum erythrorhizon* were accumulated in much higher levels in cultured cells than in the intact plants (Goldstein, 1988).

1-2-6-1: Callus cultures

The induction of callus growth and subsequent differentiation is accomplished by the differential application of growth regulators and the control of conditions in the culture medium.

With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced (Leena and Jaindra, 2003). Callus formed during *in vitro* culture, has some similarity to tissue arising *in vivo* after injury (so-called wound callus), there are differences in morphology, cellular structure, growth and metabolism, between calli derived through tissue culture and natural wounded callus cultures initiated from both dicotyledonous and monocotyledonous. Juvenile and hence physiologically the most active tissue give better callus formation; also the exogenous plant growth regulators requirement (type, concentration, auxin to cytokinin ratio) for callus formation depends upon the endogenous hormone content of the tissues (Torres, 1988). Murashige and Skoog (MS) medium supplemented with 30 g/l sucrose and 1 mg/l NAA, IAA and IBA was used for callus initiation from rosemary explants, proved to be the best medium for the production of callus among the examined growth regulators (Ozlem *et al*., 2007).

1-2-6-2: Cell suspension cultures

When callus tissue is introduced into a liquid medium and agitated, the cells disperse in the liquid to form a cell suspension culture. There are cases of cultures that over produce metabolites compared with the whole plant. Cell suspension growth is almost maximum at its 15 days after inoculation. RA increased and reached (0.42 mg/g dry wt) in *Salvia officinalis* L. during stationary phase of culture (Zeng, 1978; Hippolyte *et al*., 1992; Koik *et al*., 2005). Isolation of quinoline alkaloids in significant quantities from globular cell suspension cultures of *Cinchona ledgerian* (Scragg, 1992).

There are many types of cell suspension cultures that can be used for the production of secondary metabolites. These include continuous culture, in which a continuous supply with nutrients by the addition of fresh medium, but the culture volume remains constant. Batch cultures, where the cell suspension culture is grown in a fixed volume of nutrient culture medium (Gruel *et al*., 2002). Cell suspension cultures of rosemary were established and their ability to biosynthesize the phytochemicals; carnosic acid, carnosol, and rosmarinic acid were assessed and compared with subcultures of the same plant over time. Results showed that carnosic acid and no carnosol was detected. The amount of rosmarinic acid produced in shoot and callus cultures were obtained at higher concentrations (Annette and Claudia, 2006). Immobilization of plant cells is considered to be important for plant cell cultures, it can be provided; the extended viability of cells in the stationary phase, enabling maintenance of biomass over a prolonged time period, the promotion of differentiation, high cell density enabling a reduced bioreactor size, reduced shear sensitivity, promotion of secondary metabolites secretion, minimization of fluid viscosity increase, which causes in cell suspension mixing and aeration problems (Goldstein, 1988; Hahn *et al*., 2003). The differentiated cultures often show biochemical and genetic stability and hence offer a predictable and high productivity system which does not require extensive optimization. Secondary metabolites are synthesized or stored in organized structures such as roots, shoots, stigmas, embryos, transformed roots and transformed shoots (Aurelia *et al*., 2007).

The use of organs as opposed to cells aggregates might contribute in scaling-up of secondary metabolites (Parr, 1989; Hahn *et al*., 2003). Transformation was used for genetic manipulation of medicinal plants, using *Agrobacterium* mediated or direct transformation methods (Birch, 1997). The production of secondary metabolites was controlled by genes and plant cell cultures produce the same product in culture as produced by the intact plant (Mary, 2005).

1-2-7: Factors affecting the production of secondary metabolites

Various factors which affect the production of secondary metabolites in plant tissues were grown in culture. Initially, growth and production of secondary metabolites are optimized by manipulating the physio-chemical factors followed by selection of high-productive cells (figure 1-2).

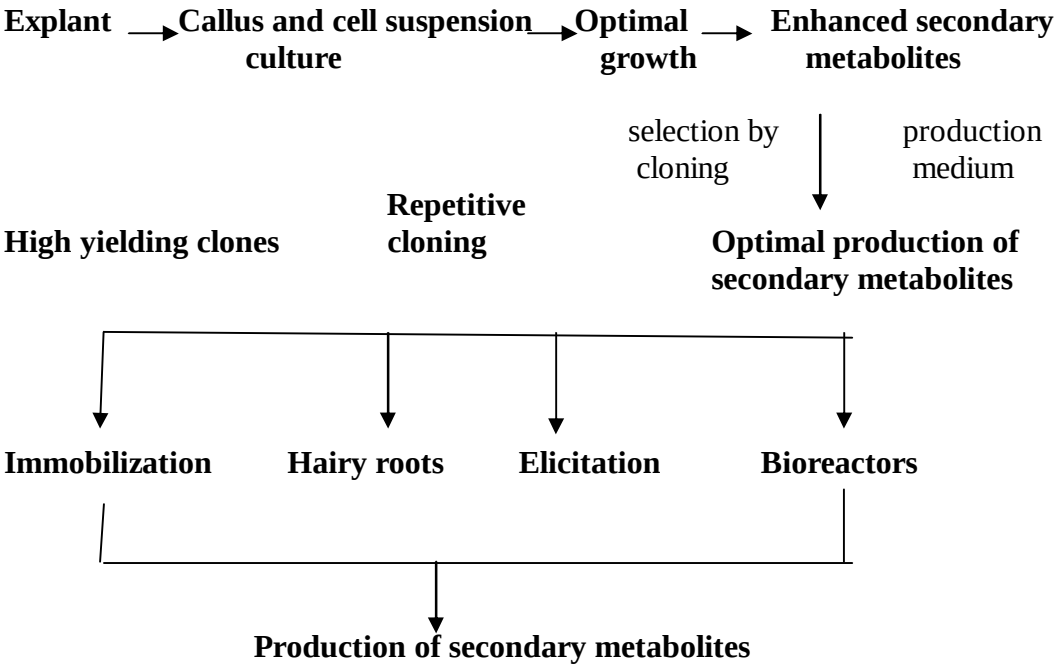


Figure 1-2: Various approaches used for the optimal production of secondary metabolites (Ramawat, 2008)

1-2-7-1: Physical factors 14

A number of physical factors affecting cultivation have been tested with various plant cells. These factors include light, pH, temperature and many others. There are many reports and patents concerning optimization of cultural conditions in order to improve growth rate of cells and/or higher yield of desirable products.

1-2-7-1-1: Effect of light

Phytochemical responses are affected by both irradiance and light quality, it was found that 4-fold increase in yield of the sesquiterpenoid azulene in the cell suspension of the liverwort (*Calypogeia granulate*) grown in white light with wave length (300-800) nm (Takeda and Katoh, 1981). Cells of *Ruta graveolens* produced several coumarins and alkaloids when grown in continuous white light. *R. graveolens* produce 2-nonanone, 2-nonanyl acetate and 2-nonamol preferentially in dark (DiCosmo and Towers, 1984).

White light induced anthocyanin synthesis in *Catharanthus roseus* and *Populus sp.*The production of chlorogenic acid in *Haploppapus gracilis* was stimulated by white, blue and red light. Blue light induced maximum anthocyanin formation in *H.gracilis* cell suspensions (Ramawat, 2008).

It is worth mentioning that as compared to normal rate of photosynthesis of field grown plants, rate of carbon fixation in tissue culture is either absent or very low. Therefore, light does not affect the primary metabolism (photosynthesis), but is involved in light mediated enzyme metabolism and photo-morphogenesis (Takeda and Katoh, 1981).

1-2-7-1-2: Effect of temperature **15**

Plant cells are usually cultured in a temperature range of 25-27 ◦C. Altered temperature range affects both the accumulated quantities of specific compound and the type of phytochemical synthesized. 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 (DiCosmo and Towers, 1984: Ushiyama, 1991). Production of indole alkaloid increased two fold when cells of *Catharanthus roseus* were incubated at 16ºC instead of 27ºC. Lower temperature growth is 3-fold slower. Change in incubation temperature of *Catharanthus sinensis* or *Nicotina tobacum* result in decreased synthesis of caffeine and nicotine, respectively (Yamada *et al*., 1980).

1-2-7-1-3: Effect of pH

The medium pH is usually adjusted between 5 and 6 before autoclaving and extremes of pH are avoided. The optimum pH is determined and controlled using a small scale bioreactor as jar fermentor with pH control equipment. A pH rang of 5-6 is required for culture of plant cells. Several reports have shown that the pH of growth medium can drastically influence the production of phytochemicals by cultured cells including, anthocyanins, anthraquinones and alkaloids (Dougall *et al*., 1983). *Daucus carota* grown at pH 5.5 produced less anthocyanin than when cultivated at pH 4.5. It has suggested that the level of anthocyanin is related to the increased degradation of the compound at higher pH. Altered pH affected the formation of tryptophol from tryptophan, at controlled pH of 6.3.

Tryptophol synthesis was stimulated to 71% over control cultures at neutral pH, but at pH 4.8 tryptophol synthesis was inhibited (DiCosmo and Towers, 1984).

1-2-7-2: Nutrient Factors

Cultured plant cells are usually grown on a medium containing all the elements required for their sustained growth**,** including essential minerals, vitamins and carbohydrate sources. Plant cell cultures are totipotent and possess all the capabilities of intact plant to synthesize primary and secondary metabolites. Various well-known basal media were tested for the production of serpentine, an indole alkaloids, the results indicated that the amount of serpentine depends on the composition of the basal medium used (Zenk *et al.*, 1977; Ushiyama, 1991).

1-2-7-2-1: Effect of carbon source

Carbohydrates are known to influence the production of phytochemicals. At 3% level sucrose proved to be the best carbon source followed by glucose, fructose and maltose for the growth and diosgenin productively by *B. aegyptica* cultures (Sasse *et al*., 1982).

Sucrose and glucose are the preferred carbon source for plant tissue cultures. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. The maximum yield of rosmarinic acid produced by cell suspension cultures of *Salvia officinalis* was 3.5 g/l when 5% of sucrose was used, but it was 0.7 g/l in the medium containing 3% sucrose. It is preferred to use sucrose concentration at 4% for rosemary to increase the rosmarinic acid content in callus cultures.

The highest levels of β-pinine and borneol were estimated to be obtained when approximately 1.9% sucrose is added to the culture medium (Azza *et al*., 2006).

1-2-7-2-2: Effect of nitrogen source

A mixture of nitrate and ammonium is used in all the standard media as a source of nitrogen. Many plant cells may not be able to tolerate a high amount of ammonium used in these media. The maximum amount of mineral nitrogen assimilated by cultured cells is used for the biosynthesis of amino acids, proteins, and nucleic acids. Availability of nitrogenous primary metabolites in high amounts has a direct effect on the synthesis of secondary metabolites (Daves, 1972; Shanjani, 2003). A lower concentration of nitrogen in the nutrient medium diminished the growth of friable callus line, but improved the growth of compact callus line. A significantly higher RA accumulation occured in a medium with one quarter of the nitrogen concentration (Natas'a *et al*., 2004). High ammonium concentrations are inhibitory to secondary metabolites and lowering of ammonium nitrogen results in increased secondary metabolites (Zenk *et al*., 1977).

1-2-7-2-3: Effect of Phosphate

Inorganic phosphate involvement in metabolic regulation in phosphosynthesis and respiration (glycolysis) is essential to maleic acid and phospholipids synthesis. Secondary products are synthesized through phosphorylated intermediates, eg. terpenes, terpenoids and phenylpropanoids, which subsequently release phosphate (Knoblocu and Berlin, 1980).

Secondary pathways are often inhibited by P_i levels which appear to be optimal for growth and low P_i concentrations are often benifical for active secondary metabolites. Therefore, P_i levels are reduced in the production medium designed for high yields of secondary metabolites (Chandler and Dodds, 1983).

1-2-7-3: Plant growth regulators

A large number of reports describe the effect of growth regulators on secondary metabolite levels of cultured cells. Two types of growth regulators are required by plant cells, namely auxins and cytokinins. Plant growth regulators do not react with intermediate compounds of biosynthesis pathway, but appear to change cytoplasmic conditions of product formation to higher or lower levels (Ramawat, 2008). 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 and low levels of BA are suitable for cell growth and rosmarinic acid production from cell culture of *A. rugosa*. The suspension cultures of *Catharanthus roseus* initiated from stem and leaf explants on a medium containing NAA and KN has been established to increase secondary products formation (Zhao *et al*., 2001). A balanced combination of cytokinin to auxin is used and therefore results should be interpreted in terms of a combined effect (Dodds and Robert, 1995).

Auxins and cytokinins have shown the most remarkable effects on growth and productivity of plant metabolites, an increase of auxin levels in the medium stimulates dedifferentiation of the cells and consequently diminishes the level of secondary metabolites (Halder and Gadgil, 1983; Komamine *et* al., 1991).

Cell manipulation and cell differentiation have to occur in cell culture when metabolite is to be increased, a better method is to use two step culture medium condition; promotion of cell growth on a nutrient medium with high levels of auxin followed by transfer of cells to medium with low level of auxin. This has led to the development of the maintenance medium and the production medium (Chu and Kurtz, 1990).

1-2-7-4: Precursors

Precursors are molecules which are directly incorporated into secondary metabolites, with some structural changes. When such molecules are fed to cultures; they are incorporated into biosynthesis pathways of secondary metabolite (Arita *et al*., 2000). Rosmarinic acid is produced from rosemary plant using two distinct precursors; Phenylalanine and Tyrosine (figure 1-3) increase rosmarinic acid concentration in callus cultures produced from leaf tissues then enhancing the RA biosynthesis pathway (Hany, 2006).

1-2-7-5: Elicitation

Elicitors are compounds of biological origin involved in plant microbe interaction. In context of product accumulation by plant cell cultures, elicitors are mediator compounds of microbial stress (biotic elicitors), or are stress agents like UV light, alkalinity, osmotic pressure, or heavy metal ions (abiotic elicitors). Fungal preparations from common pathogenic fungi (*Fusarium, Alterneria, Pythium, Colletorichum, Helminthosporium* and *Sclerotinia,* etc...) were used as elicitors (Ramawat, 2008).

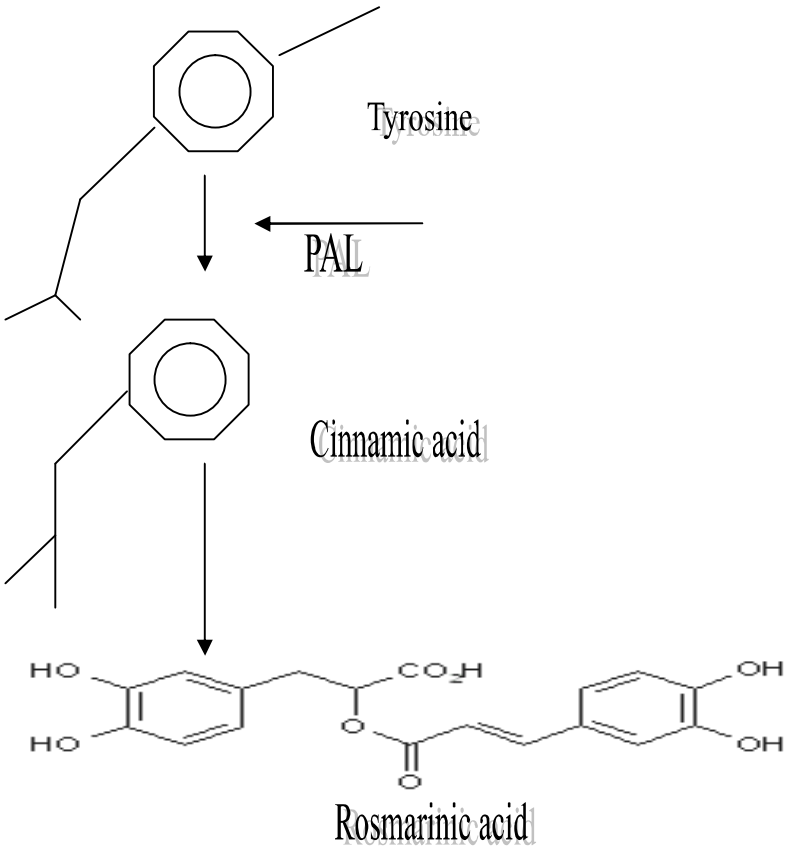


Figure 1-3. Biosynthetic pathway of rosmarinic acid (Shetty *et al.*, 1996

Various abiotic and biotic agents have been shown to mimic microorganisms and act as elicitors of the synthesis of plant compounds (Radmab *et al.*, 2003). Numerous studies on plant microbe elicitors interactions have identified microbial molecules that act as elicitors to increase the biosynthesis of plant secondary metabolites (Radmab *et al.*, 2003).

1-2-7-5-1: Biotic elicitors

The effect of elicitation on primary metabolite accumulation has largely been overlooked. However, at the transcript level, fungal elicitation altered expression of several transcripts, including representatives from the phenylpropanoid, pentose phosphate, glycolytic, and fatty acid metabolic pathways.

The response to elicitation is much more than the simple induction of biosynthetic enzymes of secondary metabolism (Batz *et al.,* 1998).

Isoflavonoids are of a particular interest, the biosynthesis pathway leading to the production of isoflavonoids can be elicited by the application of yeast cell wall extract. Biotic elicitors; autoclaved lysats of bacteria *Entrobacter sakazaki, Erwinia chrysanthemi*, fungal cell wall compound (scleroglucan) and yeast extract induced significant accumulation of shikimic acid, an early precursor to the phenylpropanoid pathway (Kessman *et al*., 1990; Corey *et al*., 2005).

A transient increased in rosmarinic acid content in cultured cells of *L. erythrohizon* after addition of yeast extract to the suspension cultures: a maximum was reached in 24 hr. When the plant cells were treated with yeast extract on the 6^{th} day of cultivation, the level of rosmarinic acid increases 2.5 times and the activity of phenylalanine ammonia-lyase in the cells rapidly increased before synthesis of romarinic acid. Bacterial elicitor showed a slightly inhibition at biomass growth, but total saponin content was increased about 1.23 times at 1ml addition (Jeong *et al*., 2005). It was observed that dry cell powder of *Candida versatilis* significantly elicited the enzyme activity 3.5 fold higher than the control treatment followed by glutathione that recorded3.4 fold and *Rhizophus oligosporus* recorded 3.0 fold (Thimmaraju *et al*., 2006). Another study showed that addition of low concentrations of quercitin or rutin to suspension cell culture of blood root plant (*Sanguinaria canadensis* L.) induced biosynthesis of sanguinarine and chelerythrine in a dose-dependent manner. Quercetin's inductive effects were similar to that of an elicitor derived from fungus *Penicillium expansum* (PE-elicitor).

Supression of quercitin and PE elicitor induced alkaloid biosynthesis by low doses of actinomycin-D (5μg/ml) and amanitin (20μg/ml) or cycloheximide (1μg/ml) demonstrate a requirement for RNA and *de novo* cytoplasmic protein synthesis and suggest that alteration in gene expression are involved in the inductive mechanism (Gail and Beecher, 1994).

1-2-7-5-2: Abiotic elicitors

Abiotic elicitors, particularly metal ions such as calcium and magnesium, and hormonal elicitors thidiazuron (TDZ), Glutathione and methyl jasmonate (MeJ) were used. Among abiotic elicitors, TDZ, Mg, Ca salts elicited 2.4, 3.0, and 2.8 fold activities respectively (Thimmaraju *et al*., 2006). Another work was concentrated on the production of secondary metabolites in callus, cell suspension of *Ammi majus* by exposing them to abiotic elicitors; SiO_2 and jasmonic acid . MeJ induced the accumulation of the triterpene β-amyrin, a precursor to the triterpene saponines. Also they indicated that the highest induction of umbelliferone was observed after treating the callus cultures with SiO_2. (Krolicka *et al*., 2008).

Irradiation with low doses of ionizing rays has been used as a stimulating agent for increasing yields and/or improving quality of various plants. Gamma irradiated progenies of black henbane (*Hyoscyamus niger* L.) were capable of synthesizing more than twice crude alkaloid compared to the parental control, high yield of biomass, double yield of crude drug than control (Sharma *et al*., 1989).

1-2-8: Essential oils (EOs) **23**

Essential oils are natural plant products which accumulate in specialized structures such as oil cells, glandular trichomes and oil or resin ducts. Chemically the essential oils are primarily composed of mono and sesquiterpenes and aromatic polypropanoids synthesized via the mevalonic acid pathway for terpenes and the shikimic acid pathway for aromatic polypropanoids. Essential oils are used regularly in healing treatments and perfumes, some of the more commonly plants used as a source for Essential oils are; lavender, sage, peppermint, eucalyptus, geranium, jasmine, rose, lemon, orange and rosemary (Simon, 1990). Essential oils composition of rosemary comprise more than sixty individual components of which major substances can constitute up to 89% of the essential oils (Burt, 2004).

Essential oils are used as additives in many common products and in the healing practice of aromatherapy. Essential oils contain compounds that the plant uses to fight infections and derive away germs and parasites. Scientists have isolated hundreds of chemicals which exist in essential oils, exhibit antibacterial, antifungal, and anti-parasitic properties (Pauli, 2006). 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 refreshing and pleasant odor (Bauer *et al.*, 1997). The main constituents in rosemary oil were α-pinene, cineole, camphor, verbenone, borneol, bornyl acetate, terpineol, camphene and geraniol (Atti-Santos *et al.*, 2005).

A comparative study of the essential oils prepared from the fresh leaves of rosemary plants were carried out, analysis of the oils by GC-MS led to the identification of 43 components, with verbenone (12.3%), camphor (11.3%) and bornyl acetate (7.6%) being the most abundant constituents (Martos *et al*., 2007). Essential oils from leaves and flowers of rosemary (collected from southern Spain) were investidated, it was found that the main components to be camphor (32.33%) and α-pinene (11.56%). Some of the more commonly lavender, sage, rosemary, eucalyptus, jasmine, lemon, orange, rose and tea tree oil (Hayashi *et al*., 1995). Another study was to test the efficacy of some essential oils from rosemary, lavender, and eucalyptus and their ingredients as repellent against the human body louse and compare their activity with the standard repellent. The results showed that the repellent activity of geraneol lasted two days and that of rosemary for one day (Kosta *et al*., 1995).

1-2-9: Methods of essential oils extraction

Essential oils are produced using several techniques. Distillation uses water and steam to remove the oils from dried or fresh plants.

The method depends on the plant material, three type of distillation were used by industrial firms: water, water with steam and direct steam (Varro and Lynn, 1988). The expression method uses machines to squeeze the oil out of the plants. Other techniques may use alcohol or solvents to remove essential oils from plant materials (Mounchid *et al*., 2004). A variety of methods can be used for essential oils extraction:

- Steam distillation depends on pressurized steam passing through the plant material. The heat of the steam forces the tiny intercellular pockets that hold the essential oils to open and release them.
- Turbodistillation method allows faster extraction of essential oils from hard-to-extract plant materials, such as bark.
- Hydrodiffusion method is less harsh than steam distillation and the resulting essential oils smell much more like the original plant.
- Supercritical carbon dioxide extraction uses carbon dioxide under extremely high pressure to extract essential oils.
- Enfleurage method does not involve heat, the essential oils dissolve in alcohol to separate the oil from the fatty substance.
- Maceration method is similar to enfleurage, the plant materials are steeped into vats of oil until the scented parts dissolve.
- Cold pressing or scarification it is also a method which results in a watery mixture of oil and liquid which will separate by centrifugation at a given time (Simon, 1990; Hayashi *et al*., 1995).

1-**2-10: Terpenes and terpenoids**

Plants produce thousands of compounds that contain one or more phenolic residues. These compounds include; phenols, phenolic acid, hydroxycinnamic acids, coumarins, lignines, lignans, tannins, and xanthones. One class of compounds that fits this description is the terpenes. The term terpenes is used to denote compounds containing an integral number of 5C units, whether or not they contain other elements such as oxygen.

Terpenoids are compounds with varying numbers of carbon atoms clearly defined from 5C units (Goodwin and Mercer, 1983). Terpenoids precursors are produced by the acetyl-CoA pathway, this pathway is probably induced by stress response in order to drive the pathway to overproduce phenolic compounds as a defense mechanism for the plant (Shetty and Labbe, 2001).

1-2-10-1: Diterpenes and triterpenes

The most abundant antioxidant in rosemary is the diterpene carnosic acid in concentrations higher than 4%. Some other less effective diterpenes are degradation products of carnosic acid, which are mostly converted to carnosol, carnosic acid-methylester, epi-rosmanol, rosmanol and 7-methyl-rosmanol (Schwarz and Ternes, 1992). Diterpenes are not found in all rosemary tissues, the leaf tissue showed the highest concentrations of diterpenes. Diterpenes are also present in the flowers of rosemary, they 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). Other terpenoid constituents in rosemary include triterpenes; oleanlic and ursolic acids at about 2-4% (Newall *et al*., 1996).

1-2-10-2: Carnosic acid and Carnosol

The principle compounds which are responsible for distinctive antioxidant activity of rosemary as well as sage, belong to the phenolic diterpenes. Carnosic acid and carnosol were shown to be the major phenolic diterpenes in leaves of *R. officinalis*. Higher concentration of carnosic acid was found ranging from 1.7 to 3.9%, and the carnosol content amounted to 0.2- 0.4% in dried rosemary leaves.

It has been demonstrated that carnosic acid is a precursor of phenolic diterpenes featuring γ and δ-lactone structure *in vitro*. In the presence of oxygen, carnosic acid is degraded within several days to carnosol and rosmanol as shown in figure 1-4 (Sergi and Leonor, 2000; Abreu *et al*., 2008). Carnosic acid is a phenolic diterpene which corresponds to the empirical formula $C_{20}H_{28}O_4$. Carnosic acid is obtained by extracting rosemary with a polar solvent (Inatani , 1982). Carnosic acid is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxyl radicals, thus preventing lipid peroxidation and disruption of biological membranes (Cuvelier *et al*., 1996; Arouma *et al*., 1998).

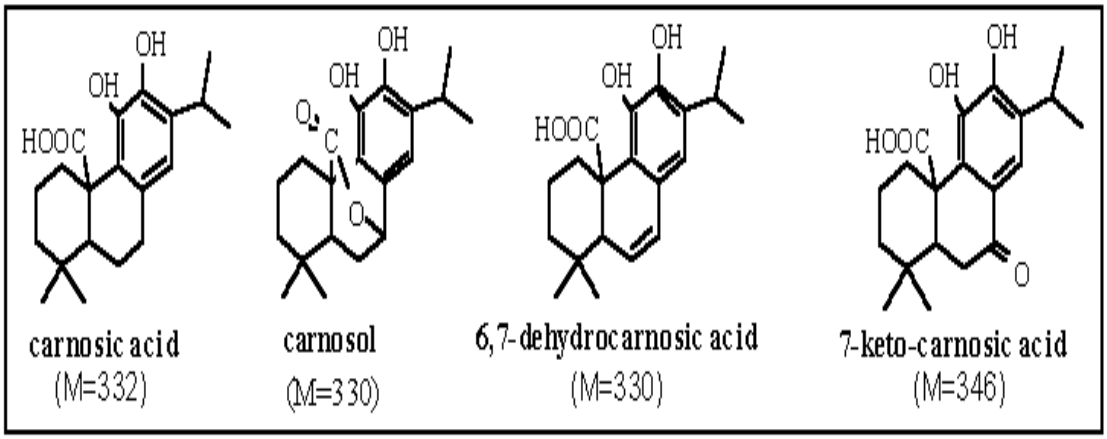


Figure 1-4. Diterpenes in rosemary leaves; carnosic acid, carnosol and their derivatives (Almela *et al*., 2006)

Oxidative stress *in vivo* induced by drought or high light stress enhances the formation of highly oxidized diterpenes due to the antioxidant activity of carnosic acid (Munne-Bosch *et al*., 1999; Munne-Bosch and Algre, 2001). Investigation of the inhibition capacities of carnosol, rosmanol and epirosmanol to oxidize low-density lipoprotein (LDL) formation in human blood.

Detection of their scavenging activities to lipid free radical and superoxide anion *in vitro* showed that carnosol, rosmanol and epirosmanol have an inhibitory activity (Zeng *et al*., 2002).

1-2-11: Phenolic compounds

Plants such as spices and herbs, are well known to inhibit the growth of bacteria, yeasts and molds. These plants contain many different types of antimicrobial compounds. One class of compounds present in plants that are responsible for this effect is the phenolic compounds which include a wide range of compounds and a broad spectrum of functional activities. Phenolic compounds are characterized by having an aromatic ring bearing one or more hydroxyl substituents, including functional derivatives. Phenolics have relatively higher acidities due to the aromatic ring tightly coupling with the oxygen and a loose bond between the oxygen and hydrogen (Fennema, 1996). Some phenols are germicidal and are used in formulating disinfectants such as, phenol, phytol, and others possess estrogenic or endocrine disrupting activities. The mode of action of this compounds include enzyme inhibition by the oxidized compounds, through the reaction with SH group or through interaction with protein. Some of phenolic compounds are classified as essential oils and often considered as antimicrobial agents such as eugenol. Rosemary is one of the many plants whose extracts have been studied expensively for antimicrobial activity. Rosemary leaves contain (2-3)% phenolic acids such as caffeic acid, chlogenic acid, labiatic acid, neoclorogenic acid and rosmarinic acid (Aeschbach *et al*., 1994; Newall *et al*., 1996; Aziza *et al*., 2008).

1-2-11-1: Flavonoids and tannins 29

Flavonoids are polyphenols which include, flavonoids, isoflavonoids and neoflavonoids, they are also referred to as bioflavonoids. They show antiallergic, anti-inflammatory and anticancer activities. Flavones contain one carbonyl group, are known to be effective antimicrobial substances against wide range of microorganisms, their activity due to their ability to complex with extracellular and soluble protein and with bacterial cell wall. Flavonoids in rosemary include diosmetin, genkwanin, eriocitrin, hesperidin and hispidulin and two common flavones apiggenin and luteolin (Almela *et al*., 2006). Three flavonoids including glucuronides, luteolin 3-o-beta-D-glucuronide and hesperidin were isolated from methanol extract of rosemary leaves, their antioxidant activities were evaluated showing great activity. Tannins are group of polyphenols, capable of tanning leather and they possess many human physiological activities such as, stimulating of fagocyte cells, host mediated tumor activity and a wide range of anti-infections. One of the molecular action is to complex with protein.

Their mode of action may be related to their ability to inactivate microbial adhesion enzymeand cell envelope, transport of protein and others (Okamura *et al*., 1994).

1-2-11-2: Rosmarinic acid

Rosmarinic acid (RA), $C_{18}H_{16}O_8$, is a natural polyphenol antioxidant carboxylic acid found in many Lamiaceae herbs (Figure 1-5).

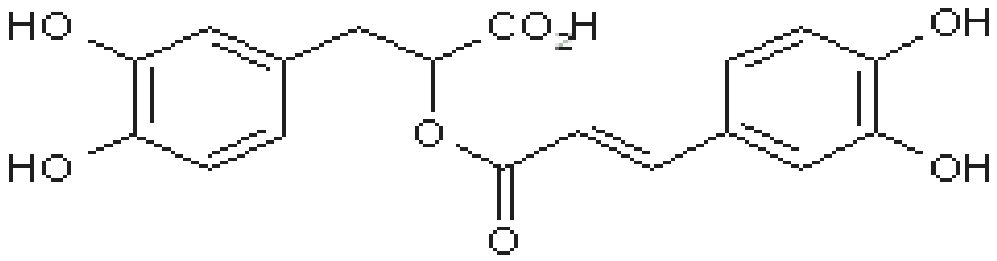


Figure 1-5. Rosmarinic acid structure (Almela *et al*., 2006)

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxy phenyllactic acid; it is isolated as a pure compound for the first time as rosmarinic acid from rosemary plant (Scarpati and Oriente, 1958). Rosmarinic acid has a multitude of biological activities, e.g. antiviral, antibacterial, anti-inflammatory and antioxidant. The presence of rosmarinic acid in medicinal plants, herbs and spices have beneficial and health promoting effects to human beings. Rosmarinic acid exhibits various pharmacological activities including prevention of oxidation of low density lipoprotein, inhibition of murine cell proliferative activity and of cyclooxygenase, and anti-allergic action. (Pamham and Kesseiring, 1985; Zelic *et al*., 2004; Ly *et al*., 2006).

Rosmarinic acid is readily accumulated in undifferentiated plant cell culture, in some cases its concentrations are much higher than in the plant itself. Rosmarinic acid is a red-orange powder that is slightly soluble in water, but well soluble in most organic solvents (Clifford,1999; Triantaphyllou *et al*., 2001).

Rosmarinic acid, together with similar compounds, has been known as "Labi-atengerbstoff" even before its chemical structure was elucidated in 1960 by Hermann. It is a tannin-like compound, sometimes described as a depside of caffeic acid.

The phenolic compound RA obtains one of its phenolic rings from phenylalanine via caffeic acid and the other from tyrosine via dihydroxyphenyl-lactic acid. Rosmarinic acid and caffeic acid, as major components of tannins, commonly occur together in many species of the Lamiaceae (Janicsak and Mathe, 1998).

1-2-12: Methods of separation and purification

1-2-12-1: High Performance Liquid Chromatography (HPLC)

HPLC can be used for the determination of phenolic acids and phenolic diterpenes present in medicinal plants (Brandsteterova and Ziakova-Caniovo, 2002). HPLC assay with C18 column was used for the determination of phenolic acids and terpenic compounds in rosemary leaves and callus extracts. The primary advantage which HPLC has over GC/MS is that it is capable of analyzing a much wider range of components (Torre *et al*., 2001). Compounds that are thermally labile, exhibit high polarity or have a high molecular mass may all be analyzed using HPLC. Compounds are separated on the basis of their relative interaction with the chemical coating of these particles (Stationary phase) and the solvent eluting through the column as a mobile phase (Deirdre, 2003; Thorsen and Hildebrandt, 2003).

Bruno *et al.*, (2004) measured rosmarinic acid levels using HPLC, determination involved the use of C18 column. HPLC method is reported for the determination of rosemary's principle phenolic antioxidants, rosmarinic and carnosic acids, providing a fast and simultaneous determination for both of them (Troncoso *et al.*, 2005). Component eluting from the chromatographic column are then introduced to the mass spectrometer via a specialized interface.

Identification of phenolic and terpenic compounds in derivatised plant extracts was established by comparing their retention times and mass spectra to the derivatised investigated compounds or by comparison of their spectral properties with literature data (Masa *et al.*, 2007). HPLC combined with diode array (DAD) and electrospray (ESI)-ion trap-MS detection was used to separate and identify the compounds present in the rosemary extracts. Rosmarinic acid, carnosic acid and their terpene-type metabolites, and some flavones were identified (Almela *et al*., 2006).

1-2-12-2: Gas Chromatography /Mass Spectrometry (GC-MS)

GC analysis separates all of the components in a sample and provides a representative spectral output. The GC instrument uses a detector to measure the different compounds as they emerge from the column (Frederic, 2007). Mass spectrometric (MS) analysis identifies substances by electrically charging the specimen molecules, accelerating them through a magnetic field, breaking the molecules into charged fragments and detecting the different charges. From the molecular mass and the mass of the fragments, reference data is compared to identify the specimen.

Capillary gas chromatography, coupled with mass spectrometry (GC-MS) provides accurate results. It gives a high degree of specificity, good sensitivity, and also permits the simultaneous quantitative determination of a wide range of phenolics and terpenes, from monoterpenes and diterpenes to sesquiterpenes and triterpenes, even at trace levels (Masa *et al.*, 2007). The volatile oil composition of the extracts is determined using GC coupled with MS, detection and an electron ionization system equipped with a capillary column.

The identification of the major compounds is based on a comparison of their mass spectra with those of data in the standard reference data series of the National Institute of Standard and Technology, their retention index. Their quantification is carried out through the relative peak areas for individual constituents (Aziza *et al.*, 2008). Mario *et al.*, (2002) examined the composition of the essential oils by GC and MS analyses. The retention indices of the oil constituents were calculated and peak identifies were determined by comparison of their retention indices with authentic standards. In another study, the chemical composition and antibacterial activity of essential oil-rich fractions from rosemary were investigated, using GC-MS analysis of these fractions result in the identification of 33 compounds of essential oils (Elena *et al.*, 1999; Santoyo e*t al.*, 2005).

1-2-13: Antioxidant activity of rosemary

Among the antioxidant compounds in rosemary leaves ~ 90% of the antioxidant activity can be attributed to the phenolic diterpenes carnosol and carnosic acid (Collin and Charles, 1987; Fadel and El-Massry, 2000). Phenolic compounds exhibit a considerable free-radical scavenging (antioxidant) activity, which is determined by their reactivity as hydrogen-or electron-donating agents; the stability of the resulting antioxidant-derived radicals, their reactivity with other antioxidants, and their metal chelating properties (Rice-Evans *et al.*, 1997; Proestos *et al.*, 2006). Many compounds have been isolated from rosemary, including flavones, diterpenes, steroids, and triterpenes. Of these, the antioxidant activity of rosemary extracts has been primarily related to carnosic acid and carnosol (Frankel, 1996).

1-2-14: Antimicrobial activity of rosemary 34

Biological active natural compounds are of interest to pharmaceutical industry. Several reports have been published on the scientifically confirmed antimicrobial activity of some natural products derived from plants (Smith *et al*., 2002; Peng, 2005).

Phytoconstituents present in rosemary ethanol extracts are producing exciting opportunity for the expansion of modern chemotherapies against wide range of microorganisms (Gachkar *et al*., 2007; Oyedemi *et al*., 2008). The antimicrobial activity of essential oils is important both for food preservation and the control of human and plant diseases of microbial origin. Essential oil is considered to be the therapeutic principle but plant phenolics, especially rosmarinic acid, are involved too. (Pattanaik *et al*., 2002; Toth *et al*., 2003). Antimicrobial activity of the essential oils was investigated by employing a microdilution method with four bacterial species; *E. coli*, *P. aeruginosa*, *S. aureus* and *B. cereus*. Results indicated that the rosemary extracts showed antibacterial activity against the G-positive bacteria (*S. aureus* and *B. cereus*). The extracts also exhibited an effect against the G-negative bacteria (*E. coli* and *P. aeruginosa*); this effect was less efficient than that presented against the G-positive bacteria, since a higher MIC value was obtained with the G-negative bacteria (Aziza *et al*., 2008).

1-2-14-1: Antibacterial activity

Bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Cohen, 1992). It was documented that among the compounds extracted from 35 plant species, twelve inhibited the growth of *Staphylococcus aureus*, and ten inhibited *Escherichia coli*.

Substances extracted from nine plant species inhibited the growth of *Bacillus sabtilis, Escherishia coli* and *Pseudomonas aeruginosa* (Alonso-Paz *et al*., 1995). Antibacterial activity is reported to be due to the essential oils extracted from rosemary and their effect against *E. coli*. *E. coli* is resistant to several antibiotics but susceptible to the essential oils of rosemary (Adams, 2002). Similar results were obtained by Valero and Salmeron (2003) for the antibacterial activity of rosemary essential oils against *Bacillus cereus* strains grown in carrot broth. Rosemary essential oils also exhibit antimicrobial activity against *E. coli* and *S aureus* (Fakoor and Rasooli, 2007).

1-2-14-2: Antifungal activity

Plant extracts from rosemary as well as its essential oils are of considerable interest because of their antifungal activity (Suhr and Neilsen, 2003; Diannella *et al*., 2004). Geslene *et al*., (2000) tested several known plant species used for therapeutic treatment, among the compounds extracted from these plants, four of them inhibited *Aspergillus niger*. Filamentous fungi and yeasts were subjected to the action of *R. officinalis, Melissa officinalis, Lavendula officinalis* essences in a steam phase; all three essences possess a similar degree of activity against the microorganisms tested (Larrondo *et al*., 2001). *In vitro* antifungal activity of water extracts of rosemary, oregano and sage were tested against five different fungi, (*Phytophthora cactorum, Verticillium dahliae, Fusarium oxysporum* sp. *Lycopersici, Phoma trachephilla* and *Botrytis cinerea*). The results showed that the extracts of rosemary have antifungal activity against all fungi tested at all concentrations used (Salamone *et al*., 2007).

The antifungal activity of the essential oils from rosemary, was tested alone or in combination. They showed antimicrobial effects against all microorganisms tested (Yujie *et al*., 2007). Mohaddes *et al*., (2008) describe the anticandidal activity of essential oils from *R. officinalis* and other plant species against clinical isolates of *Candida albicans*, they found that it possess high activity against the tested strains.

1-2-14-3: Antimicrobial susceptibility test

The researchers used the Kirby Baur disk diffusion testing method (Bauer *et al*., 1966) for antimicrobial susceptibility test for a long time, but in 2006 they switched to a broth dilution method that provides minimum inhibitory concentration (MIC) information that is not available with the Kirby Bauer method. Susceptibility tests were performed by broth dilution method (C.L.S.I., 2006).

1-2-14-3-1: Disk diffusion method

The Kirby Bauer test is a qualitative assay whereby discs of paper are impregnated with a single concentration of different antibiotics. After 18-24 hrs of incubation, the zone diameter (zone of inhibition) was measured and reference tables are used to determine if the bacteria are sensitive (S), intermediate (I) or resistant (R) to the antimicrobial agents (Donald and Ann, 2006). Preparing an extract with an organic solvent was shown to provide a better antibacterial activity (Nair *et al*., 2008).

The antifungal activity of the essential oils showed that rosemary oils which contain 1,8-cineol, eucalyptol, α-pinine and borneol, are significantly potent against *Candida albicans* strains (Mohaddese *et al*., 2008).

1-2-14-3-2: Broth dilution method **37**

The broth microdilution method is a liquid culture method whereby a standard amount of bacteria are inoculated into tubes or plates, which contain different dilutions of antimicrobial agents. The lowest concentration (highest dilution) of the agent preventing appearance of turbidity is considered to be the minimal inhibitory concentration (NCCLS, 1996; Christiane *et al*., 2007; Lodewijk and Christina, 2008). After analyzing the composition of rosemary essential oils by GC-MS; MIC against three G-positive bacteria, three G-negative bacteria and two fungi were determined for the essential oils and their mixtures. The MICs ranged from 0.125% to 1.000% (v/v). The antimicrobial activity of the rosemary oils was investigated by Georgio *et al.*, (2001) who recorded that they exhibited antibacterial activity. Results showed that G-positive bacteria were more sensitive (Georgio *et al*., 2001; Yuji *et al*., 2007).

This study conducted in attempt to increase essential oils and rosmarinic acid production in rosemary callus using some biotic and abiotic elicitors *in vitro*.

Chapter Two: 2- Materials and Methods 38

2-1: Materials

2-1-1: Equipments

Equipment	Company	Origin
Autoclave	Priorclave	England
Microwave	PenGuin	Germany
Magnetic Stirrer Hote plate	SMG-Stuart	Germany
Shaker	GFL	Germany
Incubator	Heidolph-Unimax	Germany
pH meter	Mettler-Toledo	Germany
Centrifuge	Eppendroff	Germany
Biofuge	Pico-Heraeus	Germany
Rotavapour	Buchi	Germany
Distillator	Burgwedel	Germany
Clevenger-type apparatus	Halzfeld	Germany
Uv-light	Mettler-Toledo	Germany
HPLC	Agilent	USA
GC-MS	Agilent	USA
Laminer Air Flow cabinet	Telstar-BH	Spain
Electric balance	OSK/FX	Spain
Analytical balance	OSK/Ogawa	Spain
Grinding machine	MX-110PN	Japan
Refrigerator	Alhafedh	Syria
Hemacytometer	Buchi	Germany
Soxhlet	Bushi	Germany
Gas burner	Analar	England

Instrument	Company	Origin
Test tubes	Eppendroff	Germany
Glass petri-dishes	Eppendroff	Germany
Racks	Eppendroff	Germany
Pipettes	Eppendroff	Germany
Plastic Petri-dishes	Eppendroff	Germany
Micropipettes	Eppendroff	Germany
Pistil and mortar	Eppendroff	Germany
Filter paper	Eppendroff	Germany
Conical flasks	Eppendroff	Germany
Flasks	Eppendroff	Germany
Cylinders	Eppendroff	Germany
Loops and needles	CEO	China
Forceps	CEO	China
Surgical blades	CEO	China
Surgical gloves	CEO	China
Medical cotton	Pharma	Syria
Para film	Parafilm	China

2-1-3: Chemicals

All reagents and solvents used in the experiments were of analytical grade. Rosmarinic acid (98%), carnosic acid (99%), caffeic acid (98%), carnosol (99%) and rosmanol (98%) were purchased from Sigma-Aldrich company (Germany) and brought by DHL post company, Damascus.

Other chemicals were obtained from the Molecular and Biotechnology Department, AEC- Syria, and College of Science, Damascus University-Syria (table 2-1).

Table 2-1: Chemical and biological materials used in this study

Materials	**Company**	**Origin**
Agar-Agar	Biochemica	Italy
Mueller-Hinton Agar	Duchefa	Netherland
LB broth	Duchefa	Netherland
MS medium	Duchefa	Netherland
PDA medium	Duchefa	Netherland
Ethanol	Merck	Germany
DMSO	BDH	England
$CaCl_2$	BDH	England
Petrolium ether	BDH	England
Tween 80	BDH	England
PVP	BDH	England
Inositol	BDH	England
Trypan blue	BDH	England
PBS	BDH	England
BA	BDH	England
2,4-D	BDH	England
Clorox	Jade-Box	Sudia Arabia
Bacteria	AEC	Syria
Fungi	AEC	Syria
Yeast extract	AEC	Syria

2-1-4: Culture media **41**

Preparation of culture media in a concentrated form was not recommended as some salts complexes may precipitate. Supplements that are added to the medium may affect shelf life and storage conditions.

Murashige and Skoog, 1962 medium (MS) is used in plant tissue culture experiments whose components were listed in table 2-2.

Nutrient broth (Difco) is suitable for the cultivation of fastidious bacteria; after the addition of blood and serum. It is employed for the isolation and enrichment of bacteria. It was prepared according to the company instructions.

Luria-Bertani broth medium (Difco) is used for the cultivation of *E. coli* and other bacterial strains, the medium is nutritionally rich for the growth of bacteria. LB broth was prepared without the addition of agar, the final pH was adjusted to 7.2 at 37˚C before autoclaving. In order to solidify the medium, 20 g/l of agar was added. It was prepared according to the manufacturer's instructions.

Potato Dextrose Agar (PDA) medium is recommended for pure cultures of fungi. It is not a differential medium (Downes and Ito, 2001).

Mueller-Hinton medium is a general purpose medium that may be used for the cultivation of a wide variety of fastidious and non fastidious microorganisms. It was prepared according to the manufacturer's instructions.

Table 2-2: MS medium components used as stock solutions for plant tissue culture experiments

Components		
	Chemical formula	**Weight (mg/l)**
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride anhydrate	$CaCl_2$.2H2O	440
Magnesium sulphate anhydrate	$MgSO_4.7H_2O$	370
Potassium phosphate	KH_2PO_4	170
Micronutrients		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Manganese sulphate anhydrate	$MnSO_4.4H_2O$	22.30
Zinc sulphate anhydrate	$ZnSO_4.7H_2O$	8.60
Molybdic acid (sodium salt) anhydrate	$Na_2MoO_4.2H_2O$	0.25
Capric sulphate anhydrate	$CuSO_4.6H_2O$	0.025
Cobalt chloride anhydrate	$CoCl_2.6H_2O$	0.025
Chelated Iron		
Sodium ethylene diamine tetra acetate	Na_2EDTA	33.60
Ferrous sulphate anhydrate	$FeSO_4.7H_2O$	27.80
Vitamins		
Thiamine HCl	$Cl_2H_{17}C_1N_4OS.HCl$	0.1
Nicotinic acid	$C_8H_{11}NO_3.HCl$	0.5
Pyridoxine HCl	$C_6H_5NO_2$	0.5
Glycine	$C_2H_5NO_2$	2.0
Inositol		100
Sucrose		30000

2-2: Methods

This study was conducted in the plant tissue culture laboratory, Biotechnology Department, College of Science, Al-Nahrain University, and in the plant tissue culture laboratories, Molecular and Biotechnology Department-Atomic Energy Commissions (AEC), Syria, during the period of 16/11/2008 till16/5/2009.

2-2-1: Plant material

Rosemary (*R. officinalis*), was collected from the gardens of the College of Science- Baghdad University, and the gardens of AEC-Syria, in winter (mid-January to mid-February 2009).

2-2-2: Identification of rosemary plant

Rosemary plant was identified and authenticated by Molecular and Biotechnology Department, AEC-Syria, and Prof. Dr. Ali Al-Musawi, College of Science, Baghdad University, for sample collected from Baghdad University gardens.

2-2-3: Explants sterilization

Leaf explants were surface sterilized with 70% (v/v) ethanol for 10 seconds. After rinsing three times with sterile distilled water (DH_2O), 0.5, 1.0, 1.5 or 2% (v/v) of sodium hypochlorite solution (containing 5.25% of Cl_2) was used at three periods 5, 10 or 15 min for each concentration, then rinsed three times with sterile DH_2O. All these processes were conducted inside the laminar air flow cabinet (Pierik, 1987).

2-2-4: Preparation of culture medium for callus induction

MS medium was prepared as stock solutions by dissolving the compounds listed in table 2-1, then 100 mg/l inositol, 3% sucrose were added. The pH was adjusted to 5.7-5.8 using KOH or HCl (1N), 8 g/l (Agar-Agar type) was added to solidify the medium.

The volume of the medium was completed to 1 liter. For callus induction, MS basal medium was supplemented with various concentrations of 2,4-D (0.0, 0.5, 1.0 or 2.0) mg/l.

BA was added at concentrations of 0.0, 0.2, 0.5 or 1.0 mg/l, and KIN at the concentrations of 0.0, 0.05, 0.1 or 0.2 mg/l. The medium components were placed on a hotplate magnetic stirrer till boiling. The culture media were autoclaved at 121˚C, 1.04 kg/cm^2 pressure for 15 min, then 10 ml were poured into Petri dishes, and left at room temperature to cool and become ready to use. After surface sterilization, leaf explants were cut at the ends into sections of approximately 1cm in length. Explants were placed on MS medium in Petri-dishes (100 x 15 mm). Ten replicates were used in this experiment.

2-2-5: Incubation of plant tissue cultures

Cultures were placed in a growth chamber at 25±2˚C for 16/8 hrs (light/dark) photoperiod using cool white inflorescent with a light intensity of 3000 lux as a source for light.

2-2-6: Maintenance of callus cultures

For maintenance of callus cultures, the friable callus obtained from leaf explants was dissected and cultured on MS medium containing 2,4-D and BA at 16/8 hrs light/dark photoperiods. The medium was supplemented with 2.0 mg/l of 2,4-D, 0.5 mg/l of BA, 3% sucrose and 8 g/l agar.

2-2-7: Cell count and viability

Cell suspension cultures were initiated by placing 500 mg of friable callus in 100-ml flask containing 30 ml of the maintenance MS liquid medium. Sucrose was added at concentrations 30, 40 or 50 g/l.

Suspension cultures were maintained at a gyratory shaker (120 rpm), in a growth chamber as in 2-2-5. Growth rates were determined by accounting the cultured cell number at 3 day intervals and continued for 15 days using a hemacytometer. Viable cell counts were conducted using hemacytometer. Dead cells and the viable and non-viable cells are distinguished with the use of trypan blue dye. Living cells did not take up the dye, while dead one stained blue. Aliquots of 200 µl of the cell suspension are transferred into a 15 ml centrifuge tube, then 300 µl of PBS and 500 µl of trypan blue solution were added to the cell suspension in the centrifuge tube, mixed thoroughly then allowed to stand for 5-15 min. A Pasteur pipette was used to transfer a small amount of the cell suspension to hemacytometer chamber. Viable cells were counted in 1 mm square (which was divided into 4 subsquares). The mean of the cell number in the two squares (left and right) was counted (figure 2-1).

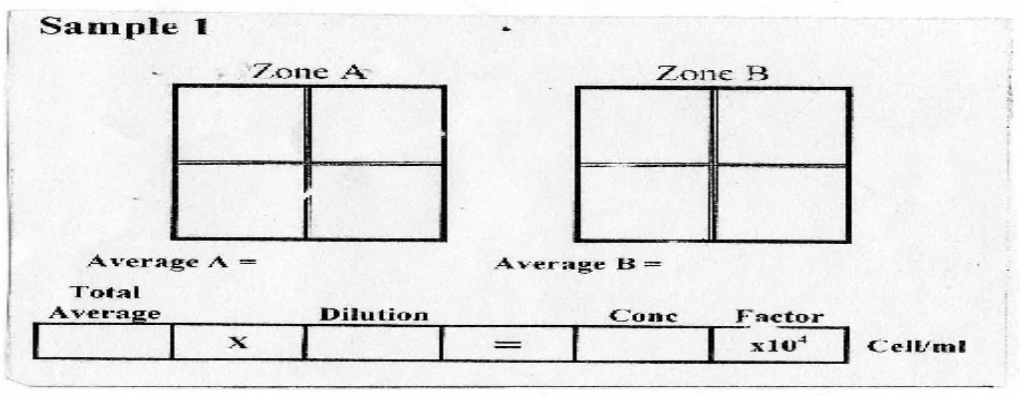


Figure 2-1: Cell count using hemacytometer squares showing the 4 left and the 4 right squares (Davis, 1996)

The cell number per ml and the total number of cells were determined using the following formula:

Cells per ml = the average cell count per square x dilution factor x 10^4 Total cell number = cells per ml x the original volume (30 ml) as mentioned by Davis (1996).

2-2-8: Precursors

In order to enhance the biosynthesis pathway of the phenolic acid (RA) and the phenolic diterpenes (CA, CAR), MS medium was supplemented with the precursors; phenylalanine, tyrosine, and different concentrations of sucrose. This was conducted for callus and cell suspension cultures of rosemary plant (Hany, 2006). A quantity of 500 mg of callus pieces were placed on the surface of MS medium supplemented with 0.1 g/l of Phe, and Tyr. Sucrose was added at the concentrations of 30, 40 or 50 g/l. Cultured vessels were incubated in growth chamber as indicated in 2-2-5.

2-2-9: Elicitors

This part of study was concentrated on the secondary metabolites production from callus tissue after exposure to biotic and abiotic elicitors at different concentrations.

2-2-9-1: Preparation of abiotic elicitors

Different abiotic elicitors were used including calcium chloride ($CaCl_2$) and gamma-rays. Different concentrations of $CaCl_2$ (0.0, 0.4 or 0.6 g/l) were added to MS medium. The pH was adjusted to 5.8 before autoclaving at 121˚C and at a pressure of 1.04 kg/cm^2 for 15 min. Pieces weight 500 mg of callus tissue at age of 4 weeks were subjected to gamma-rays at doses of 3, 5 or 7 Gray for 5 min. from the Co 60 radiation source. After irradiation, callus pieces were cultured on MS nutrient medium supplemented with 2.0 mg/l of 2,4-D and 0.5 mg/l of BA.

Culture vessels were incubated in the growth chamber as indicated in 2-2-5 (Kravchenko *et al*., 2003).

2-2-9-2: Preparation of biotic elicitors

The fungus *Fusarium oxysporum*, bacteria *Pseudomonas aeruginosa* and yeast extract were used as elicitors in this study. The strains were supplied by the department of microbiology (AEC-Syria). The fungal cultures (*F. oxysporum*) were maintained as slants on potato dextrose agar (PDA). Bacterial cultures (*P. aeruginosa*) were maintained on Mueller-Hinton Agar (MH). Liquid medium is prepared similarly without agar which was used for growing fungi or bacteria for elicitor preparation (Thimmaraju *et al*., 2006). Cultures that were maintained on agar slants, are transferred to 250-ml flasks contains 100 ml liquid medium and incubated at room temperature. The bacterial cultures were kept on a rotary shaker (90 rpm), while the fungal cultures were allowed to stand without shaking. Cultures were harvested after reaching stationary phase (5-7 days for fungal cultures and 48 hrs for bacterial cultures). Flasks are then autoclaved and the solution obtained is stored at 4˚C for future use. Yeast extract was added at concentrations of 0.0, 1.0 or 2.0 g/l. The addition of bacterial and fungal elicitors was added at the concentrations of 1.0 or 2.0 ml/l to MS medium. A quantity of 500 mg of callus pieces were placed on the surface of MS medium supplemented with 2 mg/l of 2,4-D and 0.5 mg/l of BA. Cultured vessels were incubated at the same conditions as mentioned in 2-2-5 (Kravchenko *et al*., 2003).

2-2-10: Collection of samples

Rosemary leaves were collected from field grown plants during (Mid-January to Mid-Febraury 2008). Leaves were initially rinsed

with distilled water and dried using paper towel in the laboratory. Callus cultures and cell suspension cultures grown in the growth chamber are harvested. All samples were dried at 40˚C for 24 hrs, ground into powder using the grinding machine, then subjected to extraction (Bos, 1997).

2-2-11: Preparation of leaf ethanol extract

Powdered rosemary leaves (50 g) were soaked into 250 ml of 70% ethanol. The mixture was kept for 24 hrs in tightly sealed vessels at room temperature, protected from sunlight and mixed several times with a sterile glass rod; the mixture was then filtered through Wattman no.1 filter paper. The extract was subjected to evaporation using a rotary evaporator in order to remove the solvent. The extract was then stored at 4˚C for further use in GC-Ms analysis (Akueshi *et al*., 2002).

2-2-12: Preparation of leaf aqueous extract

Samples weight 50 g of powdered rosemary leaves were soaked into 250 ml of distilled water in a glass flask and left for 24 hrs in a rotary shaker, filtered using sterile filter paper then transferred into a clean conical flask and subjected for evaporation using water bath, when the aqueous solvent was evaporated at 100˚C. The extract was then stored in a refrigerator at 4˚C for future use in HPLC analysis (Akueshi *et al*., 2002).

2-2-13: Preparation of callus ethanol extract

A quantity of 10 g of callus powder was extracted with 50 ml of 70% ethanol by soxhlet apparatus for 6 hrs at 60ºC. The solution is then evaporated to dryness using a rotary evaporator at 40 ºC.

The extract was stored at 4ºC in a refrigerator for future use in GC-MS and HPLC analysis (Harborne, 1984).

2-2-14: Extraction of rosemary essential oils

The essential oils were extracted using steam distillation method. Samples weighting 50 g of rosemary powdered leaves were soaked in 250 ml of distilled water in a conical flask, and left for 24 hrs. Then the extract was subjected to steam distillation for 3 hrs using a Clevenger-type apparatus. Essential oils were collected after decantation, then stored for further use in GC-MS analysis (Mounchid *et al.*, 2004).

2-2-15: Detection of phenols and terpenes in ethanol extracts

2-2-15-1: Chemical detection of phenols

Equal quantities of aqueous ferric chloride 1% were mixed with potassium iron cyanide 1%. Equal quantities of the reagent and alcohol plant extract were mixed. The appearance of blue-green color indicates the presence of phenols (Harborne, 1984).

2-2-15-2: Chemical detection of terpenes

One gram of dried ethanol extract was suspended with a few drops of chloroform, then a drop of 1% acetic anhydride and a drop of a concentrated sulphuric acid were added. The appearance of the brown color indicates the presence of terpenes (Harborne, 1984).

2-2-16: Preparation of standard solutions

Stock solutions of standards (rosmarinic acid, caffeic acid, carnosic acid, carnosol and rosmanol) were prepared by dissolving 10 mg of each standard in 50 ml of methanol (70%) to obtain a final concentration 200 ppm (200 mg/l). Stock solutions were then stored in a deep freezer at -20 ˚C.

Sample volume was 1µl for every injection into GC-MS and 15 µl for HPLC injection (Masa *et al.*, 2007).

2-2-17: Analysis of rosemary essential oils and ethanol extract

2-2-17-1: Gas Chromatography (GC) analysis

The essential oils were analyzed using GC apparatus (Agilent 6780-USA) equipped with EID detector and HP-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). Injector and detector temperature were set at 230 ˚C and 250˚C, respectively. Helium was the carrier gas, at flow rate of 0.8 ml/min. Samples of 1µl were injected automatically in the split mode. Quantitative data were obtained electronically from EID area.

2-2-17-2: Gas Chromatography/ Mass Spectrometry analysis (GC-MS)

Analysis of essential oils was performed using GC-MS, equipped with mass selective detector. For GC-MS detection electron ionization system with ionization energy of 70 eV was used. Samples were injected automatically in the split mode at split ratio of 1:100. All the tests were performed in triplicate.

2-2-18: Analysis of phenolic compounds

2-2-18-1: High Performance Liquid Chromatography (HPLC)

The HPLC analysis of phenolics extracted by water (aqueous extract) was performed using an HP-1100 system (Agilent-USA), consisting of a pump with degasser, multi-wave detector (MWD). Quantitative analysis of the constituents were calculated by MWD peak areas and normalized without the use of response factor correction.

2-2-18-2: HPLC analysis conditions **51**

Symmetry C18 (150 x 3.9 m, 5 μm) water column was used. A mobile phase consisting of Methanol and water (70:30), pH was adjusted to 2.5 using formic acid. The flow rate was 0.5 ml/min and injection volume 15 μl. All analyses were conducted at room temperature. MWD detector works in the range of 210-254 nm, and chromatograms were acquired at different wavelengths according to absorption maxima of analyzed compounds.

2-2-19: Identification of the compounds

The components of the oil (phenolic and terpenic compounds) were identified (in GC-MS) by comparison of recorded mass spectra with those of a computer library (Wiley 275 library). HPLC identification was confirmed by comparison of their retention indices with those of authentic standard compounds and with the data published in the literature (Shibamoto, 1987; Adams, 2002).

2-2-20: Tested microorganisms and growth conditions

The following bacterial isolates; *Bacillus cereus* representative for G-positive group, and *Pseudomonas aeruginosa* and *Escheritia coli* representative for G-negative group and two fungal isolates; *Aspergillus nigar* and *Fusarium oxysporum*, were used in this part of study. Bacterial stock cultures (*B. cereus, P. aeruginosa* and *E. coli*) were maintained on MH agar plates, a loopful of bacterial cell from each plate was inoculated into 100 ml broth in 250 ml-flask and incubated at 37 ˚C for 24 hrs in a shaker incubator. Then, the cultures were diluted with fresh sterile medium to give OD_{600nm} of 0.5 (McFarland standards) which is equivalent to 1.5 x 10^5 CFU/ml. Fungal colonies (*A. nigar* and *F. oxysporum*) are maintained on potato dextrose agar, then suspended in sterile distilled water.

The suspension was filtered once through sterile gauze (0.5 mm) to remove hyphae. The turbidity was adjusted to McFarland standard of 0.5 which is equal to $1x10^4$ CFU/ml.

2-2-21: Antimicrobial assay

This was designed to determine the activity of ethanol leaf extracts of rosemary as a potential antibacterial and antifungal activity against the tested microorganisms. MIC determination was conducted by broth dilution, agar dilution and well diffusion methods, using MH agar for growth of bacteria and LB agar for growth of fungi. DMSO was used to facilitate mixing of the extracts with the broth, as long as oils are immiscible with water which could affect its antimicrobial activity (Donald and Clyde, 1974).

2-2-21-1: Broth dilution method (Donald and Clyde, 1974**)**

Aliquots of 100 µl of bacterial and/or fungal suspensions were inoculated into tubes containing MH and LB broth respectively, supplemented with different concentrations of 10, 20, 40, 80, 160, 320 or 640 µl/ml of ethanol extracts. The tubes were then incubated at 37˚C for 24 hrs for bacterial cultures and 30˚C for 72 hrs for fungal cultivation. The MIC for each sample was determined by measuring the optical density (OD) at 600 nm using spectrophotometer. Control (non inoculated) samples were also included.

2-2-21-2: Agar dilution method (Josep *et al*., 2000**)**

Different concentrations of ethanol leaf extracts (10, 20, 40, 80, 160, 320 or 640) µl/ml were added to sterile petri-dishes. Then melted MH agar and PDA media (at approximately 50 ˚C) for growth of bacterial and fungal isolates, respectively, were poured into the plates and swirled to mix the components well.

Then left to solidify, and 100 µl of the suspensions containing the tested microorganisms were spread using sterile glass rod and incubated at 37˚C for 24 hrs and 30˚C for 72 hrs for growth of bacterial and fungal isolates, respectively. Colonies are then counted, and the MIC was taken as the lowest concentration of extract at which the tested organism does not show visible growth.

2-2-21-3: Well diffusion method (Bauer *et al*., 1966)

The same media and growth conditions were followed, except wells are made in the solidified medium using cork borer. Aliquots of 100 µl of the inoculum were applied to the wells, then incubated as above. Diameters of inhibition zones were measured in mm.

2-2-22: Statistical analysis

The presented data are an average of ten replicates for all experiments except samples used in HPLC and GC-MS experiments since they used as triplicate. A completely randomized design was used. Least significant differences (LSD) were calculated for all data, whereas Qi-square was used only for explants survival rate and significant differences between means were compared. Statistical analysis of data was conducted using SAS package (2001).

Chapter three: 3- Results and Discussion 54

3-1: Surface sterilization of explants

The effect of NaOCl on the survival percent of rosemary explants at different concentrations and different periods of time was studied. Table 3-1 shows that 1% NaOCl for 10 min. is the most effective concentration for surface sterilization since it gave the highest percent (100%) survival. However, the concentration of 2% for 10 min. caused a damage to plant tissues whereas the lower concentration (0.5 %) led to a reduction in survival percent.

Table 3-1: Survival (%) of rosemary explants after surface sterilization with different concentrations of NaOCl and three time periods

NaOCl%	Time (Min.)	Survival %
0.5	5	10
	10	80
	15	70
1.0	5	30
	10	100
	15	80
1.5	5	20
	10	80
	15	60
2.0	5	5
	10	30
	15	40
Qi-square $p \leq 0.01$		11.45**

Treatment with NaOCl for 5 min at the concentrations of 0.5, 1.0, 1.5 or 2.0% gave the lowest survival percent (10, 30, 20 and 5)% respectively, while treatment for 15 min. increased the survival percent to 70, 80, 60 and 40% at the concentrations of 0.5, 1.0, 1.5 or

2.0% respectively. NaOCl is used widely for explants surface sterilization, it was found to be effective in eliminating bacterial and fungal contaminants, especially when explants are rinsed with 70% ethanol before NaOCl treatment. The selection of sterilizing material depends on the source of the explant, roughness of its surface and other factors. The sterilization material should be easy to remove from explants when washed with DDH_2O (Yeoman and Macleod, 1977; Sateesh, 2003). The importance of NaOCl in explants sterilization was reported by Pierik (1987), who found that increasing the surface sterilization period and concentration often leads to serious reduction in survival rate. Sodium hypochlorite exhibits a dynamic balance as is shown by the reaction:

$$NaOCl + H_2O \leftrightarrow NaOH + HOCl \leftrightarrow Na^+ + OH^- + H^+ + OCl^-$$

Hypochlorous acid, a substance present in sodium hypochlorite solution, when it contact with organic tissue acts as a solvent, releases chlorine that combined with protein amino group (Pecora *et al*., 1999). The antimicrobial effect of sodium hypochlorite based on its high pH due to hydroxyl ions action, the high pH interferes with the cytoplasmic membrane integrity with an irreversible enzymatic inhibition, alterations in cellular metabolism and phospholipid degradation observed in lipidic peroxidation (Carlos-Estrela, 2002).

3-2: Induction of callus cultures

Table 3-2 shows that addition of 2,4-D at the concentration 2.0 mg/l led to a significant increase in the response percentage of callus induction achieving 82.5% as compared with the control treatment which reached 9.5% only.

It was shown that reducing 2,4-D concentration resulted in green and compact callus, whereas higher concentrations produced light friable callus (figure 3-1).

Table 3-2: Effect of different concentrations of 2,4-D and BA and their combinations on the response percentages of callus induction on rosemary leaf explants after 4 weeks of incubation

2,4-D (mg/l)	BA (mg/l)				
	0.0	0.2	0.5	1.0	Mean of 2,4-D
0.0	2	10	12	14	9.5
0.5	54	74	68	28	56.0
1.0	58	76	84	66	71.0
2.0	60	88	100	82	82.5
Mean of BA	43.7	62.0	66.0	47.5	
LSD ≤ 0.05	BA= 2.656*		2,4-D= 2.656*		BA x 2,4-D= 5.312*

* Significant

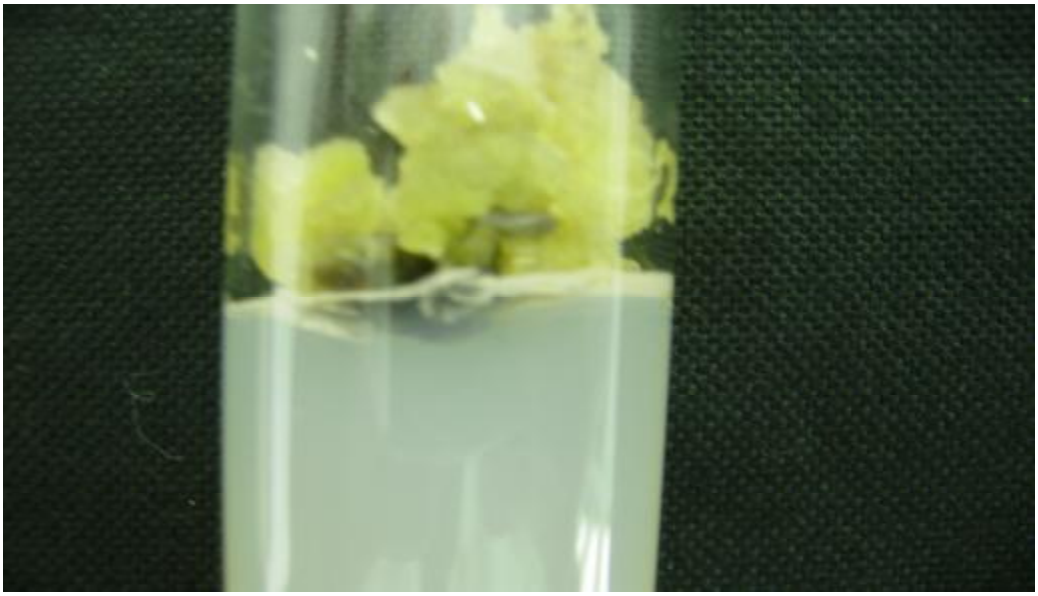


Figure 3-1: Callus induction on rosemary leaf explants grown on MS medium containing a combination of 2.0 mg/l 2,4-D and 0.5 mg/l BA after four weeks of incubation at 16/8 hrs (light/dark) photoperiod

Addition of BA at the concentration of 0.5 mg/l led to a significant increase in the percentage of explants showing callus induction recording 66.0%. The interaction between 2,4-D and BA achieved 100% response in the combination of 2.0 mg/l 2,4-D and 0.5 mg/l of BA as compared to the control treatment that gave 2%. The effect of different concentrations of 2,4-D and KIN and their combinations on callus induction were investigated in order to evaluate the callus induction percent on leaf explants. Table 3-3 shows that the addition of 2,4-D at the concentration of 2.0 mg/l significantly increased the response percentage of callus induction (75.0%) as compared to the control treatment which recorded 8.2%.

Addition of KIN at the concentration of 0.1 mg/l led to a significant increase in the percentage of explants showed callus induction reaching 58.0%. All concentrations of KIN significantly increased the response percentage of callus induction compared to the control treatment which produced a response of 38%. Interaction 2,4-D and KIN achieved 90% response in a combination of 2.0 m/l of 2,4-D and 0.1 mg/l of KIN (figure 3-2). Four weeks after incubation, 2,4-D treatment was most effective in callus induction, reaching 100% when the medium contains 2.0 mg/l 2,4-D and 0.5 mg/l BA, while it reached 90% when 2,4-D was added at the concentration of 2.0 mg/l in combination with 0.1 mg/l of KIN. It was concluded that 2,4-D at the concentration of 2.0 mg/l gave the best response for callus formation. Results of the present study also showed that 2,4-D containing media responded well for callusing. 2,4-D induces cell division and it is among the widely used auxins for *in vitro* callus induction (Salman, 1988).

Table 3-3: Effect of different concentrations of 2,4-D and KIN and their combinations on the response percentage of callus induction on rosemary leaf explants after 4 weeks of incubation

2,4-D (mg/l)	KIN (mg/l)				
	0.0	0.05	0.1	0.2	Mean of 2,4-D
0.0	2	9	12	10	8.2
0.5	40	40	50	20	37.5
1.0	50	60	80	70	65.0
2.0	60	70	90	80	75.0
Mean of KIN	38.0	44.7	58.0	45.0	
LSD ≤ 0.05	KIN= 2.737*		2,4-D= 2.737*	KIN x 2,4-D= 5.472*	

- Significant

Establishment of a callus from the explants was illustrated by Dodds and Roberts (1995) who divided the process into three stages; induction, cell division and differentiation. The period of these phases depend mainly on the physiological status of explant cells as well as the culture conditions, including the appropriate combination of plant growth regulators. Variation in callus induction might be the result of endogenous hormone types and hormone levels in each explant. Calli varied not only in weight but also in phenotypes. They found to be green to white or yellow to white in color. Hard, dense, fragile or mucilaginous calli were produced depending on hormone concentration or combination. Similar results were obtained by Natas *et al.*, (2004), who found that MS medium supplemented with 1.0 mg/l of 2,4-D and 0.1 mg/l KIN formed friable yellow callus which were maintained and grew fast.

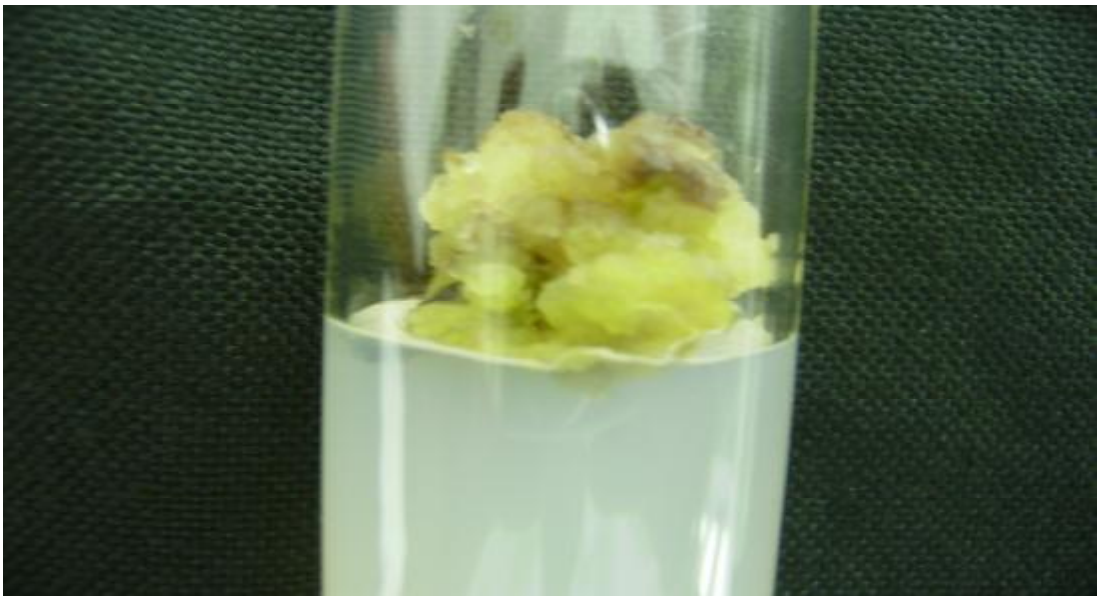


Figure 3-2: Callus induction on rosemary leaf explants grown on MS medium containing a combination of 2.0 mg/l 2,4-D and 0.1 mg/l KIN after four weeks of incubation at 16/8 hrs (light/dark) photoperiod

3-3: Maintenance of callus cultures

Table 3-4 revealed that the addition of 2,4-D exhibited a positive effect on rosemary callus growth at the concentration of 2.0 mg/l 2,4-D in combination with 0.5 mg/l of BA. Inclusion of 2,4-D at the concentration of 2.0 mg/l gave significantly higher callus fresh weight (1099.2) mg than other concentrations, while the lowest fresh weight (75.5) mg was obtained in 2,4-D free medium.

The highest callus fresh weight obtained from BA treatment (974.3) mg occurred at the concentration 0.5 mg/l.

The interaction between 2,4-D and BA at the treatments 2.0 mg/l of 2,4-D with 0.5 mg/l of BA resulted in maximum callus fresh weight (1780.0) mg (figure 3-3), this treatment was significantly higher than the control treatment which produced 52.8 mg.

Table 3-4: Effect of different concentrations of 2,4-D and BA and their interactions on rosemary callus fresh weight (mg) grown on MS medium after four weeks of incubation at 16/8 hrs (light/dark) photoperiod (initial callus weight was 50 mg)

2,4-D (mg/l)	BA (mg/l)				
	0.0	0.2	0.5	1.0	Mean of 2,4-D
0.0	52.8	56.4	112.0	81.0	75.5
0.5	406.4	492.1	776.4	127.	450.7
1.0	559.2	878.5	1228.9	590.2	814.2
2.0	667.1	1103.5	1780.0	846.4	1099.2
Mean of BA	421.4	632.6	974.3	411.3	
LSD ≤ 0.05	BA: 21.205*	2,4-D: 21.205*		BA x 2,4-D: 42.41*	

* Significant

Caruso *et al*. (2000) stated that rosemary callus grown on solid MS medium containing 2.0 mg/l 2,4-D increased in weight and size.

Chandra and Bhanja (2002) found that induction and maintenance of callus was obtained in MS basal medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l BA, and the leaf segment showed the best response for callus induction of *Flacourtia jangomas*. Cultures grown on MS medium under a photoperiod of 16/8 hrs were mostly, friable, grew fast and large in size. The daylight fluorescent tubes give important emission bands at red and blue spectral wavelength. These conditions have been found to give the best culture growth responses (Roland *et al*., 1992). Table 3-5 shows that using 2,4-D at the concentration of 2.0 mg/l showed significantly higher callus fresh weight (963.2) mg as compared to other concentrations, while the lowest fresh weight was (56.0) mg obtained from the treatment of 2,4-D free medium.

The highest callus fresh weight (804.6) mg was obtained at the treatment 0.1 mg/l of KIN. The interaction between 2,4-D and KIN at the treatments of 2.0 mg/l of 2,4-D and 0.1 mg/l of KIN, revealed the maximum callus fresh weight of 1442.5 mg (Figure 3-4). The combinations between 2,4-D and KIN showed a significant increase in callus fresh weight. All treatments were higher than the control treatment that produced fresh weight of 52.8 mg.

Table 3-5: Effect of different concentrations of 2,4-D and KIN and their interactions on rosemary callus fresh weight (mg) grown on MS maintenance medium after four weeks of incubation at 16/8 hrs (light/dark) photoperiod (initial callus weight was 50 mg)

2,4-D (mg/l)	KIN (mg/l)				
	0.0	0.05	0.1	0.2	Mean of 2,4-D
0.0	52.8	54.5	60.5	55.5	56.0
0.5	159.1	481.7	754.2	216.8	402.9
1.0	521.4	757.1	961.4	678.8	729.6
2.0	721.4	877.1	1442.5	811.8	963.2
Mean of KIN	363.9	542.6	804.6	440.7	
LSD ≤ 0.05	KIN: 8.978*		2,4-D: 8.978*		KIN x 2,4-D: 17.956*

* Significant

Hui *et al.*, (2008), also reported similar results with callus growth and maintenance on MS medium containing 2.0 mg/l 2,4-D. Callus induction and maintenance requires a balanced ratio from auxin and cytokinin as mentioned by Skoog and Miller (1957); Shain-Dow and Shang-FA (1999).

Jayasree *et al.*, (2004), observed optimum callus induction and maintenance on MS medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l KIN.

They noticed swelling of leaf explants after 2 weeks of culture, followed by callus initiation between 40-50 days. Rosemary leaf explants may contain some levels of endogenous auxins that make a balanced ratio with the exogenous auxin and may be even cytokinin. The increase in callus mass was important for the production of secondary metabolites since they are proportionally related. It would be convenient from the practical point of view to induce and maintain callus on the same growth nutrient and plant growth regulators (Ramawat, 2008). Production of a large quantity of callus fresh weight as recorded in table 3-4 and figure 3-3 is advantageous, since the yield of secondary metabolites is a proportional to biomass yield. Quick callus induction and fast growth response is favorable in tissue culture experiments for production of secondary metabolites.

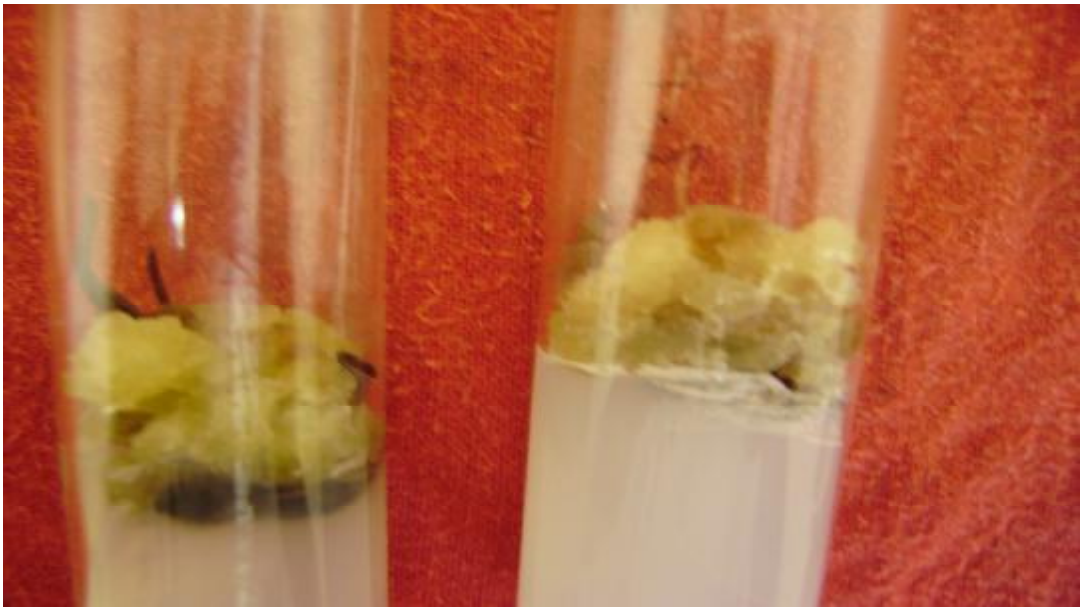


Figure 3-3: Callus production from rosemary leaf explants grown on MS Medium supplemented with a combination of 2.0 mg/l 2,4-D and 0.5 mg/l BA after four weeks of incubation at 16/8 hrs (light/dark) photoperiod

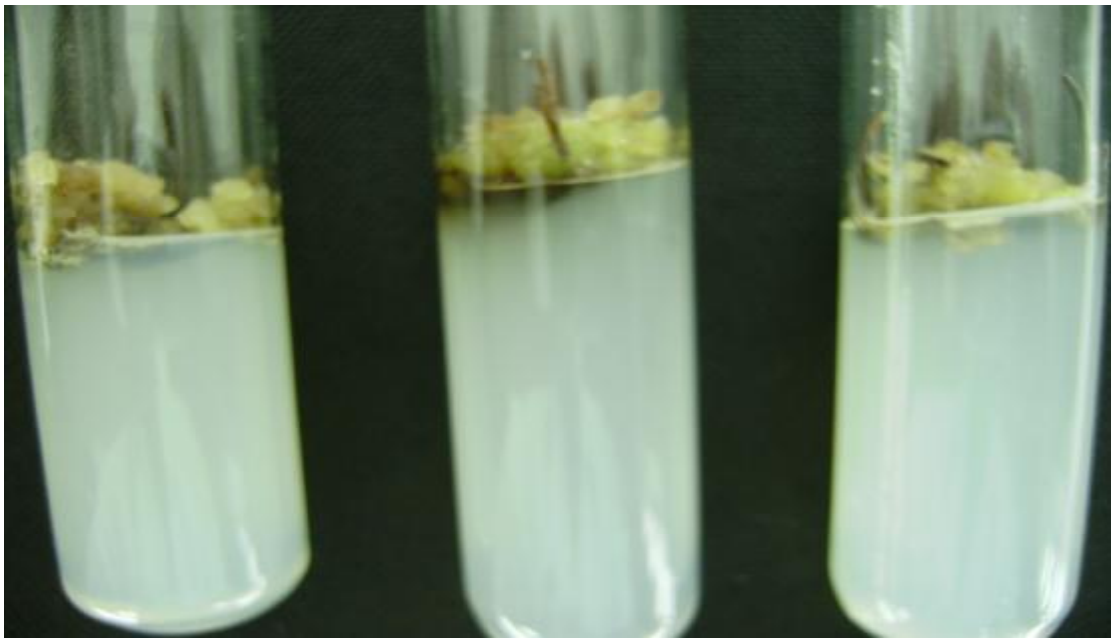


Figure 3-4: Callus production from rosemary leaf explants grown on MS medium containing a combination of 2.0 mg/l 2,4-D and 0.1 mg/l KIN after four weeks of incubation at 16/8 hrs (light/dark) photoperiod

3-4: Effect of gamma-rays on callus fresh weight

After four weeks of incubation period of the callus tissues irradiated with the three doses of gamma-rays (3, 5 or 7) Gray, the effect of radiation was shown in table 3-6 and figure 3-5.

The highest fresh weight (2138.3) mg was obtained at the dose of 5 Gray, while the lowest fresh weight (1582.5) mg was obtained at the dose of 3 Gray. All treatments were significantly higher than the control which gave 1026.3 mg of callus fresh weight. There was a reduction in callus fresh weight at highest dose (7 Gray) of gamma-rays as compared to the lower dose (5 Gray).

Tatiana *et al.*,(1999) found that treatment of lavender callus with high doses (20-60 Gy) of gamma-rays inhibited callus growth, while the lower dose (10Gy) improved embryo conversion into plants.

It can be concluded that the reduction in callus fresh weight may be caused by the reduced amount of endogenous growth regulators, especially the cytokinins due to the higher dose of irradiation (Singh, 1996).

Table 3-6: Effect of different doses of Gamma-rays (Gy) on callus fresh weight (initial callus weight was 500 mg) after four weeks of incubation period

Dose (Gy)	**Fresh weight (mg)**
0	1026.3
3	1582.5
5	2138.6
7	1824.8
LSD ≤ 0.05	27.82*

* Significant

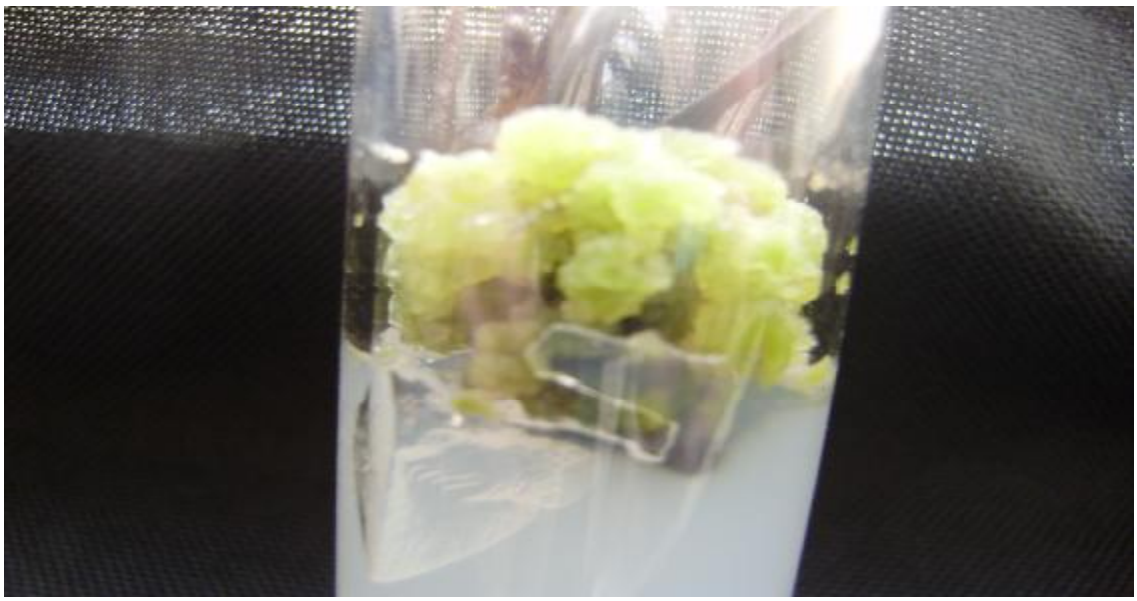


Figure 3-5: Callus growth after exposure to gamma-rays at a dose of 5 Gy after four weeks of growth on MS medium supplemented with 2.0 mg/l of 2,4-D and 0.5 mg/l of BA.

3-5: Effect of sucrose concentrations on cell suspension cultures

The effect of sucrose concentrations (30, 40) g/l on cell number per milliliter in cell suspension cultures was shown in table 3-7. The data revealed that the addition of sucrose at 40 g/l with 0.1 g/l of Phe to MS medium was significantly increased the number of cells/ml; it reached 86.2 x 10^5 after 15 days of incubation period, while the less number was 12.8 x 10^4 at 3 days of incubation. This means, that the high number of cells produced by cell suspension cultures when 40 g/l of sucrose was added, gave the highest level of secondary products compared to the control (30 g/l).

These results are in agreement with those recorded by Torres (1988), who found that the cell number reached more than 15 x 10^3 cell/ml from cell suspension cultures. Higher sucrose concentrations induced rosmarinic acid synthesis in *Coleus blumei, Anchusa officinalis, Salvia officinalis* and *Rosmarinus officinalis* cell cultures. Highest rosmarinic acid content 36% of cell dry weight was obtained in suspension cultures of *Salvia officinalis* at 5% sucrose (Ulbrich *et al.,* 1985; Shetty *et al*., 1996) . Addition of phenylalanine (Phe) and tyrosine (Tyr) to the nutrient medium stimulated rosmarinic acid production. Increased sucrose concentrations in MS nutrient medium diminished the growth of all species. Media with 4% and 5% of sucrose stimulated RA accumulation more than medium with 3% sucrose (control).

Medium supplemented with 0.1 g/l Phe and Tyr increased rosmarinic acid level in callus, while the higher concentration decreased RA accumulation in comparison to the medium free of them (Su and Humphrey, 1990; Pavlov and Hieva, 1999).

Table 3-7: Cell number of rosemary cell suspension cultures calculated in 1 ml after addition of 30, 40 g/l sucrose and 0.1 g/l Phe

Incubation period (day)	**Cell number/ml**		
	Sucrose 30 g	**Sucrose 40 g**	
3	12.6×10^4	12.8×10^4	
6	22.5×10^4	26.5×10^4	
9	42.8×10^4	48.6 x 104	
12	58.5×10^4	64.4×10^4	
15	74.5×10^4	86.2×10^5	
LSD ≤ 0.05	7.36*	9.28*	

* Significant

3-6: GC-MS analysis of essential oils

Chemical analysis of essential oils extracted from rosemary leaves revealed 22 compounds different in quantity as determined by percent in total oil composition. The main components of the oil were shown in table 3-8 and figure 3-6. The components of the essential oils were identified by comparison of recorded mass spectra with those of a computer library. The identification was confirmed by comparison of their retention indices with data published in the literature (Adams, 2002).

Rosemary oil was shown to contain the highest composition of camphor (20.54%), cineole (13.30%), verbenone (12.71%), bornyl acetate (12.22%), carene (7.73%), camphene (4.32%), lemonene (4.29%), α-pinene (2.28%) and β-pinene (1.77%), while α-amorphene recorded the lowest percentage (0.20%), these compounds are of a big medicinal value.

Table 3-8: Retention time and peak area (%) of the different compounds found in rosemary essential oil analyzed by GC-MS

Compound	**RT (min.)**	**Peak area%**
Carene	6.19	7.73
Camphene	6.51	4.32
A-pinene	7.16	2.28
B-pinene	7.69	1.77
B-phellandrene	8.00	2.10
Cineole	9.38	13.30
3-carene	10.34	1.49
Terpinolene	11.37	1.58
Camphor	14.98	20.54
Bornyl acetate	15.89	12.22
Verbenone	17.89	12.71
Linderol	18.73	1.70
Lemonene	20.21	4.29
Thymol	20.89	0.54
Eugenol	23.89	0.78
Eucarvone	24.77	1.44
P-cymene	25.14	1.61
Humulene (α-caryophyllene)	26.64	0.65
A-amorphene	27.54	0.20
Caryophyllene oxide	31.70	0.63
Ledene	34.88	0.95
A-bisabolol	35.69	0.26

These data contrast with values on essential oil from rosemary grown in southern Spain collected by Chalchate *et al*., (1993) who found them to be rich in α-pinene (24.7%) and camphor (18.9%), they also contained some borneol (4.5%). The Essential oils from the fresh leaves of rosemary were isolated and analyzed by Porte *et al.*,(2000), 45 constituents were identified, the major constituents of the oil being camphor (26.0%) and α-pinene (11.5%), while the results of GC-MS analysis of essential oils in this study showed that camphor, cineole,

verbenone, bornyl acetate, carene and camphene were the most abundant components of rosemary essential oils which have a great therapeutic value.

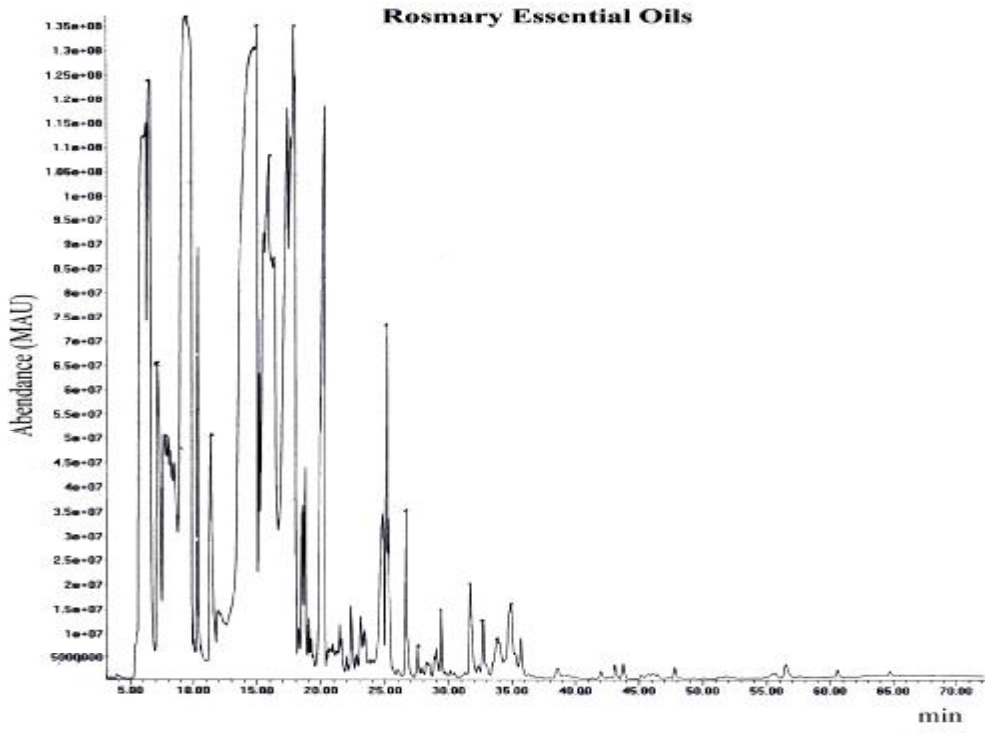


Figure 3-6: Chromatogram showing the chemical composition of essential oils extracted from rosemary leaves analyzed by GC

There is a great variability in the chemical composition of essential oils obtained, such variability depend on several factors including climate, seasons, geographical location, soil structure and texture, part of the plant and the method used to obtain the essential oil. Results showed that the most available constituents of the essential oil of rosemary were terpenoids, monoterpenes, sesquiterpenes and diterpenes, and they also contain aliphatic and aromatic esters, phenolic compounds and substituted benzene hydrocarbons.

The content of EOs depends considerably upon extrinsic and intrinsic factors, optimal harvest time to guarantee high content of EOs and high quality was reported to be at the plant development phase just before flowering (Toth *et al*., 2003).

3-7: GC-MS analysis of rosemary leaf ethanol extract

Table 3-9 and figure 3-7 showed 13 compounds at different percentages in leaf extract, the most represented compounds were isocarnosol (5.85%), camphor (3.88%), ferruginol (4.53%), cineole (2.66%), verbenone (2.50%), borneol (1.56%) and bornyl acetate (0.57%). The lowest percentage (0.09%) was recorded for eugenol. Other compounds were presented at only trace levels with some non-identified compounds.

Table 3-9: Retention time and peak area (%) of the different compounds found in leaf ethanol extract analyzed by GC-MS

Compound	**RT (min)**	**Peak area%**
Cineole	8.80	2.66
A-caryophyllene	11.71	0.40
Camphor	13.36	3.88
Borneol	14.44	1.56
Terpinen-4-ol	14.88	0.25
P-menth-1-en-8-ol	15.59	0.59
Verbenone	16.26	2.50
Bornyl acetate	19.49	0.57
2-methyl-4-vinylphenol	21.09	0.15
Eugenol	22.85	0.09
Pyrazolobis (bbn) thiolium	57.58	9.65
Ferruginol	58.70	4.53
Isocarnosol	61.69	5.85

Results of the composition of rosemary leaf extracts resemble to those obtained by Panizzi *et al*., 1993 and Aziza *et al*., 2008 but with some quantitative variations. These differences in the chemical composition may be due to different chemotypes and the nutrient status of the plants. The effect of harvest time on chemical composition also studied by Martos *et al*., (2007) who stated that the highest yield was recorded during summer. Cineole concentration was approximately constant throughout the year. Other constituent levels varied randomly with the plant life cycle.

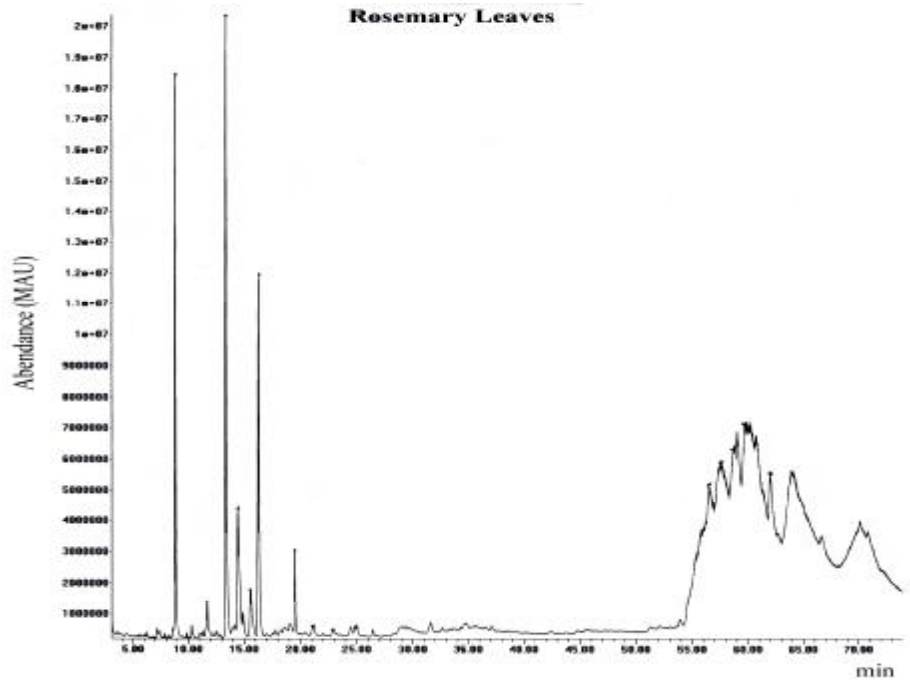


Figure 3-7: Chromatogram showing the chemical composition of rosemary leaf ethanol extract analyzed by GC

3-8: GC-MS analysis of rosemary callus ethanol extract

The GC-MS analysis of untreated callus from leaf explants of rosemary revealed the main constituents, their relative percentage of the total peak area and retention times as shown in Table 3-10 and figure 3-8.

The data indicate that 18 compounds were identified in callus ethanol extract and some non-identified compounds were fount at different percentages. These higher percent (16.78%) of quinoline was recorded followed by 9.12% of phytol . Callus extract also contained cineole (4.44%), camphor (2.51%), verbenone (2.30%), borneol (1.91%), Ferruginol (1.59%), isocarnosol (1.27%). Stearic acid (0.71%) and caryophyllene (0.64%) were found at the lowest levels.

Table 3-10: Retention time and peak area (%) of the different compounds found in rosemary callus extract analyzed by GC-MS

Compound	**RT (min)**	**Peak area%**
Cineole	9.36	4.44
Camphor	15.10	2.51
Borneol	16.58	1.91
Verbenone	19.04	2.30
5-oxymethylfurfurol	22.29	0.72
Bornyl acetate	23.66	1.66
Caryophyllene	31.59	0.64
Ferruginol	58.07	1.59
Isocarnosol	61.89	1.27
Stearic acid	62.35	0.71
Payarzolobis (bbn) thiolium	64.41	1.66
Phytol	64.82	9.12
Tetrahydroxy-tetramethyl-dihydrophenalenol-furan-3-one	65.33	14.45
Tetramethylbenzaldehyde 2,4-dinitrophenylhydrazone	65.68	17.78
Quinoline	67.11	16.78
Dihydronomorphinone	67.47	3.73
Sclareol	67.78	0.71
Lidoflazine	71.68	1.85

These data showed that callus extract produced high percent for some constituents as compared with the same constituents in leaf extract. Cineole percentage reached 4.44% in callus extract of rosemary, while it was recorded 2.66% in leaf extract. Bornyl acetate level increased more than two fold in callus as compared to leaf extract. Eugenol was found only in leaf extract at trace level. Other constituents such as, quinoline, phytol and sclareol were found only in callus extract while they were absent in leaf extract. On the other hand, Ferrugenol was reduced to 1.59% in callus extract, while it reached 4.53% in leaf extract. Data showed that callus extract of rosemary plant contains more constituents than that presented in leaf extracts and relatively at higher percentages.

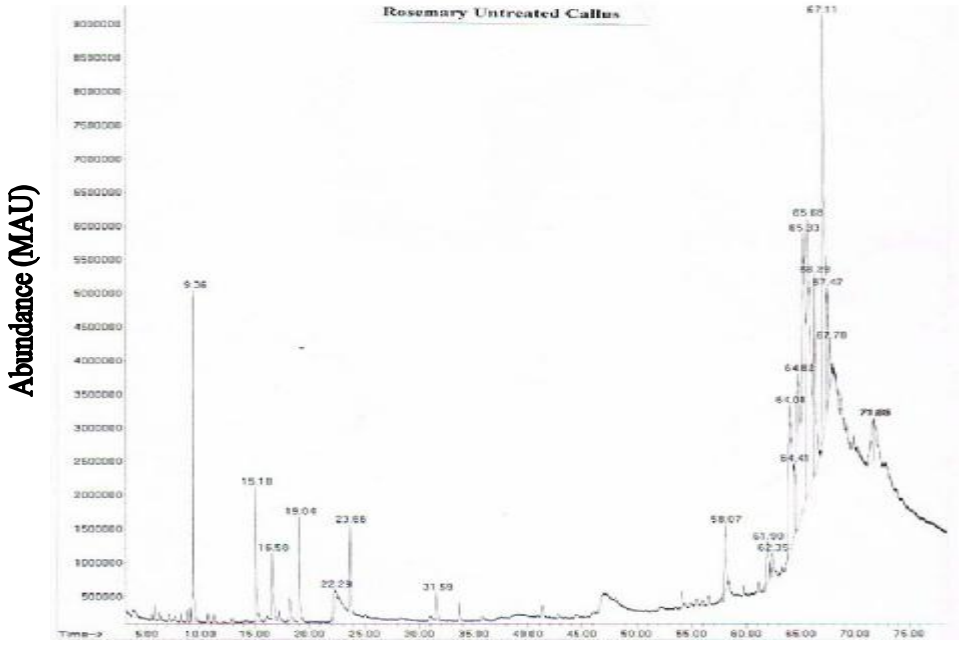


Figure 3-8: Chromatogram showing the chemical composition of rosemary callus ethanol extract analyzed by GC

Quinoline alkaloids which found in callus have been of a great therapeutic value, the antimalarial activity of these alkaloids have been documented, besides their pharmaceutical uses (Ramawat, 2008).Thymol is an active ingredient in pesticide products registered for the use as repellents, fungicides, medicinal disinfectants and virucides (Robert, 1993). Various stress factors impact on the qualitative and quantitative accumulation of valuable secondary products in nature (Lila, 2006). This stress is known to cause many physiological, biochemical and molecular changes in plant metabolism and possibly alter the secondary metabolite production and composition in plants (Chapin *et al*., 1987; Mooney *et al*., 1991).

3-9: Elicitation

In this part of study several biotic and abiotic elicitors were performed to improve the productivity of useful metabolites in rosemary callus cultures.

3-9-1: Effect of gamma-rays

The chemical analysis of callus tissues treated with 3 Gray of gamma-rays revealed the most represented compounds as shown in table 3-11 and Figure 3-9.

A number of 15 compounds were identified at different percentages, the most abundant compounds were, cineole (4.37%), camphor (3.18%), borneol (4.39%), bornyl acetate (3.71%), **verbenone** (1.65%), ferruginol (2.39%), isocarnosol (1.13%), phytol (3.28%). The data showed the presence of other compounds such as, isoquioline carbonitrile (3.42%), sclareol (7.08%) and several non-identified compounds, some of them were found at trace levels.

Table 3-11: Retention time and peak area (%) of the different compounds found in rosemary callus cultures treated with 3 Gray of gamma-rays, analyzed by GC-MS

Compound	RT (min)	Peak area%
Cineole	9.35	4.37
Camphor	15.09	3.18
Borneol	16.56	4.39
Verbenone	19.01	1.65
Bornyl acetate	23.64	3.71
Benzene (1-ethylnonyl)	55.01	1.32
Isoquinoline carbonitrile	65.15	3.42
Ferruginol	58.07	2.39
Isocarnosol	61.92	1.13
Phytol	64.04	3.28
1,2,3- Tris (trimethylsiloxy) benzene	64.41	0.77
Tetramethyl-dinitrophenalenol-furan-3-one	64.78	4.49
Trimethyl chalcone	65.33	16.84
Dihydronormorphinone	67.10	15.39
Sclareol	67.50	7.08

The chemical analysis of callus treated with 5 Gray of gamma-rays revealed the main constituents, their retention time and their relative percentage of the total peak area as shown in table 3-12 and Figure 3-10. A number of 12 compounds were identified, phytol (15.45%), cineole (4.30%), camphor (2.04%), bornyl acetate (4.52%) and linderol (3.22%). Other compounds were presented at different percentages and some non-identified compounds were found at trace levels. These data showed the presence of linderol for the first time, while it was absent at the lowest dose (3 Gray) of gamma-rays.

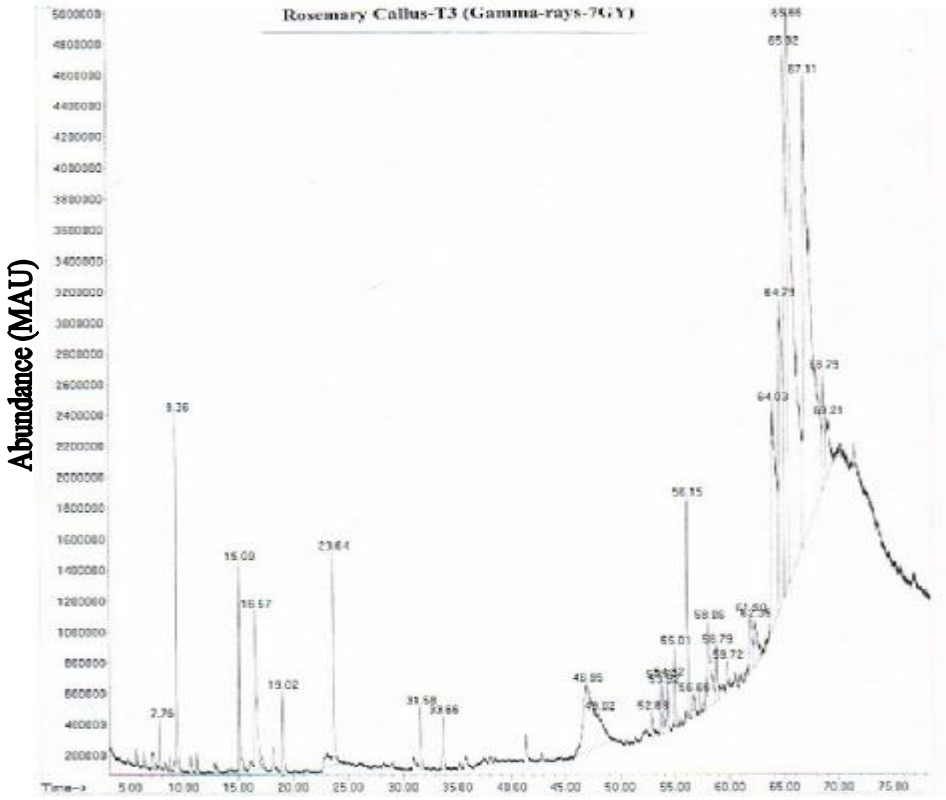


Figure 3-9: Chromatogram showing the chemical composition of rosemary callus cultures treated with 3 Gray of gamma-rays, analyzed by GC

Phytol was recorded the highest percentage at the dose 5 Gray as compared with the lowest dose (3 Gray). Decrease in camphor level was observed at 5 Gray of irradiation. Isocarnosol and ferruginol percentages relatively increased when the dose increased up to 5 Gray, which reached 1.26% and 2.49%, while it recorded 1.13% and 2.39% respectively at the lowest dose (3 Gray) of gamma-rays.

Table 3-12: Retention time and peak area (%) of the different compounds found in rosemary callus cultures treated with 5 Gray of gamma-rays, analyzed by GC-MS

Compound	RT (min)	Peak area %
Cineole	9.35	4.30
Camphor	15.08	2.04
Linderol	16.56	3.22
Bornyl acetate	23.63	4.52
Ferruginol	58.07	2.49
Isocarnosol	61.87	1.26
Phytol	63.98	15.45
Tetrahydroxy-tetramethyl-dinitrophenalenol-furan-3-one		
Trimethoxy chalcone	65.28	23.18
2-methylimidazolo-1,2-pyridin	67.16	6.00
Dihydronormorphinone	67.48	2.25
Hexahydro-3-acridinyl- benzoic acid	67.79	1.54

The chemical analysis of callus tissues treated with 7 Gray of gamma-rays showed the presence of 15 compounds (table 3-13 and Figure 3-11). The most abundant compounds were, quinoline (11.45%), phytol (7.79%), cineole (3.01%), bornyl acetate (3.19%), camphor (1.86%), ferruginol (1.37%), sclareol (2.58%) and linderol (2.45%). Other compounds were found at lower levels such as, verbenone (0.88%), caryophyllene (0.83%) and isocarnosol (0.60%). Quinoline alkaloids only recorded the highest percentage when the dose increased to 7 Gray, while it was absent with the lower doses of irradiation.

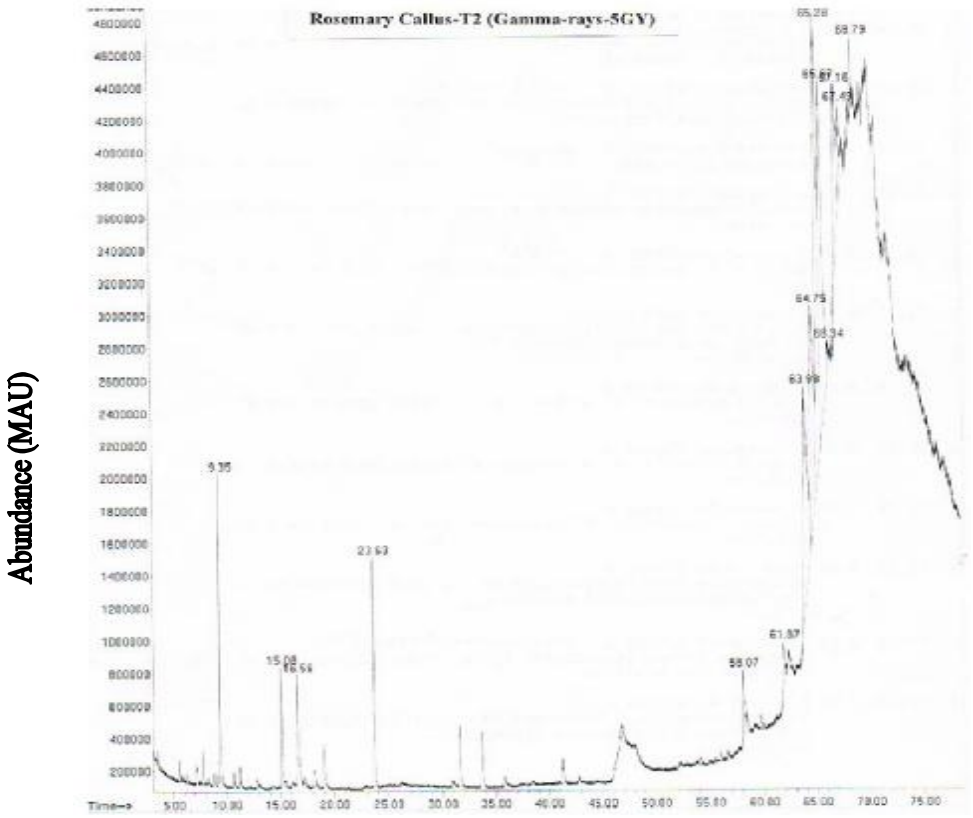


Figure 3-10: Chromatogram showing the chemical composition of rosemary callus cultures treated with 5 Gray of gamma-rays, analyzed by GC

Results indicated that callus treated with 3 and 5 Gy of gamma-rays increased essential oil yield and its components as compared to the highest dose (7 Gray) of gamma-rays and untreated callus. It was clearly noticed that the lower doses of gamma-rays stimulated essential oil production from rosemary callus, more than the highest dose. These results are in agreement with Monica *et al*., (2006), who found that radiation at higher doses of gamma-rays 10, 20 and 30 Gray reduced the total phenolic content in rosemary plant, the alterations in the active principles following increasing doses of radiation were analyzed employing various methods of extraction and chromatography.

Table 3-13: Retention time and peak area (%) of the different compounds found in rosemary callus tissues treated with 7 Gy of gamma-rays, analyzed by GC-MS

Compound	RT (min)	Peak area %
Cineole	9.35	3.01
Camphor	15.09	1.86
Linderol	16.56	2.45
Verbenone	19.01	0.88
Bornyl acetate	23.64	3.19
Caryophyllene	31.57	0.83
Ethyl-α-d-glucopyrnoid	47.82	0.06
Ferruginol	58.07	1.37
Isocarnosol	61.87	0.60
Phytol	64.03	7.79
Pyrazolobis (bbn) thiolium	64.41	3.90
1,3,5-Tris (trimethylsiloxy)benzene	64.79	9.30
4-ethoxy-3,4,5-trimethoxy chalcone	65.31	14.42
Tetramethylbenzaldehyde, 2,4-dinitrophenylhydrazone	65.64	21.62
Quinoline	67.09	11.45
Dihydronormorphinone	67.38	8.27
Sclareol	67.89	2.58

Al-Rumaih and Al-Rumaih (2008) stated that changes in the activity of the key enzymes which confer tolerance to irradiation stress were found when high levels of gamma irradiation used.

It can be concluded that the reduction in essential oils from callus may be caused by the reduced amount of endogenous growth regulators, especially the cytokinins due to irradiation. Gamma-rays causes oxidative stress and affects biomolecules by causing conformational changes, oxidation, rupture of covalent bonds, and formation of free radicals (Singh, 1996).

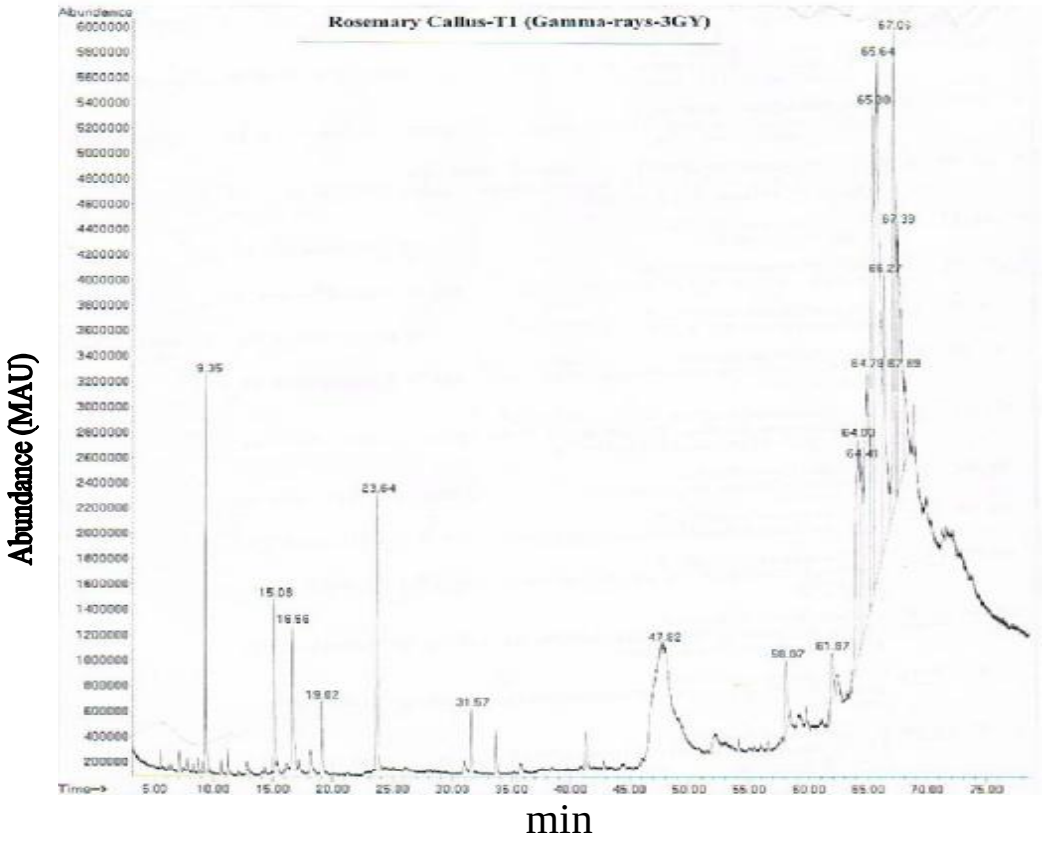


Figure 3-11: Chromatogram showing the chemical composition of rosemary callus cultures treated with 7 Gy of gamma-rays, analyzed by GC

3-9-2: Effect of calcium chloride ($CaCl_2$)

Table 3-14 and figure 3-12 showed that 16 compounds were identified, phytol was the most represented compound in rosemary treated callus with 0.4 g/l of $CaCl_2$ which recorded 8.34%. Other compounds were, cineole (3.48%), camphor (2.06%), borneol (2.27%), bornyl acetate (2.47%), verbenone (1.28%), Ferruginol (1.85%) and isocarnosol (1.53%). Several other components were found at lower percentages produced from callus cultures treated with 0.4 g/l of $CaCl_2$.Also higher percentages were recorded for some non-identified compounds.

Table 3-14: Retention time and peak area (%) of different compounds found in rosemary callus treated with 0.4 g/l of $CaCl_2$ analyzed by GC-MS

Compound	RT (min)	Peak area %
Cineole	9.35	3.48
Camphor	15.10	2.06
Borneol	16.57	2.27
Verbenone	19.02	1.28
Bornyl acetate	23.65	2.47
Caryophyllene	31.59	0.93
α-caryophyllene	33.66	0.78
Ethyl-alpha-d-glucopyranoside	46.95	1.55
Ferruginol	58.07	1.85
Isocarnosol	61.89	1.53
Stearic acid	62.35	0.58
Phytol	64.80	8.34
Trans-tetramethylbenzaldehyde-2,4-dinitrophenylhydrazone	65.33	12.77
Tetramethylbenaldehyde-2,4-dinitrophenylhydrazone	65.65	18.06
Trans-4-methoxy-trimethoxy chalcone	66.29	6.36
Dibenz-cycloheptanone-trimethyi-chalcone	67.10	24.37

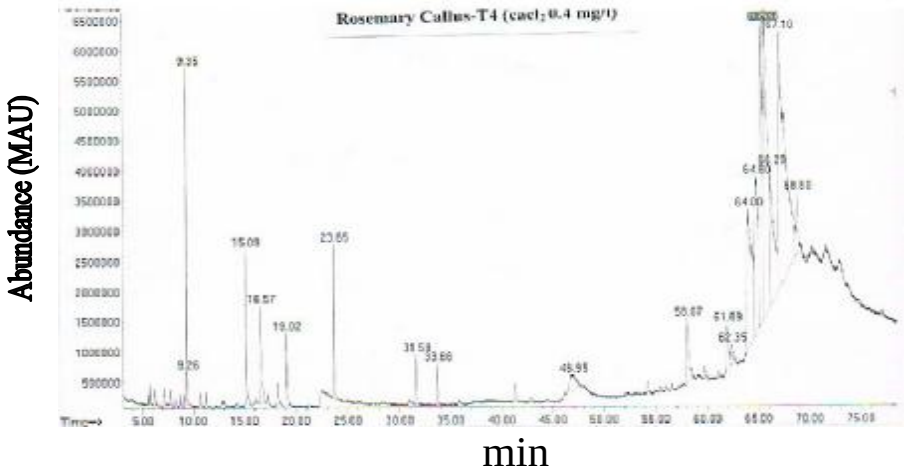


Figure 3-12: Chromatogram showed the chemical composition of rosemary callus cultures treated with 0.4 g/l of $CaCl_2$ analyzed by GC

Table 3-15 and figure 3-13 showed that 20 compounds were identified when $CaCl_2$ was added at 0.6 g/l to the callus culture medium.

Table 3-15: Retention time and peak area (%) of different compounds found in rosemary callus cultures treated with 0.6 g/l of $CaCl_2$ analyzed by GC-MS

Compound	**RT (min)**	**Peak area %**
Cineole	9.35	2.70
Camphor	15.09	1.72
Borneol	16.56	2.27
3-cyclohexene-1-methanol-alpha-trimethyl	18.15	0.38
Verbenone	19.01	1.19
Bornyl acetate	23.65	2.56
Caryophyllene	31.58	0.94
α-caryophyllene	33.66	0.91
Caryophyllene oxide	41.28	0.46
Ethyl-alpha-d-glucopyranoside	46.97	1.38
Ferruginol	58.07	1.90
Isocarnosol	61.89	1.22
Stearic acid	62.35	0.52
Spirostan-3-one-lactone	64.00	8.44
1,3,5-Tris (trimethylsiloxy) benzene	65.34	18.27
Tetramethylbenzaldehyde-2-imidazol-2-yl	65.67	19.94
Phytol	64.82	8.45
Benopyran-4-one-3,4-dimethoxyphenyl	67.11	10.09
Thiazol-4-chlorophenylamine-2-methylimidazol-(1,2-pyridin)	67.51	14.12
Lidoflazine	70.36	0.93

The most represented compounds were, cineole (2.70%), camphor

(1.72%), borneol (2.27%), bornyl acetate (2.56%), verbenone (1.19%) and isocarnosol (1.22%). These data revealed that cineole and camphor percentages were reduced, while phytol, borneol and isocarnosol were not affected when $CaCl_2$ concentration increased.

Addition of 0.4 g/l $CaCl_2$ to the callus cultures medium enhance accumulation of cineole, camphor approximately 1.3 fold. $CaCl_2$ affected the oil yield and some components produced from callus tissue. The highest yield was produced from fresh callus induced on a medium supplement with the lowest concentration (0.4 g/l) of $CaCl_2$. These data are similar to those obtained by Azza *et al*. (2006), who found that the lowest concentration of calcium chloride produced the higher yield from rosemary callus and increased the monoterpenes. These results demonstrated that an external source of calcium was required for elicitor-induced secondary metabolites accumulation and suggest that calcium may be involved in the signal transduction system that mediated the process (Ramawat, 2008).

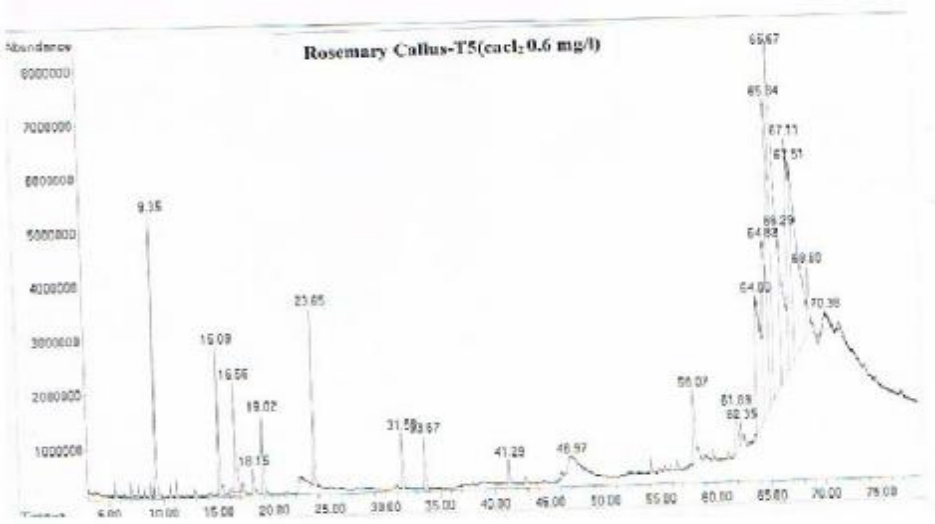


Figure 3-13: Chromatogram showed the chemical composition of rosemary callus treated with 0.6 g/l of $CaCl_2$ analyzed by GC

3-9-3: Effect of sucrose 82

The GC-MS analysis of callus cultures treated with 40 g/l of sucrose showed the presence of 18 compounds as shown in table 3-16 and figure 3-14, cineole (0.94%), camphor (1.55%), borneol (2.74%), verbenone (1.76%), palmitic acid (1.78%), bornyl acetate (2.69%), bromolauric acid (1.47%) and caryophyllene oxide (0.89%).

Table 3-16: Retention time and peak area (%) of different compounds found in rosemary callus cultures treated with 40 g/l of sucrose, analyzed by GC-MS

Compound	RT (min)	Peak area %
Cineole	9.36	0.94
Camphor	15.10	1.55
Borneol	16.58	2.74
Verbenone	19.04	1.76
Bornyl acetate	23.66	2.69
Caryophyllene oxide	41.31	0.89
Bromolauric acid	46.81	1.47
Palmitic acid	58.09	1.78
3-methyl-1-phenyl-2-azafluorenone	64.06	7.69
Benzopyran-4-one-dihydro(3,4,5-trimethoxyphenyl	64.42	1.43
Tetrahydroxy-tetramethyl-dihydrophenalenol-furan-3-one	64.81	10.94
1,2,3-tris(trimethylsiloxy)benzene	65.33	14.34
Phenol-methylene-bis-methylethyl (Ethyl antioxidant)	67.12	10.52
Dichromium tetra acetate	67.51	7.84
Benzopyran-4-one (3,4-dimethoxyphenyl)-3,4-dimethoxyphenyl	68.24	0.67
1,3,5-Tris(trimethylsiloxy)benzene	68.81	0.86
Diphenyl-1,2-carbazoledicarboximide	70.35	2.98

Borneol and bornyl acetate percentages were increased with the increase in sucrose concentration up to 40 g/l, while cineole and camphor were decreased. Verbenone also decreased with the increasing in sucrose concentration. New compounds were obtained for the first time when 40 g/l of sucrose was added to the callus cultures such as, palmitic acid, bromolauric acid and caryophyllene oxide. Also some non-identified compounds were found at higher levels.

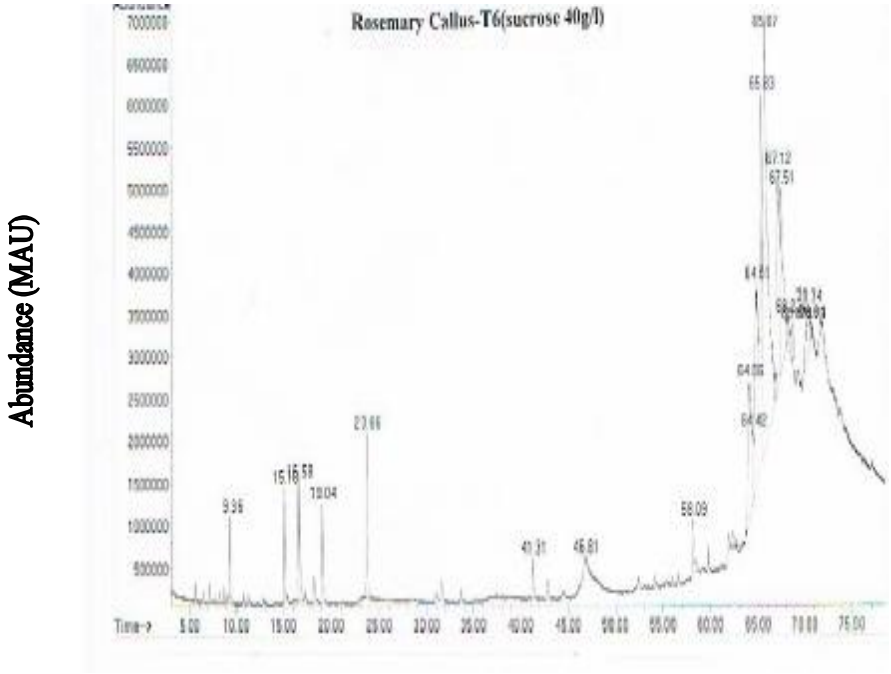


Figure 3-14: Chromatogram showed the chemical composition of rosemary callus cultures treated with 40 g/l sucrose analyzed by GC

Table 3-17 and figure 3-15 shows the presence of 15 compounds when sucrose concentration was increased to 50 g/l into culture medium, the most abundant components were, cineole (2.20%), camphor (1.90%), borneol (2.01%), verbenone (1.03%) and bornyl acetate (1.68%).

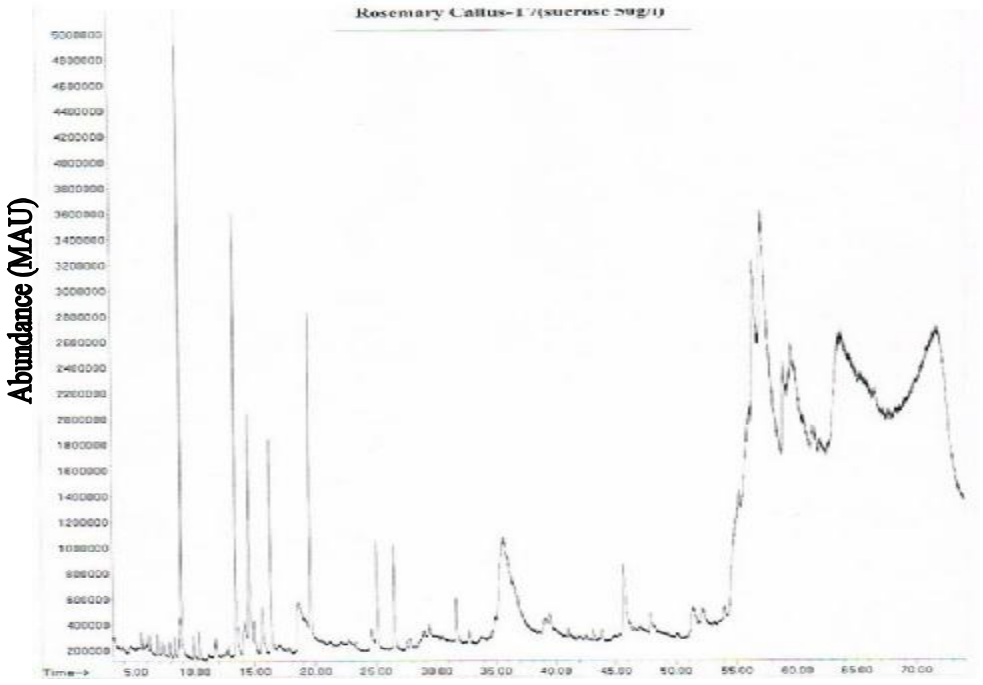
Other compounds were found at lower levels such as, Terpineol (0.13%), α-terpineol (0.34%), caryophyllene (0.60%), α-caryophyllene (0.57%) and caryophyllene oxide (0.27%). Also non-identified compounds were found at different levels.

Table 3-17: Retention time and peak area (%) of different compounds found in rosemary callus treated with 50 g/l sucrose analyzed by GC-MS

Compound	**RT (min)**	**Peak area %**
Cineole	8.68	2.20
Terpineol	10.39	0.13
Camphor	13.39	1.90
Borneol	14.50	2.01
A-terpineol	15.64	0.34
Verbenone	16.29	1.03
2-Furancarboxaldehyde-5-(hydroxymethyl)	18.60	1.40
Bornyl acetate	19.54	1.68
Caryophyllene	25.08	0.60
A-caryophyllene	26.50	0.57
Caryophyllene oxide	31.69	0.27
Palmitic acid	45.66	1.09
3-Hydroxy-3-trifluoromethyl-2-oxaspiro-undecan-5-one	51.40	0.51
Podocarpa-12-hydroxy-isopropyl-ethyl	56.60	12.35
Tetramethylbenzaldehyde-2,4-dinitrophenylhydrazone	57.31	16.71

Addition of 4% or 5% sucrose stimulated secondary products synthesis in callus cultures of *Coleus blumei* and decreased callus growth (Natas *et al*., (2004). It was found that the increase of sucrose concentration up to 50 g/l caused a reduction in borneol, bornyl acetate and verbenone levels as compared to the control treatment,

while cineole and camphor percentages were relatively increased with increasing in sucrose concentrations.



min

Figure 3-15: Chromatogram showed the chemical composition of rosemary callus cultures treated with 50 g/l of sucrose analyzed by GC

Komamine *et al*. (1991) reported that sucrose and glucose are preferred carbon sources for plant tissue cultures, the concentration of the carbon source affect cell growth and yield of secondary metabolites in many cases. Media components play a vital role in stimulating the secondary metabolite production, the general consensus is that sucrose is better than all other carbohydrate sources, and concentrations above 3% enhance the biosynthesis of phytochemicals.

3-9-4: Effect of yeast extract 86

The effect of yeast extract on secondary products from rosemary callus cultures was determined by comparing the product percentage after 4 weeks of exposure, to that of non-elicited controls. Table 3-18 and figure 3-16 shows the presented of 10 compounds produced from callus treated with 1 g/l of yeast extract.

Table 3-18: Retention time and peak area (%) of different compounds found in rosemary callus cultures treated with 1 g/l of yeast extract analyzed by GC-MS

Compound	RT (min0	Peak area %
Cineole	8.83	1.49
Camphor	13.36	0.98
Borneol	14.46	0.82
Terpineol	15.60	0.32
Verbenone	16.26	0.89
Bornyl acetate	19.51	0.59
Caryophyllene	25.04	0.32
A-caryophyllene	26.46	0.28
Palmitic acid	45.67	0.61
Stearic acid	52.21	0.34

The most abundant compound was cineole (1.49%), while other compounds were found in small amounts, camphor (0.98%), borneol (0.82%), terpineol (0.32%), verbenone (0.89%) and bornyl acetate (0.59%), palmitic acid (061%) and stearic acid (0.34%). The data showed that most of the components fail to show a clear response to yeast extract after exposure to 1 g/l concentration.

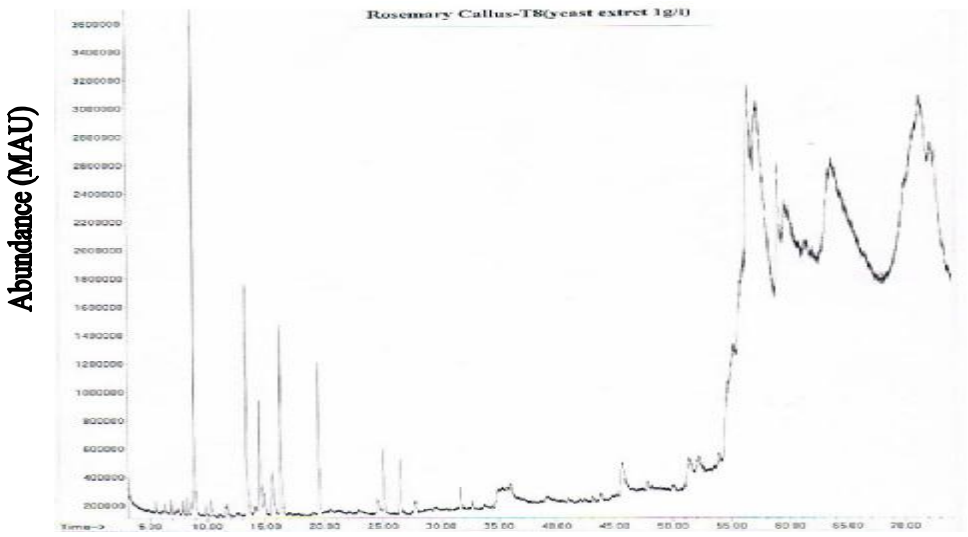


Figure 3-16: Chromatogram showed the chemical composition of rosemary callus cultures treated with 1 g/l of yeast extract analyzed by GC

Table 3-19 and figure 3-17 shows that 19 compounds were identified in callus extract after exposure to 2 g/l of yeast extract, the most represented compounds were, cineole (1.89%), camphor (1.95%), borneol (1.95%), verbenone (1.24%) and bornyl acetate (1.57%). α-phellandrene recorded the lowest percent (0.09%). At 2 g/l of yeast extract addition to the callus cultures, total contents were relatively increased compared to 1 g/l treatment. Borneol, bornyl acetate and camphor levels were increased when rosemary callus treated with 2 g/l of yeast extract. New compounds appeared after increasing yeast extract concentration such as, α-phellandrene, β-phellandrene, carene and terpinene, but in small amounts, while they were absent with the lower concentration. These results may be attributed to shikimic acid; a precursor of phenylpropanoid pathway may be accumulated following YE-elicitation.

Several end-products of this pathway such as, phenolic compounds may also be accumulated with maximal elicitation.

Table 3-19: Retention time and peak area (%) of different compounds found in rosemary callus cultures treated with 2 g/l of yeast extract analyzed by GC-MS

Compound	**RT (min)**	**Peak area %**
β-phellandrene	6.87	0.09
α-phellandrene	7.88	0.10
Carene	8.31	0.11
Cineole	8.83	1.89
Terpinene	9.89	0.12
Terpineol	10.34	0.11
Camphor	13.35	1.95
Borneol	14.45	1.95
α-terpineol	15.58	0.50
Verbenone	16.24	1.24
2-Furancarboxaldehyde-5-(hydroxymethyl)	18.43	1.2
Bornyl acetate	19.50	1.57
Caryophyllene	25.03	0.72
α-caryophyllene	26.45	0.67
Caryophyllene oxide	31.63	0.30
Palmitic acid	45.62	1.08
Stearic acid	52.13	0.32
Podocarp-12-hydroxy-isopropyl-ethylester	56.52	13.70
Cholestan-3-ol	64.01	18.40

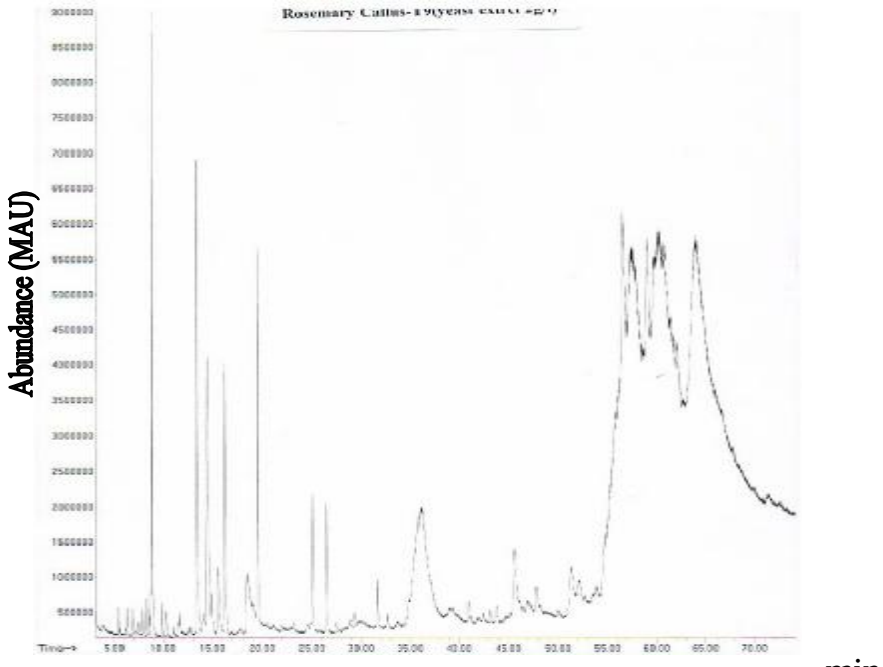


Figure 3-17: Chromatogram showed the chemical composition of rosemary callus cultures treated with 2 g/l of yeast extract analyzed by GC

These results are similar to those obtained by Coery *et al.* (2005) who found an increase in secondary metabolites levels following exposure to YE at moderate and high concentrations.

3-9-5: Effect of *Fusarium oxysporum*

Table 3-20 and figure 3-18 showed that 25 compounds were identified in rosemary callus cultures treated with $1x10^4$ CFU/ml of *Fusarium oxysporum* as elicitor.

The most abundant compounds being, cineole (4.47%), camphor (2.42%), borneol (1.88%), verbenone (1.875) and bornyl acetate (1.76%). It was observed that several compounds were found in small amounts, these compounds were, α-pinene (0.30%), β-pinene (0.24%), camphene (0.17%), caryophyllene (0.84%), -caryophyllene (0.71%) and palmitic acid (0.34%).

Table 3-20: Retention time and peak area (%) of different compounds present in rosemary callus cultures treated with $1x10^4$ CFU/ml of *Fusarium oxysporum* as elicitor analyzed by GC-MS

Compound	**RT (min)**	**Peak area %**
α-pinene	5.72	0.30
Camphene	6.19	0.17
β-pinene	7.14	0.24
Cineole	9.37	4.47
Camphor	15.12	2.42
Borneol	16.59	1.88
P-menth-1-en-8-ol	18.18	0.61
Verbenone	19.04	1.87
Bornyl acetate	23.67	1.76
Caryophyllene	31.61	0.84
α-caryophyllene	33.68	0.71
Palmitic acid	58.09	0.34
Hexadecatrienoic acid-methylester	61.93	0.21
Chromen-2-one-methoxy-naphthol-imidazol-2-yl	64.01	6.22
Pyrazolobis (bbn)thiolium	64.26	1.49
Pyrszolobis(bbn)thiolium	64.41	1.90
1,3,5-Tris (trimethylsiloxy)benzene	64.74	3.38
1,3,5-Tris (trimethylsiloxy)bezene	64.80	4.20
Nickel, pentamethylcyclopenta dienyl-o-phenyleediamine-N-o	65.35	13.36
Chromen-2-one-naphtho-imidazol-2-yl	65.64	19.01
1,3,5-Tris (trimethylsiloxy)benzene	66.30	6.75
Dibenz-cycloheptanone-trimethoxy	67.11	11.69
Cymarin	67.53	6.93
Norpregna-carboxylic acid-hydroxy-methylester	67.86	5.22
Pyrrolo-quinoline-4-carboxylic acid-ethylester	68.80	4.04

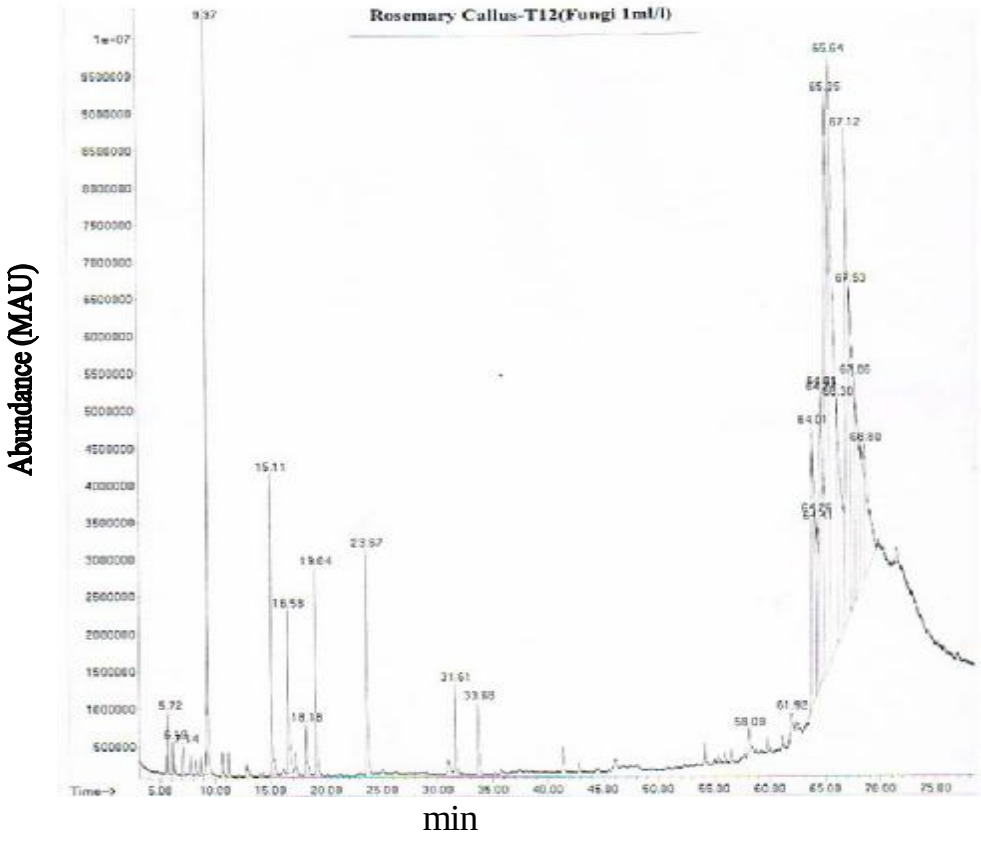


Figure 3-18: Chromatogram showed the chemical composition of rosemary callus cultures treated with $1x10^4$ CFU/ml of *Fusarium oxysporum* as elicitor analyzed by GC

New compounds were recorded with higher percentages such as, cymarin (6.93 %) and also non-identified compounds were found with different percentages. Contents of phenolic and terpenic compounds in rosemary samples are comparable to those reported in literature, but it has to be emphasized that results may differ because there are several factors that can influence on these compounds in rosemary plant, Time of extraction, temperature, pH and the type of solvent used, were put into consideration to avoid chemical modification, degradation and other biochemical changes of the components in the sample (Masa *et al.*, 2007).

These compounds may represent novel compounds not usually synthesized by rosemary callus and may be due to elicitation by fungus *F. oxysporum*. It is noteworthy to mention that these compounds may have great therapeutic values as indicated by Azza (1992). Table 3-21 and figure 3-19 showed that 19 constituents were identified in rosemary callus cultures treated with $2x10^4$ CFU/ml of *F. oxysporum*, and the most abundant compound were cineole (5.61%), camphor (4.12%) and borneol (3.68%).

Table 3-21: Retention time and peak area (%) of different compounds found in rosemary callus cultures treated with $2x\ 10^4$ CFU/ml of *Fusarium oxysporum* as elicitor analyzed by GC-MS

Compound	**RT (min)**	**Peak area %**
α-pinene	5.73	0.76
Camphene	6.19	0.56
β-pinene	7.15	0.51
Bornylene	9.27	0.49
Cineole	9.37	5.61
Camphor	15.12	4.12
Borneol	16.59	3.68
Terpineol	18.18	0.58
Verbenone	19.05	2.36
Bornyl acetate	23.67	2.80
Caryophyllene	31.61	1.33
α-caryophyllene	33.69	1.19
Stearic acid	46.84	1.71
Palmitic acid	58.10	0.56
Podocarpa-isopropyl-ethylester	64.00	3.91
Pyrazolobis (bbn)thiolium	64.43	1.14
Tetrahydroxy-dihydrophenalenol-furan-3-one	64.80	9.38
Cymarin	67.53	3.58
Octamethyl-octadecahydro-picen-3-one	71.90	3.36

The data showed the presence of other compounds such as, cymarin (3.58%), verbenone (2.36%), bornyl acetate (2.80%), caryophyllene (1.33%), α-caryophyllene (1.19%) and stearic acid (1.71%). Treatment with $2x10^4$ CFU/ml enhanced the production of α-pinene, β-pinene, camphene, bornylene, terpineol and palmitic acid, but at lower levels. These data showed that addition of *F. oxysporum* elicitor at the higher concentration induced the production of phenolic compounds and some fatty acids such as, palmitic acid and stearic acid which were absent or found at low levels as compared with control treatment. Addition of *F. oxysporum* at the concentration of $1x10^4$, $2x10^4$ CFU/ml to callus cultures induced production of these compounds.

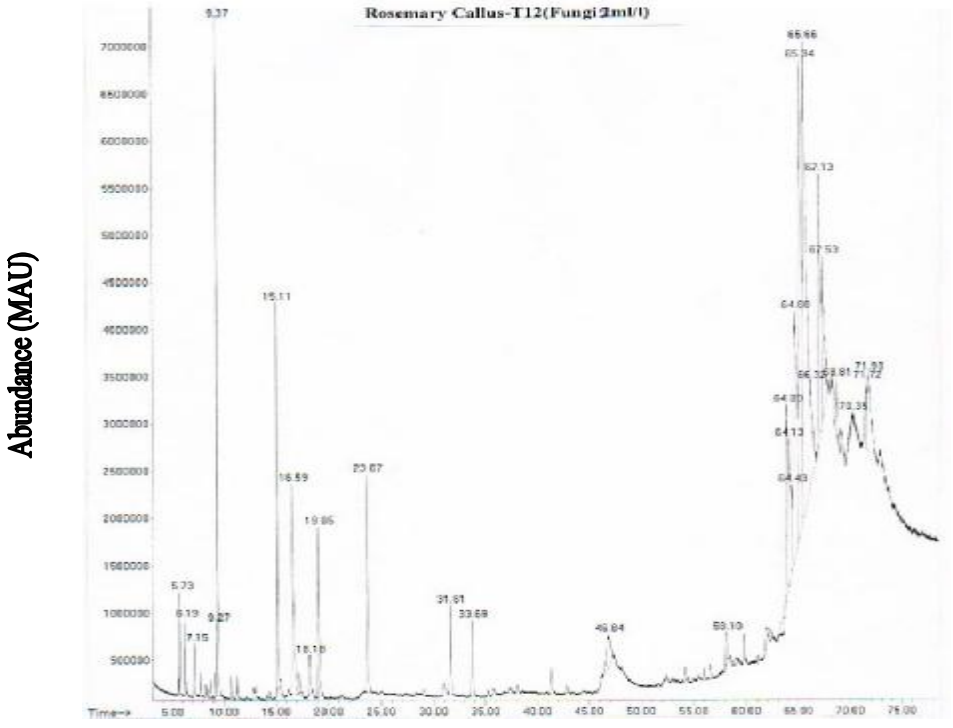


Figure 3-19: Chromatogram showed the chemical composition of rosemary callus cultures treated with $2x10^4$ CFU/ml of *Fusarium oxysporum* as elicitor analyzed by GC

It was concluded that the host defense mechanism is based upon induction of phenylalanine ammonialyase (PAL) activity resulting in overall increase in the phenolic profile. These results are in harmony with those obtained by Gerold and Ralf (1996), who found that autoclaved fungal cells induced most of the compounds production, and the compound elicited reached maximum concentration after addition of 2 ml/l of autoclaved fungal cells to the callus culture medium.

3-9-6: Effect of *Pseudomonas aeruginosa*

Table 3-22 and figure 3-20 showed the effect of treatment with $1.5x10^5$ CFU/ml of *Pseudomonas aeruginosa* on secondary products following 4 weeks of rosemary callus cultures treatment.

Table 3-22: Retention time and peak area (%) of different compounds found in rosemary callus cultures treated with $1.5x10^5$ CFU/ml of *Pseudomonas aeruginosa* as elicitor analyzed by GC-MS

Compound	RT (min)	Peak area %
Cineole	9.35	2.27
Camphor	15.12	2.49
Borneol	16.57	2.15
Terpineol	18.16	0.60
Verbenone	19.03	2.52
Bornyl acetate	23.65	1.48
Caryophyllene	31.60	0.56
Chromen-2-one-naphtho-imidazol-2-yl	63.96	4.17
Perimidine-2-acetoxyphenyl-1-methyl	64.02	5.85
Estra-trien-16-one-3-(trimethylsiloxy)	64.38	1.48
Tetrahydroxy-tetramethyl-dinitrophenalenol-furan-3-one	64.72	9.99
Pyrazolobis (bbn) thiolium	65.33	18.36
Zapotin	67.11	22.76
Sclareol	68.43	0.28

The most abundant compounds produced from rosemary callus after treatment with $1.5x10^5$ CFU/ml of *P. aeruginosa* as elicitor were, cineole (2.27%), camphor (2.49%),borneol (2.15%), verbenone (2.52%), bornyl acetate (1.48%) and zapotin (22.76%).

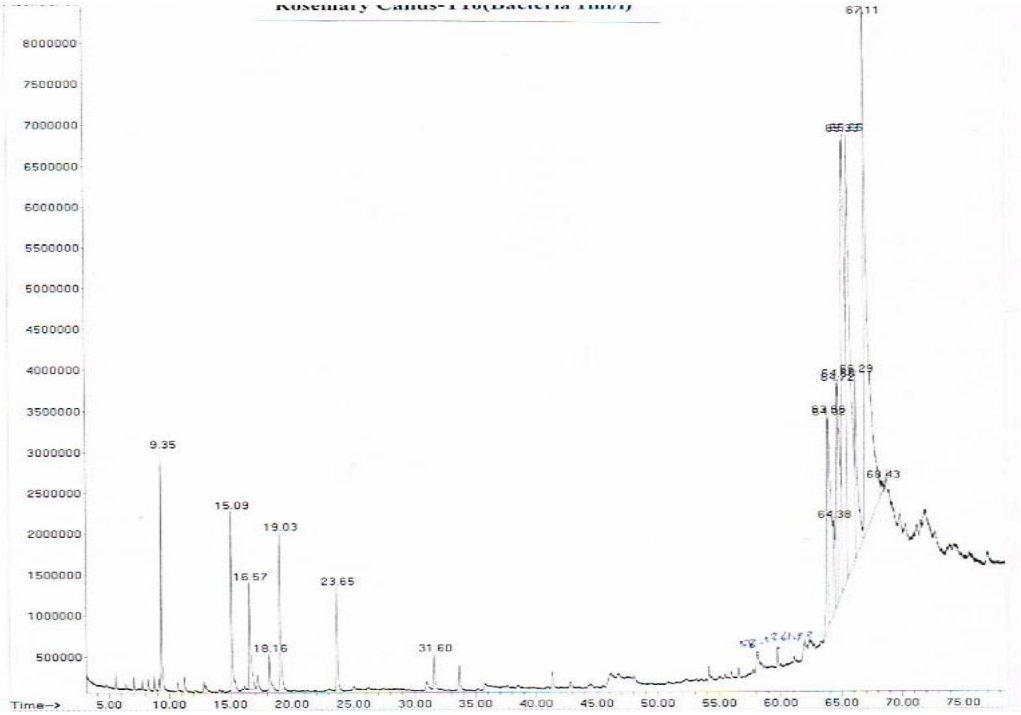


Figure 3-20: Chromatogram showing the chemical composition of rosemary callus cultures treated with $1.5x10^5$ CFU/ml of *Pseudomonas aeruginosa* as elicitor analyzed by GC

Other compounds were found in small amounts such as, Terpineol (0.60%), caryophyllene (0.56%) and sclareol (0.28%). The data showed the presence of non-identified compounds at higher percentages.

Table 3-23 and figure 3-21 showed that the most represented compounds obtained from the treatment with $3x10^5$ CFU/ml of *P. aeruginosa* were, cineole (2.08%), camphor (2.47%), borneol (1.96%), verbenone (2.15%) and bornyl acetate (1.31%), sclareol (5.97%) and zapotin (4.68%). Other compounds were obtained at lower levels, caryophyllene (0.72%), α-caryophyllene (0.63%), terpineol (0.57%), palmitic acid (0.40%) and valeric acid (0.96%).

Table 3-23: Retention time and peak area (%) of different compounds found in rosemary callus cultures treated with $3x10^5$ CFU/ml of *Pseudomonas aeruginosa* as elicitor analyzed by GC-MS

Compound	**RT (min)**	**Peak area %**
Cineole	9.37	2.08
Camphor	15.12	2.47
Borneol	16.60	1.96
Terpineol	18.18	0.57
Verbenone	19.05	2.15
Bornyl acetate	23.67	1.31
Caryophyllene	31.61	0.72
α-caryophyllene	33.69	0.63
Valeric acid	46.83	0.96
Palmitic acid	58.13	0.40
Hexadecatrienoic acid-methylester	61.92	0.12
Tetramethylbenzaldehyde-2-4-dinitrophenylhydrazone	64.78	29.65
Trans-4-Ethoxy-trimethoxy chalcone	65.35	14.99
Zapotin	66.31	4.68
Dibenz-cycloheptanone-trimethoxy	67.13	10.01
Sclareol	67.51	5.97
Octamethyl-octadecahydro-picen-3-one	71.94	3.96

The data showed higher percentages were recorded for non-identified compounds. It was noticed that the addition of the lower concentration ($1.5x10^5$ CFU/ml) of *P. aeruginosa* led to increase most of the components percentages, while the addition of the higher concentration ($3x10^5$ CFU/ml) did not affect the production percentages as compared to the control treatment.

Gwi-Taek *et al*., (2005) found similar results with *Panax ginseng* plant, bacterial elicitor showed a slight inhibition at biomass, but total saponin content increases about 1.23 times at 1 ml/l addition. Phenolic metabolites are known to be stimulated in response to microbial elicitors, such as *pseudomonas* spp. (Yang *et al*., 1997; Shetty *et al*., 1996).

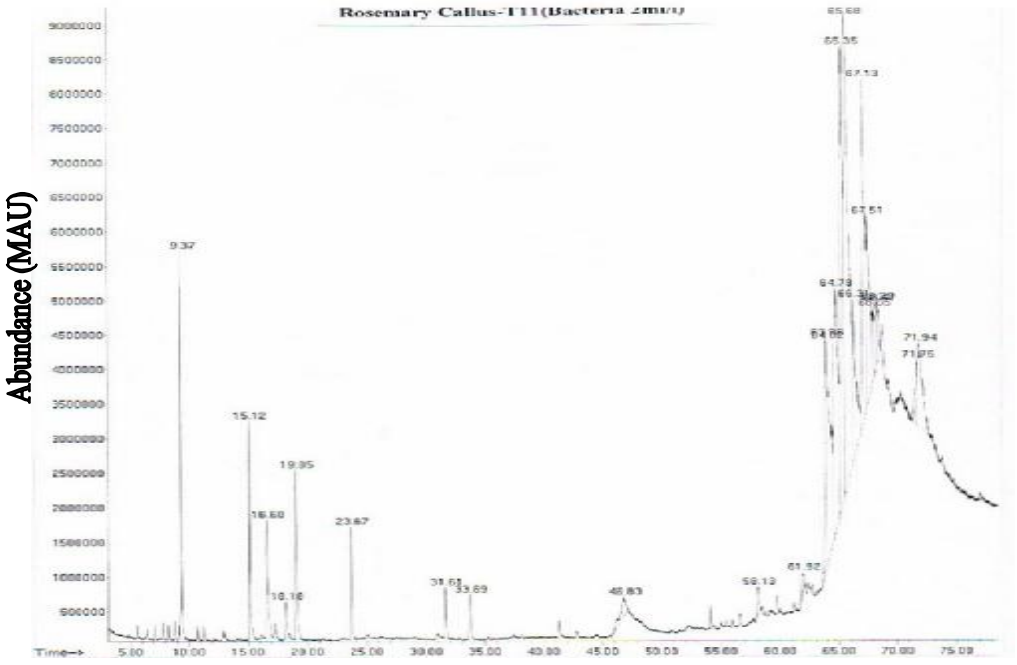


Figure 3- 21: Chromatogram showing the chemical composition of rosemary callus cultures treated with $3x10^5$ CFU/ml of *Pseudomonas aeruginosa* as elicitor analyzed by GC

3-10: HPLC analysis of rosemary extracts **98**

The average content of phenolic and terpenic compounds in rosemary leaves and callus cultures treated with different concentrations of biotic and abiotic elicitors were presented in table 3-24.

Table 3-24: Effect of some biotic and abiotic elicitors on phenolic and terpenic compounds quantity (ug/ml) of rosemary aqueous extracts analyzed by HPLC*

Compound (µg/ml)	Concentration (µg/ml)							
	Leaf	Untreated Callus	$CaCl_2$		*P. Aeruginosa*		*F. oxysporum*	
			0.4 g/l	0.6 g/l	1 ml/l	2 ml/l	1 ml/l	2 ml/l
RA	3.3	3.7	4.3	3.2	3.9	3.1	4.1	4.5
CAF	0.4	0.5	0.3	0.2	2.3	2.2	2.5	2.7
CA	2.8	2.6	2.3	1.9	3.1	2.3	2.4	3.3
CAR	1.7	1.9	0.6	0.5	2.4	1.7	2.1	2.8
ROL	4.3	4.2	4.6	3.2	0.8	0.5	0.6	0.9

*RA= Rosmarinic Acid, CAF=Caffeic Acid, CA=Carnosic Acid, CAR=Carnosol,
ROL= Rosmanol

Treatment with 2 ml/l ($2x10^4$ CFU/ml) of *F. oxysporum* as elicitor produced the highest concentration (4.5 µg/ml) of rosmarinic acid as compared to other treatments and control (untreated callus), while RA concentration in leaves was 3.3 µg/ml.

From these results, $CaCl_2$ at the lower concentration (0.4 g/l) increased RA level in rosemary callus compared with untreated callus. Also *P. aeruginosa* at $1.5x10^5$ CFU/ml was more effective than $3x10^5$ CFU/ml, and the amounts of RA compound were found to be more than those found in leaves. Callus accumulated more RA when treated with these elicitors. The most effective stimulator was

F. oxysporum at the concentration of $2x10^4$ CFU/ml. The retention times and peak area (%) for RA production following treatment with biotic and abiotic elicitors were shown in table 3-25 and figures 3-22, 3-23, 3-24 and 3-25 as compared with the standard rosmarinic acid (figure 3-26). It was found that fungal elicitor at the concentration of $2x10^4$ CFU/ml was more effective among other elicitors, which produced 15.5891% of RA as compared to the control treatment which recorded 13.0921%.

Table 3-25: Retention time and peak area (%) for rosmarinic acid obtained from rosemary callus cultures treated with some biotic and abiotic elicitor analyzed by HPLC

RT (min)	**RA Peak area%**	**Treatment**
15.112	13.0921	Untreated callus
15.092	14.8751	0.4 mg/l $CaCl_2$
15.112	13.3219	$1.5x10^5$ CFU/ml *P. aeruginosa*
15.092	15.5891	$2x10^4$ CFU/ml *F. Oxysporum*

It has to be noted that tannin-like phenolics are considered to be plant defense metabolites and their production in the plant increases under stress conditions. This fact is used to stimulate the production of RA in cell cultures with considerable results (Holzmannova, 1996; Petersen and Simmonds, 2003). It was found that RA represented the main phenolic acid detected in all investigated samples, the other phenolics were presented in lower concentrations, and this is in accordance with Bradsteterova and Ziakova-Caniova (2003) who stated that rosmarinic acid was the most abundant phenolic acid in all

tested samples. It was observed that the treatment with biotic elicitors (*P. aeruginosa*, *F. oxysporum*), and abiotic elicitors ($CaCl_2$), increased also the productivity of callus tissue from phenolic diterpenes. Caffeic acid (figure 3-27) was presented in all tested samples but at lower concentrations than RA compounds.

The highest CAF productivity was increased following treatment with *F. oxysporum as* elicitor, it reached 2.7 µg/ml when $2x10^4$ CFU/ml of *F. oxysporum* was added to the culture medium, while the analysis of leaf and callus extracts produced 0.4, 0.5 µg/ml of CAF respectively.

The highest concentration (3.3 µg/ml) of carnosic acid (figure 3-28) was obtained when callus treated with *F. oxysporum* at $2x10^4$ CFU/ml, while CA concentrations recorded 2.8, 2.6 µg/ml in leaves and callus extracts respectively.

The highest productivity of carnosol (figure 3-29) reached 2.8 µg/ml when callus was treated with $2x10^4$ CFU/ml of *F. oxysporum* as compared to leaf and callus extracts which yielded 1.7, 1.9 µg/ml respectively.

The highest productivity of rosmanol (figure 3-30) recorded 4.3, 4.2 ug/ml from leaf and untreated callus respectively, while the treatment with $2x10^4$ CFU/ml of *F. oxysporum* produced 0.9 ug/ml of ROL which was lower than that found in leaf and untreated callus.

Although numerous phenolics, flavonoids and diterpenes have been reported in rosemary extract, only RA, CAF, CA, CAR and ROL were presented in sufficient amount to be identified and quantified in this study (figure 3-31).

Biotic and abiotic factors led to an increase in the production of secondary metabolites in rosemary callus cultures. *F. oxysporum* enhanced accumulation of RA and the phenolic diterpenes in callus tissue. Results from this research compared the results showed that carnosic acid in leaves which are photosynthetic tissue, with those non-photosynthetic tissues, such as callus, the biosynthesis of secondary metabolites is often affected by concentration of growth regulators (Su and Humphrey, 1995; Swagata and Jha, 2001).

In the present study unorganized callus of rosemary can synthesize carnosol, but in low concentrations. This finding raised the following question, whether this compound (CAR) is a true compound of callus cultures or a product of degradation of carnosic acid. This study provided the evidence that this compound is present in undifferentiated cultures. So far, neither CAR nor CA has been found in undifferentiated rosemary callus cultures by Caruso *et al*., (2000).

These compounds as determined were similar in content and concentration to the data reported in previous studies showed that rosmarinic acid and carnosic acid were the most abundant compounds in rosemary extracts (Cuvelier *et al*., 1996; Zeng *et al.*, 2002).

Phenolic compounds are synthesized in plant cells via the phenylpropanoid pathway localized in the cytosol, with the final stages of biosynthesis and accumulation in the vacuole (Luis and Johnson, 2005). They are present in leaves, petals and callus which is consistent with the results obtained for RA in this study. On the other hand, diterpenes such as CA are synthesized in plants via the non-mevalonate isopentenyl diphosphate pathway (McGarvey and Croteau, 1995).

It is reported by Masa *et al*., (2007) that there is strong seasonal variation in concentrations of phenolic acids and phenolic diterpenes in rosemary. Solar radiation during summer resulted in water and light stress, decreases concentrations of some phenolics, while they are increased during winter. Carnosic acid may give rise to carnosol after enzymetic dehydrogenation or to highly oxidized diterpenes such as rosmanol, isorosmanol.

Oxidative stress *in vivo* induced by drought or high light stress enhances the formation of highly oxidized diterpenes due to the antioxidant activity of CA (Sergi *et al*., 2001).

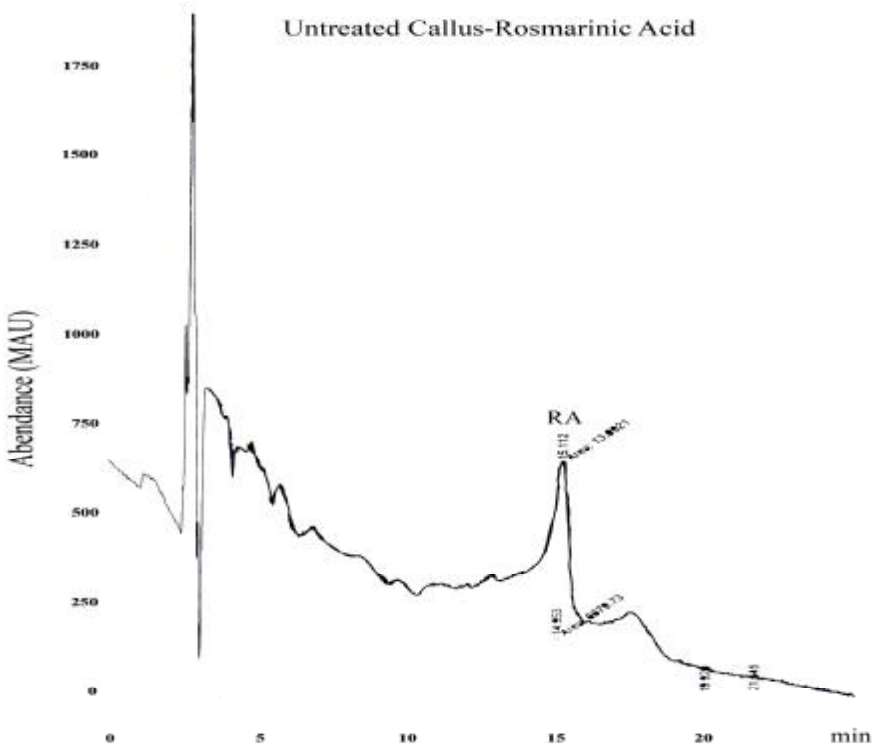


Figure 3-22: Chromatogram showed the retention time and abundance (MAU) of rosmarinic acid produced from rosemary untreated callus analyzed by HPLC

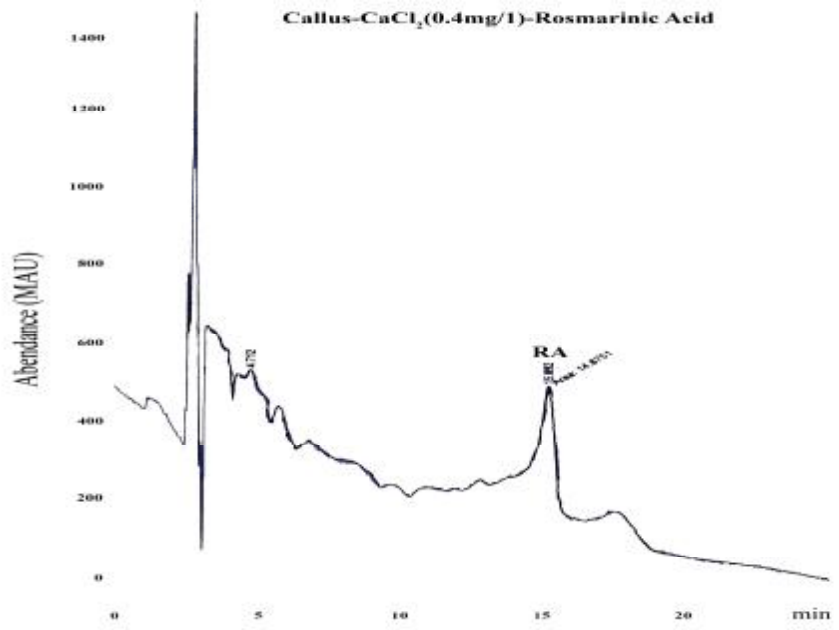


Figure 3-23: Chromatogram showed the retention time and abundance (MAU) of rosmarinic acid produced from rosemary callus treated with 0.4 g/l of $CaCl_2$ analyzed by HPLC

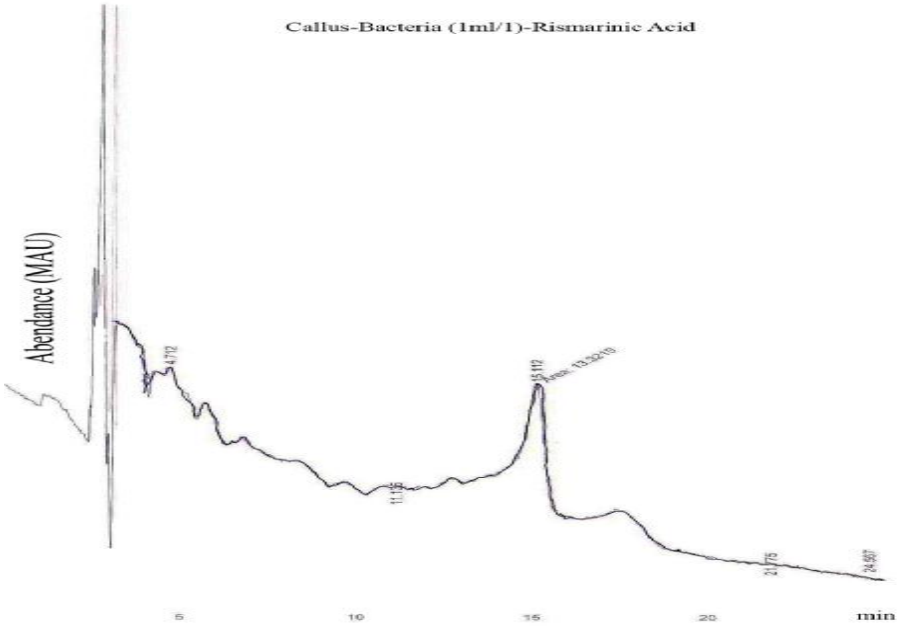


Figure 3-24: Chromatogram showed the retention time and abundance (MAU) of rosmarinic acid produced from callus cultures treated with $1.5x10^5$ CFU/ml of *P. aeruginosa* analyzed by HPLC

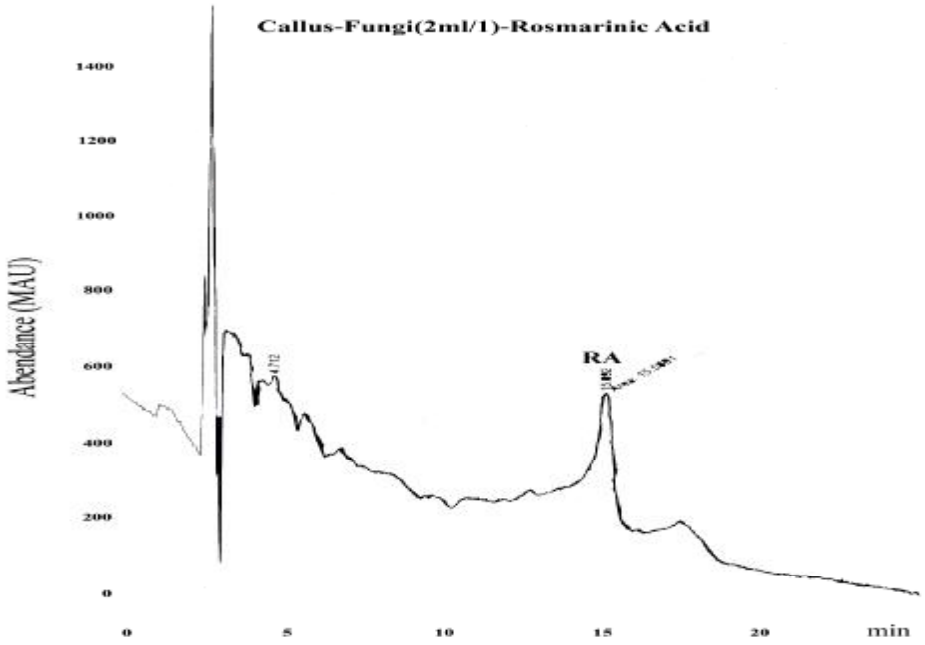


Figure 3-25: Chromatogram showed the retention time and abundance (MAU) of rosmarinic acid produced from callus cultures treated with $2x10^4$ CFU/m of *F. oxysporum* analyzed by HPLC

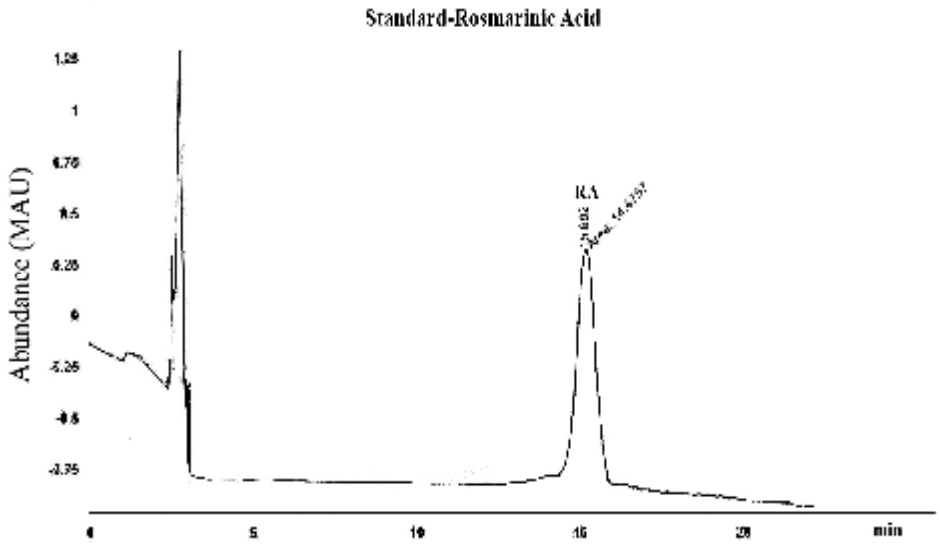


Figure 3-26: Chromatogram showed the retention time and abundance (MAU) of standard rosmarinic acid (RA) analyzed by HPLC

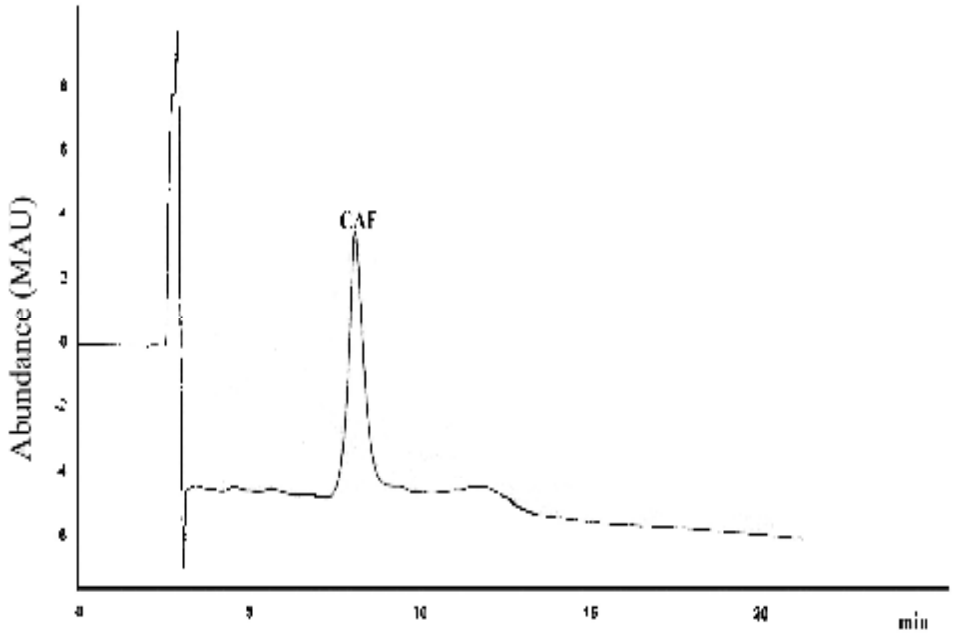


Figure 3-27: Chromatogram showed the retention time and abundance (MAU) of standard caffeic acid (CAF) analyzed by HPLC

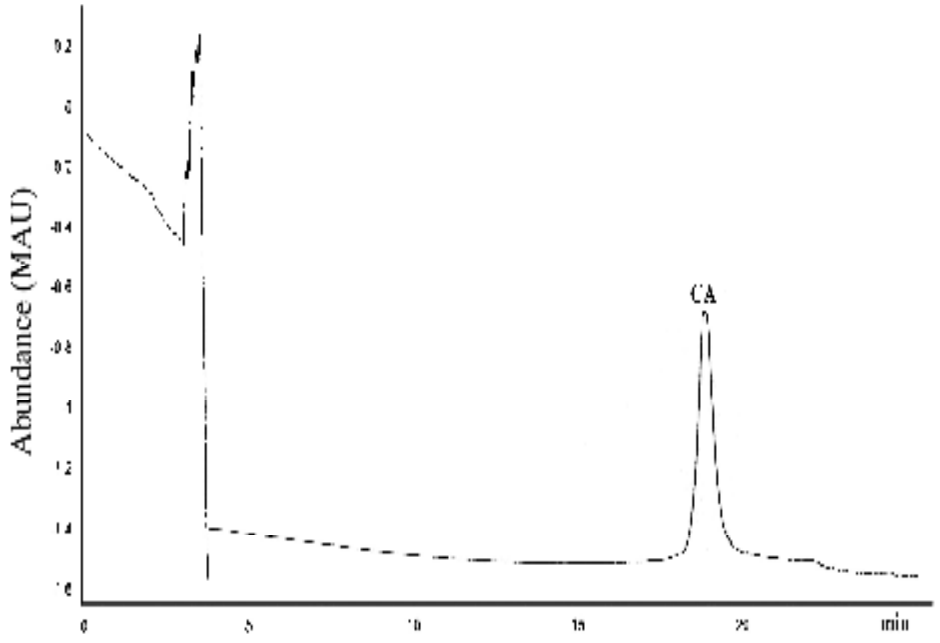


Figure 3-28: Chromatogram showed the retention time and abundance (MAU) of standard carnosic acid (CA) analyzed by HPLC

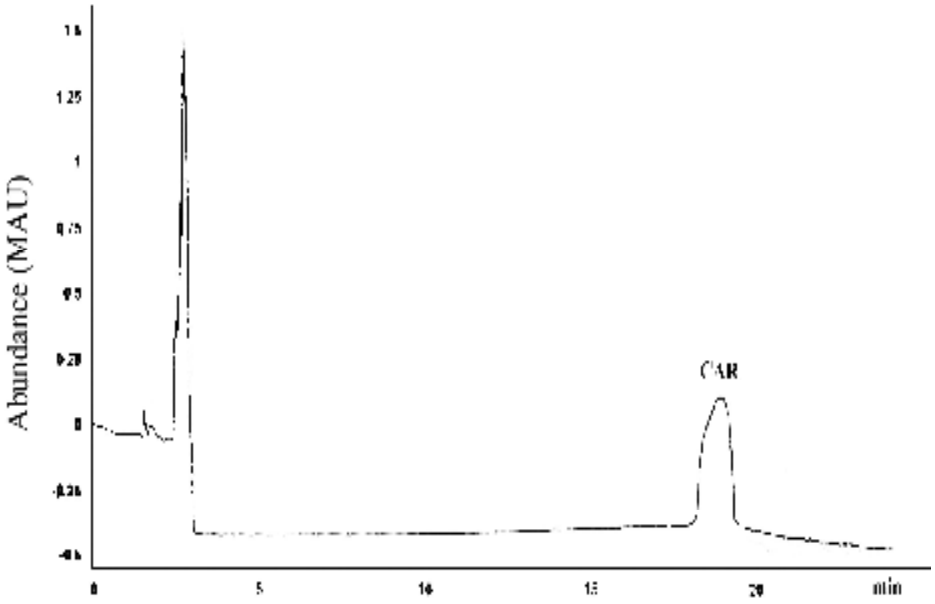


Figure 3-29: Chromatogram showed the retention time and abundance (MAU) of standard carnosol (CAR) analyzed by HPLC

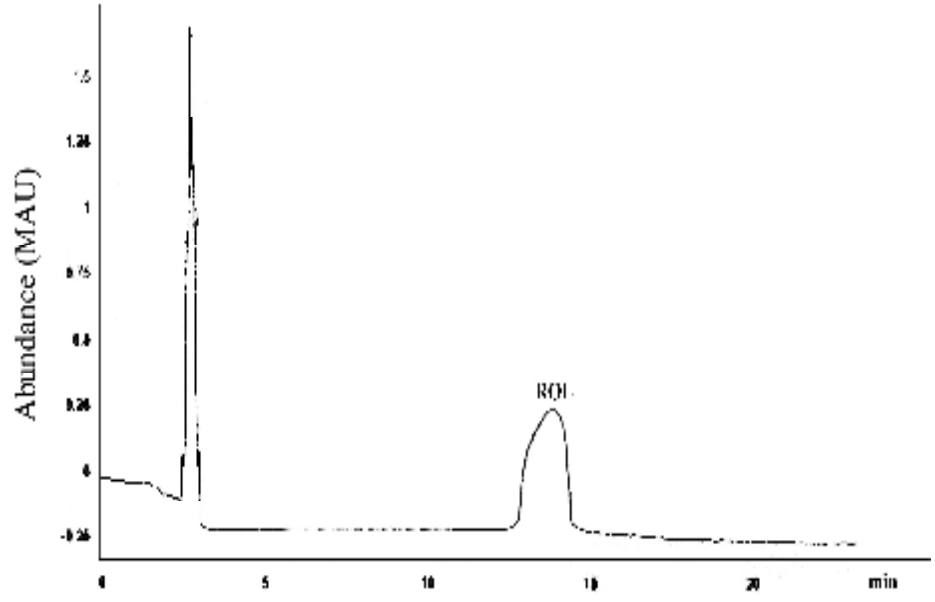


Figure 3-30: Chromatogram showed the retention time and abundance (MAU) of standard rosmanol (ROL) analyzed by HPLC

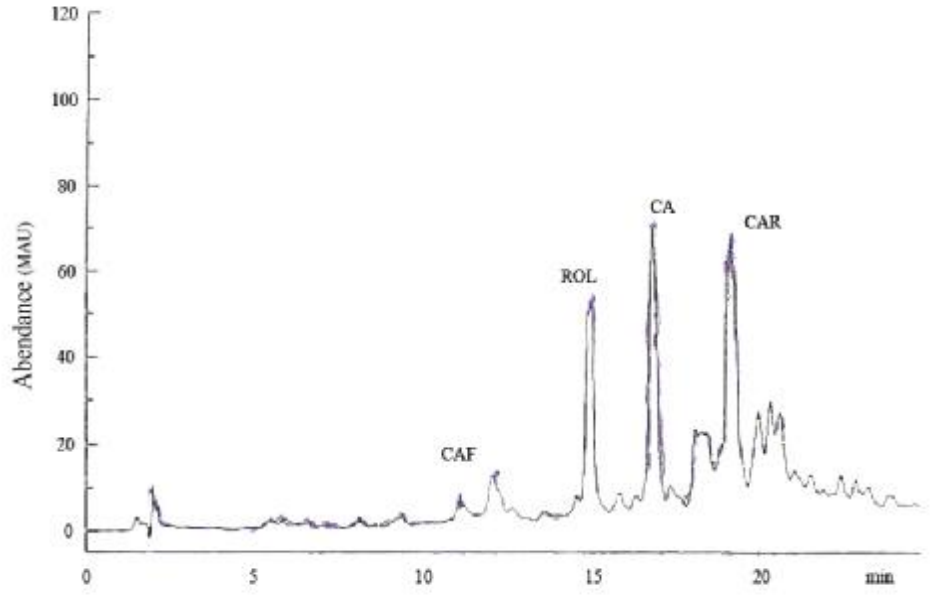


Figure 3-31: Chromatogram showed the retention time and abundance (MAU) of caffeic acid (CAF), rosmanol (ROL), carnosic acid (CA) and carnosol (CAR) produced from untreated callus of rosemary analyzed by HPLC

3-11: Antimicrobial assay

The ethanol extracts of rosemary were tested for their antibacterial and antifungal activity toward five selected isolates which represent two bacterial isolates; *E. coli*, *P. aeruginosa* as representative for G-negative and *B. cereus* representative for G-positive and two other fungal isolates; *A. nigar*, *F. oxysporum*.

In this study, minimum inhibitory concentration of the extracts (MIC) were considered as the lowest concentration which inhibit growth of microbial cells. Results shown in table 3-26 and figure 3-32 indicated that higher concentrations of the extract were required to inhibit G-negative bacteria. It was found that 320 µl/ml was enough to inhibit *E. coli* while 640 µl/ml was required to inhibit *P. aeruginosa*. Ethanol extracts were more active against G-positive; *B. cereus* as long as 160 µl/ml was enough to inhibit their growth.

Table 3-26: Effect of different concentrations of rosemary ethanol extract on microbial growth as tested by agar dilution method*

Microorganism	Ethanol extract (µl/ml)							
	10	20	40	80	160	320	640	Control
E. coli	+	+	+	+	+	-	-	+
P. aeruginosa	+	+	+	+	+	+	-	+
B. cereus	+	+	+	+	-	-	-	+
A. nigar	+	+	+	+	+	-	-	+
F. oxysporum	+	+	+	+	-	-	-	+

* (+) visible growth, (-) no growth

The existence of antimicrobial activity in the crude ethanol extract of rosemary leaves was obvious. It was observed that the extract was more active against *B. cereus* than those of other bacterial species as long as (160-640) µl/ml was enough to inhibit their growth. However, *E. coli* was found more resistant at that concentration. On the other hand, *P. aeruginosa* was found the most resistant bacteria, it was not inhibited till a concentration of 640 µl /ml.

It was also found that rosemary ethanol extract exhibits antifungal activity against the tested fungal species (*A. nigar* and *F. oxysporum*). Data shown in table 3-26, figures 3-32 and 3-33 indicated that at the lower concentrations (10-80) µl/ml, fungal growth was visible, while at the higher concentrations (160-640) µl/ml growth inhibition was clear. The MIC values of ethanol extract against *F. oxysporum* and *A. nigar* were 160 and 320 µg/ml respectively. Accordingly *F. oxysporum* seems to be more sensitive than *A. nigar*. Growth of tested microorganisms during exposure to different concentrations of rosemary leaf ethanol extracts were also monitored and expressed as colony count in CFU/ml as shown in table 3-27.

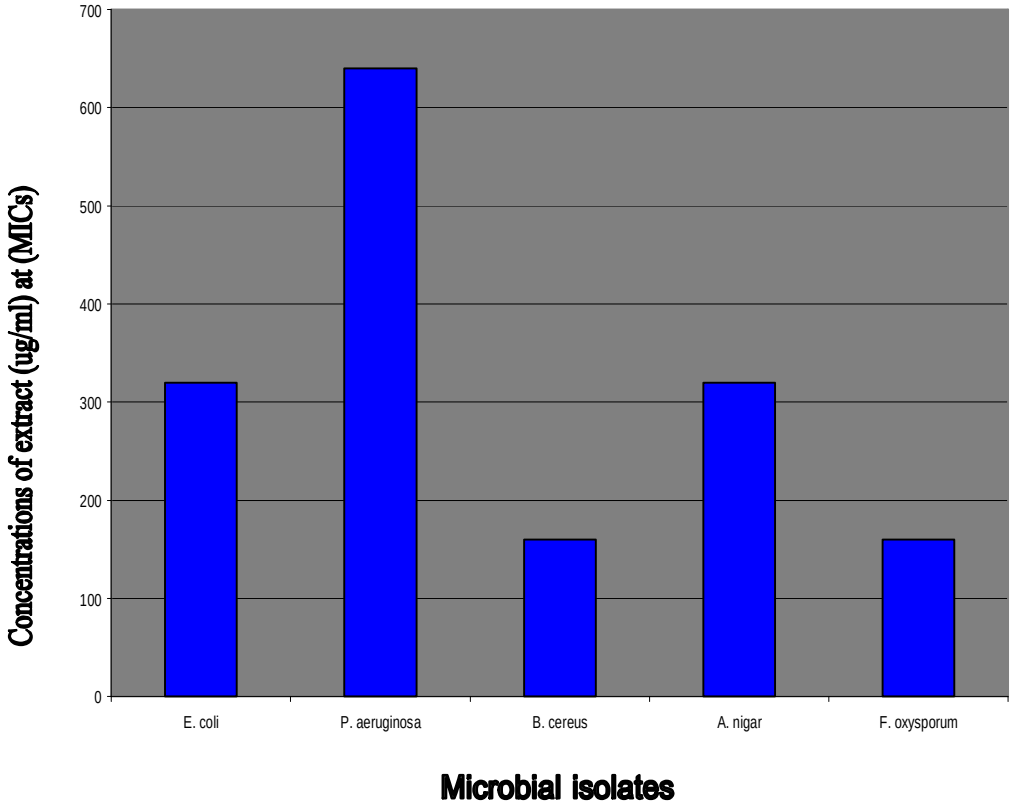


Figure 3-32: Minimum inhibitory concentration of rosemary ethanol extract (µl/ml) against some microbial isolates.

It was observed that a reduction in cell numbers was obvious with increase concentration of ethanol extract. For instance at lower concentrations (10-40) µl/ml as much as $3x10^5$ CFU/ml was recorded. However, when the concentration doubled more than 3-fold, reduction in cell number of *E. coli* was obvious. Concerning fungal isolates, a (23.6) CFU/ml for *A. nigar* and identical number for *F. oxysporum* were obtained when the concentrations of the extracts were 320 and 160 µl/ml respectively.

As mentioned previously rosemary extract contains caffeic acid, carnosic acid, carnosol, and rosmarinic acid, these compounds were found to be effective antimicrobial agents against several fungal species.

Accordingly, the antimicrobial efficacy may be associated with their specific phenolic composition.

Table 3-27: Growth inhibition (represent colonies number) of tested microorganisms using different concentrations of rosemary ethanol extract*

Microorganism	**Ethanolic extract (µl/ml)**							
	10	**20**	**40**	**80**	**160**	**320**	**640**	**Control**
E. coli	+	+	+	86.3	33.6	22.3	23	+
P. aeruginosa	+	+	+	+	46.6	34.3	19.6	+
B. cereus	+	+	+	63.6	28.3	29.6	28.3	+
A. nigar	+	+	+	76.3	35.3	23.6	22.3	+
F. oxysporum	+	+	+	66.3	21.6	22.3	21.3	+
LSD ≤ 0.05				39.4*	9.25*	5.34*	5.27*	

* (+) visible growth

These results are in agreement with Grosvenor *et al.*, (1995), who found that G-positive bacteria were the most sensitive as compared with other bacterial species. The antimicrobial activity demonstrated by ethanol extracts against the microorganisms tested in this study was comparable with those of other spices reported in the literature which inhibited the growth of G-positive and G-negative (Shelef *et al.*, 1980; Jain, 1994). Higher activity of plant extracts as well as their constituents against bacterial species was reported by Recio and Rios (1989), and by Shah (2004), they found that the differences in the antimicrobial activity might be due to the difference in bacterial cell wall constituents. According to literature (Glownaik *et al.*, 2006; Shah, 2004), the components exert antimicrobial effects through the

disruption of bacterial or fungal membrane integrity and the inhibition of respiration and ion transport processes. Timothy and Nathalie (2006) found that the leaf extracts were effective in reducing germination of zoospores of *Phytophthora* spp. concerning rosemary, the active compounds of the extract was found to be rosmarinic acid and caffeic acid which could completely inhibit germination of *Phytophthora* fungus at some concentrations, and can be used as antifungal agents after tested with another fungal species.

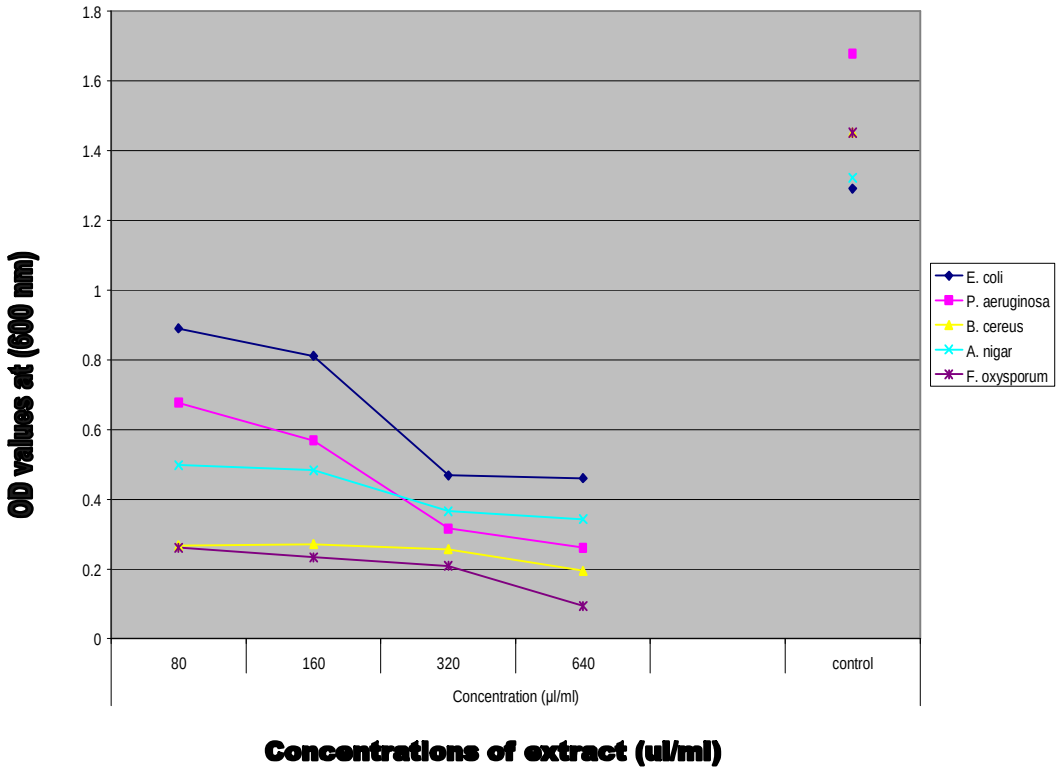


Figure 3-33: The Optical density (OD) values obtained from different concentrations of rosemary ethanol extract inoculation with microbial isolates (at wave length of 600 nm)

α-pinene, β-pinene, camphor, cineole, verbenone and bornyl acetate which are presented at high concentrations in rosemary extract can play a role in its bacterial and fungal activity.

Results obtained might be different from previous reports due to the difference in active compound composition which can probably be attributed to the nutritional status of the plant as well as the seasonal variations and the type of explants. Antimicrobial activity against known food pathogens, such as, *E. coli, P. aeruginosa* and *B. cereus* which might cause food spoilage and poisoning and subsequently cause human diseases, the use of plants to heal diseases including infectious ones, has been extensively applied by people. Data from the literature as well as these results reveal the great potential of plants for therapeutic treatment. Therefore, more studies need to be conducted to search for new compounds. Once extracted, and before being used in new therapeutic treatments, they should have their toxicity tested *in vivo*. Bioassay have demonstrated the toxicity of extracts from different plants (Carvalho *et al*., 1988; Nascimento *et al*., 1990).

1: Conclusions

1-Callus cultures of rosemary can be induced on leaf explants and maintained on MS medium containing 2.0 mg/l 2,4-D and 0.5 mg/l BA. The combinations of 2,4-D and BA produce a maximum fresh weight of callus.

2-Essential oils can be produced and increased in callus cultures of rosemary after the addition of biotic and abiotic elicitors.

3-Fungal elicitors at a concentration of 2.0 mg/l were more effective than other studied elicitors in stimulating essential oil and rosmarinic acid production.

4-Cineole, camphor, borneol, bornyl actate, isocornosol, ferruginol, α-pinene, β-pinene and phytol were the most abundant compounds in leaves and callus of rosemary plant.

5-It has been reported for the first time the existence of quinoline in rosemary callus, since previous literature has not indicated the presence of quinoline, which has been mentioned to be of a great therapeutic value.

2-Recommendations

1-More detailed research is recommended to investigate other medicinal plants as a source for phytochemicals using tissue culture techniques.

2-Further studies are recommended to explore the detailed analysis of chemical composition, anticancer and anti-inflammatory effect of rosemary and to characterize the antioxidant properties of rosemary essential oils.

3-Application of bioreactors to increase the production of secondary metabolites using differentiated and non-differentiated cultures.

4-Further studies are needed to investigate biotic and abiotic elicitors for increasing secondary metabolites production using tissue culture technique.

5-Examination of plant extracts on other types of pathogenic bacteria and fungi and a wide range of infections.

6-Using genetic engineering approaches for the manipulation of genes responsible for the higher production of secondary metabolites.

References: 115

Abdel-Hady, M. S. and Abou-Deif, F. (2001). The effect of gamma radiation on callus induction and plant regeneration of maize. Bull. NRC, Egypt, 26(3): 383-394.

Abreu, M. E.; Muller, M.; Alegre, L. and Munne-Bosch, S. (2008). Phenolic diterpenes and α-tocopherol contents in leaf extracts of 60 *Salvis* species. J. of Sci. and Agric., 88(15): 2648-2653.

Adams, R. P. (2002). Identification of essential oil compounds by Gas Chromatography / Mass Spectroscopy. Allured, Carol Stream, Illinois,: 1-15.

Aeschbach, R.; Loliger, J.; Scott, B. C.; Muracia, A.; Butler, J. B. and Aruoma, O. L. (1994). Antioxidant action of thymol, carvacrol, 6-gingerol, zingerone, and hydroxytyrosol. Food Chem. Toxicol., 32: 31-36.

Akueshi, C. O.; Kadiri, C. O.; Akueshi, E. U.; Agina, S. E. and Gurukwem, B. (2002). Antimicrobial potential of *Hyptis sauvedens* Poit (Lamiaceae), Nigeria. J. Bot., 15: 37-41.

Alex, D.; Bach, T. J. and Chye, M. L. (2000). Expression of *Brassica* juncea 3-hydroxy-3-methylglutaryl CoA synthase is developmentally regulated and stress-responsive. The Plant J., 22: 415-426.

Alica, Z. and Eva, B. (2003). Validation of HPLC determination of phenolic acids present in some Lamiaceae family plants. J. of Liquid Chromatog. and Related Technol., 26(3): 443-453.

Almela, L.; Sanchez-Munoz, B.; Fernandez-Lopez, J.; Roca, M. and Rabe, V. (2006). Liquid Chromatographic-Mass Spectrometric analysis of phenolic and free radical scavenging activity of rosemary extract from different raw materials. J. Chromatog., 1120 (1-2): 22-229.

Alonso-Paz, E.; Cerderiras, M. P.; Fernandez, J.; Ferreira, F.; Moyna, P.; Soubes, M.; Vazquez, A.; Veros, S. and Zunno, L. (1995). Screening of Uruguayan, medicinal plants for antimicrobial activity. J. Ethnopharm., 45: 67-70.

Al-Rumaih, M. M. and Al-Rumaih, M. M. (2008). Influence of ionizing radiation on antioxidant enzymes in three species of *Trigonella.* Amer. J. Environ. Sci. 4(2): 151-156.

Al-Sereitia, M. and Abu- Amer, K. (1996). Therapeutic potential and pharmacology of medicinal plants. In 40th Annual of the Egy. Soc. of Pharmac. and Therap., Cairo,44.

Al-Sereitia, M.; Abu-Amer, K. M. and Sena, P. (1999). Pharmacology of rosemary (*Rosmarinus officinalis* L.) and its therapeutic potentials. Al-fateh University of Medicinal Science, Fac. of Med., Tripoli, Libya.

Amin, A. A. (2003). Physiological effect of gamma irradiation and kinetin on growth, yield and chemical constituents of wheat. Egy. J. Appl. Sci., 18(6): 34-49.

Ammo, S. O. and Ayisire, B. E. (2005). Induction of callus and somatic embryogenesis from cotyledons explants of *Parkia biglobosa* (Jacq), Benth. Afr. J. Biotechnol., 4: 68-71.

Angioni, A.; Barra, A.; Cereiti, E.; Barile, D.; Coisson, J. D. and Ariorio, A. (2004). Chemical composition, plant genetic differential and antifungal activity investigation of the essential oil of *Rosmarinus officinalis* L. J. Agric, Food Chem., 52: 37-42.

Annette, K. and Claudia, R. (2006). Phenolic antioxidant compounds produced by *in vitro* cultures of rosemary (*Rosmarinus officinalis* L.) and their anti-inflammatory effect on lipopolysaccharide-activated microglia. Pharmaceutical biology (Formerly international J. of Pharma., 44: 401-410.

Arita, M.; Asai, K. and Nishioka, T. (2000). Finding precursor compounds in secondary metabolites. Kyoto University, Kyoto. Japan, Review, 3(2): 606-856.

Arouma, O. I.; Spencer, J. P. and Rossi, R.(1998). An evaluation of the antioxidant and antiviral action of extracts of rosemary herbs. Food Chem. Toxicol., 34(5): 449-456.

Arouma, O. I. ; Haiwell, B. ; Aeschbach, R. and Loliger, J. (1992). Rosemary constituents. : Carnosol and carnosic acid. Xenobiotica, 22: 257-268.

Atti-Santos, A. C.; Rossato, M.; Pauletti, G. F.; Rota, L. D.; Rech, J. C.; Pansera, M. R.; Agostini, F.; Atti-Serafini, L. and Moyna, P. (2005). Physio-chemical evaluation of *Rosmarinus officinalis* L. essential oil. Braz. Arch. of Biol. and Tech. Int. J., 48(6): 1035-1039.

Aurelia, S. P.; Lusarkiewicz, P. J.; Aleksandro, P. and Zygmunt, K. (2007). Influence of cultivar, explants source on *in vitro* growth of *Cannabis sativa* L. Plant Genet., 47: 145-151.

Azza, A. T.; Paul, E. R. and Susan, L.C. (2006). Effect of some nutritional factors on monoterpene synthesis in *Rosmarinus officinalis* cultured *in vitro*. Acta Horticult. J., 319: 1-3.

Aziza, K. G.; Haiko, H.; Artur, S. J. and Simone, M. S. (2008). Rosemary (*Rosmarinus officinalis*) a study of composition, antioxidant and antimicrobial activities of extracts obtained with supercritical carbon-dioxide. Cienc. Technol. Ailment., Campinas, 28(2): 463-469.

Batz, O. L.; Reinold, S. and Hahlbrock, K. (1998). Extensive reprogramming of primary and secondary metabolism by fungal elicitoror infection in parsley cells. Biol. Chem., 379: 1127-1135.

Bauer, A. W.; Kirby, W. M.; Sheris, J. C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disc method. AM. J. Clin. Pathol., 45: 149-158.

Bauer, K.; Garbe, D. and Surburg, H. (1997). Common Fragrance and Flavor Materials. 3rd ed. Germany: Wiley-VCH.

Birch, R. G. (1997). Plant transformation Problems. Plant Mol. Biol., 48: 297-326.

Bos, R. (1997). Analytical and phytochemical studies on valerian and valerian based preparations [Dissertation]. Groningen: Rijks universiteit Groningen. Dept. of Pharma. Biol., 184-193.

Brandsteterova, E. and Ziakova-Caniova, A. (2003). Phenolic acids in natural plants, analysis by HPLC. Encyclopedia of Chromatography, Cazes, J. ed., Marcel Dekker Inc.

BricKell, C. (1990). The RHS Gardener's Encyclopedia of Plant Flowers. Dorling Kindersley Publishers Ltd.

Bruno, Z.; Majda, H.; Davorin, B. and Durda, V. (2004). Recovery and purification of rosmarinic acid from rosemary using electro dialysis. Act. Chem. Slov., 52: 126-130.

Burt, S. (2004). Essential oils; their antibacterial properties and potential application in foods. A review. Int. J. of Food Microbiol., 94: 223-253.

Carlos-Estrela, R. (2002). Mechanism of action of sodium hypochlorite. Braz. Dent. J., (2): 113-117.

Caruso, J. L.; Callahan, J.; DeChant, C.; Jayasimhulu, K. and Winget, G. D. (2000). Carnosic acid in green callus and regenerated shoots of *Rosmarinus officinalis.* Plant Cell Rep., 19: 500-503.

Carvalho, V.; Melo, V. M.; Aguiar, A. and Matos, F. S. (1988). Toxicity evaluation of medicinal plant extracts by the brine shrump (*Arthenus salina* Leah.) bioassay. Cienciae cult., 40: 1109-1111.

Chalchate, J. C. O.; Garry, R. P.; Michet, A.; Benjilali, B. and Chabart, J. I. (1993). Essential oils of rosemary (*Rosmarinus officinalis* L.). The chemical composition of oils of various origins (Morocco, Spain, France). J. of Essential Oil, 5: 613-618.

Chandler, S.F. and Dodds, J. H. (1983). The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum laciniatum* . Plant Cell Rep., (2): 105-108.

Chandra, I. and Bhanja, P. (2002). Study of organogenesis i*n vitro* from callus tissue of *Flacourtia jangomas* Lour. Burdwan University, India. Current Sci., 83.

Chapin, F,; Bloom, A.; Field, C. and Waring, R. (1987). Plant responses to multiple environmental factors. Biosci., (37): 49-57.

Christiane, G.; Yves, G.; Louise, R. and Catherine, T. (2007). Comparison of disk diffusion and agar dilution methods for erythromycin and ciprofloxacin susceptibility testing of *Campylobacter jejuni* subsp. *jejuni*. Antimicrobial Agent and Chem., 2: 1524-1526.

Chu, I. Y. and Kurtz, S. L. (1990). Commercialization of plant micropropagation. In; Ammirat, P. V., Evans, D. R., Sharp, W. R., Bajaj, Y. P. S.; eds.: Handbook of Plant Cell Culture. New York: McGraw-Hill Pub. Co., 5: 120-164.

Clifrord, M. N. (1999). Chlorogenic acids and other cinnamates. Nature, occurance of dietary burden. J. Sci. Food Agric., 79: 362-372.

Clinical and Laboratory Standards Institute. (2006). Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. Approved standard M45-A. Clinical and Laboratory Standards Institute, Wayne, PA.

Cohen, N. L. (1992). Epidemiology of drug resistance: implications for a post-antimicrobial era. Sci., 2657: 1050-1055.

Collin, M. A. and Charles, H. P. (1987). Antibacterial activity of carnosol and carnosic acid: two antioxidant constituents of *Rosmarinus officinalis* L. Food Microbial., 4: 311-315.

Corey, D. B.; David, V. H.; Mohamed, A. F.; Joel, T.; Pedro, M. R. and Lioyd, W. S. (2005). Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. J. of Exp. Bot., 56(410): 323-336.

Cuvelier, M. E.; Richard, H. and Berset, C. (1996). Antioxidative activity and phenolic composition of pilot-plant and commercial extract of sage and rosemary. J. Amer. Oil Chem., 73: 645-652.

Daves, M. E. (1972). Polyphenol synthesis in cell suspension cultures of paul's scarlet Ros. Plant., 104: 50-56.

Davis, J. M. (1996). Basic Cell Culture. Oxford University Press, New York, 93-134.

Deans, S. G. and Svoboda, K. P. (1993). Biotechnology of aromatic and medicinal plants. New York ; Langman Scientific and Technical., 1: 113-136.

Deirdre, O. F. (2003). HPLC Principles and Applications. Chem.. V1: 1-6.

Dianella, S.; Claudio, A.; Tiziano, A.; Emanula, C.; Graziano, G. and Fernando, D. (2004). Antimicrobial Activity of Euplotin C. The sesquiterpene taxonomic marker from the marine Euplotes crassus. University of Pisa, Italy.

DiCosmo, F. and Towers, G. (1984). Stress and secondary metabolites in cultured plant cells. In: Resent Adva. In Phytochem. Plenum Pub., 18: 154-160.

Dodds, H. J. and Robert, L. W. (1995). Experiments in Plant Tissue Culture. 4th Edition. Cambridge Univ. Press. U.K.

Donald, C. S. and Ann, V. B. (2006). Antimicrobial susceptibility testing. Wisconsin Veterinary Diagnostic Laboratory.

Donald, R. S. and Clyde, T. (1974). Broth-dilution method for determining the antibiotic susceptibility of bacteria. Antimicrobial Agent and Chemtherapy. Amer. Soc. for Microbiol., 7: 15-21.

Dornenburg, H. and Knor, D. (1995). Strategies for the improvement of secondary metabolite production in plant cultures. Enzyme. Microb. Technol., 17: 674-684.

Dougall, D. K.; Labrake, S. and Whitten, G. H. (1983). Growth and anthocyanin accumulation rates of carrot suspension cultures grow with excess nutrients rates, pHs, and temperatures. Biotechnol. Bioeng., 25: 581-594.

Downes and Ito. (2001). Compendium of Methods for the Microbiological Examination of Foods, 4th ed. American Public Health Association, Washington.

Elena, E. S.; Martha, C.; Yajaria, C.; Hernan, F. and Jairo, R. M. (1999). HRGC/FID and HRGC/MSD analysis of the secondary metabolites obtaining by different extraction from *Lepechinia sciedeana* and *In vitro* evaluation of its antioxidant activity. Wiley Interscience. Fed. Rep. of Germany.

Ellof, J. N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants. J. Ethnopharm., 60: 1-6.

Eva, S. B.; Maria, H. T.; Attila, H.; Csilla, R.; and Szollosi, V. (2003). Antioxidant effect of various rosemary **(***Rosmarinus officinalis* L.) clones. Acta Biol., 47(1-4): 111-113.

Fadel, H. M. and El-Massry, K. F. (2000). *Rosmarinus officinalis* L.; effect of drying on the volatile oil of fresh leaves and antioxidant activity of their extracts. J. Essential Oil Bearing plants, 3: 5-19.

Fakoor, M. H. and Rasooli, I. (2007). Pathogen Control by antioxidative characteristics of *Cuminun cyminum* and *Rosmarinus officinalis* essential oils. (URL://WWW.actahort.org/)

Fennema, O. R. (1996). Food Chemistry, 3rd ed. Marcel Dekker, Inc. New York, 962.

Foster, S. and Tyler, V. (1999). The Honest Herbal; A sensible guide for the use of herbs and related remedies. 4th ed. Haworth Herbal Press, pp: 321-322.

Frankel, E. N. (1996). Antioxidant activity of rosemary extract and its constituent, carnosic acid, cornosol and rosmarinic acid, in bulk oil and oil-in-water emulsion. J. of Agric. and Food Chem., 44: 131-135.

Frederic, D. (2007). GC/MS Analysis. Patentattorney@law.com. http://sites.netscape.net/dougfrm.

Gail, B. M. and Beecher, C. W. W. (1994). Quercetin-induced benzophenanthridine alkaloid production in suspension cell cultures of *Sanguinaria Canadensis*. Umfang, Siete, pp: 553-557.

Gachkar, L. (2007). Chemical and biological characteristics of *Cuminum cyminum* and *Rosmarinus officinalis* essential oils. Food Chem., 102: 898-902.

Genders, R. (1994). Scented Flora of the World. Robert Hale. ISBN 0-7090-5440-8.

Gerold, R. and Ralf, G. B. (1996). Elicitation of volatile compounds in photomixotrophic cell culture of *Petroselinum crispum.*Plant Cell, Tissue and Org. Cult., 46:1-6.

Geslene, G. F.; Juliana, L.; Paolo, C. F. and Giuliana, L. S. (2000). Antibacterialactivit of plant extracs and phytochemicals on antibiotic resisance baceria. Brazilian J. of Microbiol., 31: 247-256.

Georgio, P.; Marianna, U.; Pascal, B.; Claudia, J.; Gianpiero, B.; Felix, T.; Mario, C.; Riccardo, C. and Joseph, C. (2001). Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. from sarinia and Corsica. Universita di Sassari, Italy.

Glownaik, P. (2006). Activity of *Crithmum maritimum* L. (Apiaceae) against Gram-positive bacteria. Annales UMCS, Sectio, 19: 122-123.

Goodwin, T. W. and Mercer, E. I. (1983). Introduction to Plant Biochemistry, 2nd ed. Pergamon Press, New York, 424-425.

Goldstein, W. (1988). In "Plant Tissue Culture as A source of Biochemicals" ed. Staba, E.J. C.R. C. Press. Boca. Raton Florida, pp: 191-234.

Grosvenor, P.; Supriona, A. and Gray, D. (1995). Medicinal Plants from Riau Province, Sumatra, Indonesia. Part 2: Antibacterial and antifungal activity. J. of Ethnopharm., 45: 97-111.

Gruel, S.; Gruel, E. and Kaya, Z. (2002). Establishment of cell suspension cultures and plant regeneration in sugar beet (*Beta vulgaris* L.), Turck, J. Bot., 26: 197-205.

Gwi-Taek, J.; Don-Hee, P.; Hwa-Won, R.; Doman, K.; Baik, H.; Je-Chang, W. and Si-Wouk, K. (2005). Enhanced production of secondary metabolite in hairy root cultures of *Panax ginseng* C. A.

Meyer by elicitation. Chonnam National University. Kwangju. Korea, pp 500-557.

Halder, T. and Gadgil, V. (1983). Fatty acids of callus tissue of six species of cucurbitaceae. Phytochem., 22: 1965-1967.

Haas, R.; Meyer, T. and Van-Putten, J. (1993). A flagellated mutants for Helicobacter phyori generated by genetic transformation of naturally component strains using transposon shuttle mutagenesis. Mol. Microbiol., 8: 753-760.

Hahn, E. T.; Kim, Y. S.; Yu, K. W.; Jeong, C. S. and Paek, K. Y. (2003). Adventitious root cultures of *Panax ginseng* ginsedoside production through large-scale bioreactor system. J. Plant Biotechnol., 5: 1-6.

Hany, M. E. (2006). Using biotechnology to improve the production of rosmarinic acid from rosemary plants. University of Nebraska-Lincoln. Dissertations/ AAI 3217531.

Harborne, J. B. (1984). Phytochemical Methods. A guide to Modern Technique of Plant Analysis, Chapman Hall, London.

Hayashi, K.; Kamiya, M. and Hayashi, T. (1995). Virucidal effects of the steam distillate from *Houttuynia cardata* and its components on HSV-1, influenza virus and HIV. Plant. Med., 61: 237-241.

Hermann, K. (1960). Uber den "gerbstoff" der Labiatenblatter. Arch. Pharm., 293: 1043-1048.

Hippolyte, I.; Marin, B.; Baccou,, J.C. and Jonard, R. (1992). Growth and rosmarinic acid production in cell suspension cultures of *Salvia officinalis* L. Plant Cell Rep., 11: 109-112.

Holzmannova, V. (1996). Rosmarinic acid and its biological activity (in Czech), 90: 486-496.

Hui, X.; Kim, Y. K.; Jin, X.; Lee, S. Y. and Park, S. U. (2008). Rosmarinic acid biosynthesis in callus and cell cultures of *Agastache rugosa Kuntze*. Journal of Med. Plant Res., 2(9): 237-241.

Inatani, K. (1982). Structure of a new antioxidative phenolic diterpenes isolated from rosemary (*Rosmarinus officinalis* L.). Agric. Biol. Chem., 46(6): 1,2,14.

I.S.H.S. (1990). International Society for Horticultural Science. Wageningen, M. A. N. M. F. The Hague, Elsevier's Dictionary of Horticultural and Agricultural Plant Production in Ten Languages, Elsevier Science Publishers B. V. Amsterdam, the Netherlands.

Jain, R. C. (1994). Antibacterial activity of garlic extract. Indian J. of Med. Microbiol., 11: 26-31.

Jamzad, Z.; Ingrouille, M. and Simmonds, M S. J. (2003). Three new species of *Nepeta* (Lamiaceae) from Iran. Taxon., 52: 34-38.

Janicsak, G. and Mathe, I. (1998). Densitometric method for parallel measurement of rosmarinic and caffeic acids in plant samples. Acta Pharm. Hung., 68(5): 259-260.

Jayasree, P. K.; Asokan, M. P.; Sobha, S.; Ammal, L.; Rekha, K.; Kala, R. G.; Jayasree, R. and Thulaseedharan, A. (2004). Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* Muell. Arg-x. Biotech. Div. Rubber Research Institute of India.

Jeong, G. T.; Don-Hee, P.; Hwa-won, R.; Doman, K.; Baik, H.; Je-Chang, W. and Si-Wouk, K. (2005). Enhanced production of secondary metabolite in hairy root culture of *Panx ginesing* CA. Meyer by elicitation. Chonnam National University. Kwangju, Korea, 500-757.

Josep, G.; Isabel, P.; Carmen, A. and Montserrat, O. (2000). *In vitro* antifungal susceptibility of nondermatophytic keratinophilic fungi. Unitat de Microbiologia, Facultat de Medicina, Universitat. Rovira; Virgili, Tarragona, Spain.

Katzung, B. G. (1995). Basic and Clinical Pharmacology, ed. 6, London: Prentice Hall International (UK) Limited.

Kessmann, H.; Edwards, R.; Gero, P. W. and Dixon, R.A. (1990). Stress response in alfalfa (*Nedicago sativa* L.) V, constitutive and

elicitoe-induced accumulation of isoflavonoid conjugation in cell suspension cultures. Plant Physiol., 94: 227-232.

Knoblocu, K. H, and Berlin, J. (1980). Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* L. G. Zantur forsch., 35: 551-556.

Komamine, A.; Misawa, M. and DiCosmo, F. (1991). Plant Cell Culture in Japan. Progress in production of useful plant metabolites by Japanese, Enterprises Using Plant Cell Culture Technology. CMC Co. Ltd., Tokyo, Japan.

Koik, W. K.; Asada, Y. and Yoshikawa, T. (2005). Rosmaric acid production by *Coleus forskohlii* hairy root culture. Spring. Art., 80: 151-155.

Kosta, Y. M.; Rachel, G.; Uri, J. M. and Shlomo, M. (1995). Repellency of essential oils and their components to the human louse, *Pediculus humanus*. London: Prentice Hall International (UK) Limited.

Kravchenko, O. A.; Lysikov, V. N. and Palii, A. F. (2003). Effect of gamma radiation on callus formation and somatic embryogenesis of wax maize. (http:www.agron.missouri.edu).

Krolicka, A.; Lojkowska, E.; staniszewska, I.; malinski, E. and Szafranek, J. (2008). Identification of secondary metabolites in *in vitro* culture of *Ammi majus* treated with elicitors. ISHS Acta Horticul., 560: 1-4.

Larrondo, J. V.; Agut, M. and Calvo-Torras, M. A. (2001). Antimicrobial activity of essences from labiates. Faculty of Veterinary Sciences, Universided Autonoma de Barcelona, Spain.

Leena, T. and Jaindra, N. T. (2003). Role of biotechnology in medicinal plants. Tropical J. of Pharma. Res., 2(2): 243-253.

Lemos, T. L.; Monte, F. G.; Matos, F. J.; Alencar, J. W.; Craveiro. A. A.; Barbosa, R. C. and Lima, E. D. (1992). Chemical composition

and antimicrobial activity of essential oils from Brazilian plants. Fitoterap., 63: 266-268.

Lila, M. (2006). The nature-versus nurture debate on bioactive phytochemicals: the genome versus terror, J. Sci. Food Agric., (86): 2510-2515.

Lodewijk, S. and Christina, M. J. E. (2008). Activity of Daptomycin against *Listeria monocytogenes* isolates from Cerebrospinal fluid. Antimicrobial Agents and Chem., 52(5): 1850-1851.

Luis, J. and Johnson, C. B. (2005). Seasonal variations of rosmarinic acid and carnosic acid in rosemary extracts. Analysis of their *in vitro* antiradical activity. Spanish J. of Agric. Res., 3(1): 106-112.

Ly, T. N.; Shimoyamada, M. and Yamanchi, R. (2006). Isolation and characterization of rosmarinic acid oligomers in *Celastrus hindsii* bench leaves and their antioxidative activity. Food Chem., 54: 3786-3793.

Maganga, A. (2004). Influence of variety and organic cultural practices on yield and essential oil content of lavender and rosemary in Interior BC. Ecorational Technologies, Kamloops, 1-23.

Mario, D. L.; Giovanni, S. P.; Stefania, D. and Emanuela, B. (2002). Essential oil formation, useful as a new tool for insect pest control. AAPS Pharm. Sci. Technol., 3(2): 13.

Martos, M. V.; Navajas, Y. R.; Fernandez-Lopez, J. and Perez Alvarez, J. A. (2007). Chemical composition of the essential oils obtained from me spices widely used in Mediterranean region. Acta Chim. Slov., 54: 921-926.

Mary, A. L. (2005). Valuable Secondary Products from *in vitro* Culture. CRC, Press. LLC., 285-288.

Marzouk, Z.; Neffati, A.; Marzouk, B.; Chraief, I.; Fathia, K.; Chekir, G. and Boukef, K. (2006). Chemical composition and antibacterial and antimutagenic activity of Tunisian *Rosmarinus officinalis* L. oil from Kasrine. J. of Food , Agric. and Environ., 4(3-4): 61-65.

Masa, I. R.; Darinka, B.V.; Valter, D. and Eenest, V. (2007). Determination of major phenolic acids, phenolic diterpenes and triterpenes in rosemary (*Rosmarinus officinalis* L.) by gas chromatography and mass spectrometry. Acta Chem. Slov., 54: 60-67.

Mazza, C. A. ; Boccalandro, H. E.; Giordano, C. V.; Battista, D.; Scopel, A. L. and Salome, P. A. (2000). Functional significance and induction by solar radiation of ultraviolet-absorbing sunscreens in field-grown soybean crops. Plant Physiol., 122: 117-126.

McGarvey, D. and Croteau, R. (1995). Terpenoid metabolism. Plant Cell, 7: 1015-1026.

Mehrabani, A. T.; Mohammadreza, A.; Alireza, G.; Nasrlah, D. and Seyyed, J. (2005). Production of rosmarinic acid in *Echium amoenum* Fish. C. A. Mey. Cell Cultures Sch. Pharm-Shaheed Beheshti, Uni. Med. Sci. and Health services.

Misra, P. and Chaturvedi, H. (1984). Micropropagation of *Rosmarinus officinalis* L. Plant Cell Tissue Org. Cult., 3: 163-168.

Mohaddese, M.; Mohammad, M. F. and Mahin, S. (2008). Antifungal activity of essential oils from *Zataria multiflora*, *Rosmarinus officinalis*, *Lavandula stoechas*, *Artemissia sieberi* of *Candida albicans*. PhCog. Mag., 4: 14-15.

Monica, B. P,; Natalia, L. C. and Clara, A. C. (2006). Radiation-induced enhancement of antioxidant activity in extracts of rosemary (*Rosmarinus officinalis* L.). Universidad Nacional del Sur Avenida Alem, Bahia Blanca, Argentina.

Mooney, H.; Winner, W. and Pell, E. (1991). Response of plants to multiple stresses. New York: Academic Press. 121-141.

Mounchid, K.; Bourjilat, F.; Dersi, N.; Aboussaauira, T.; Rachidai, A.; Tantaoul-Elaraki, A. and Alaoui-Ismaili, M. (2004). Toxicity of south Morocco *Rosmarinus officinalis* essential oil: antibacterial and histopathological effects. Les Actes del'institut Agron. Veter., (2-3): 139-144.

Mounchid, K.; Bourjilat, F.; Dersi, N.; Aboussaauira, T.; Rachidai, A.; Tantaoul,-Elaraki, A. and Alaoui-Ismaili, M. (2005). The susceptibility of *Escheritia coli* strains to essential oils of *Rosmarinus officinalis* and *Eucalyptus globules.* African J. of Biotechnol., 4(10): 1175-1185.

Munne-Bosch, S.; Schwarz, K. and Alegre, L. (1999). Enhanced formation of α-tocopherol and highly oxidized abiatane diterpenes in water-stressed rosemary plants. Plant Physiol., 121: 1047-1052.

Munne-Bosch, S. and Alegre, L. (2001). Changes in carotenoids, tocopherol, and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in *Rosmarinus officinalis* plants. Plantae, 210: 925-931.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with *Tobacco* tissue culture. Physiol. Plant., 15: 473-497.

Nair, R.; Kalariya, T. and Sumitra, C. (2008). Antibacterial activity of some selected Indian medicinal flora. Turk. J. Biol., 29: 41-47.

Namdeo, A. (2007). Plant cell elicitation for production of secondary metabolites: A review. Pharmaco. Rev., 1: 69-79.

Nascimento, S. C.; Chiappeta, A. and Lima, R. M. O. (1990). Antimicrobial cytotoxic activities in plants from Pemambuco, Brazil. Fitoterap., 61: 353-355.

Natas'a, B.; Dunia, L. L. and Sibila, J. (2004). Rosmarinic acid synthesis in transformed callus culture of *Coleus blumei* Benth. Zagreb, Croatia.

NCCLS, (1993). Performance Standards for Antimicrobial Disc Susceptibility Tests. Approved Standard NCCLS Publications. M2-A5. Uillanova, PA, US.

Newall, C.; Anderson, L. and Phillipson, J. (1996). Herbal Medicines: A guide for Health Care Proffessionals. London: Pharma. Press, pp: 229-230.

Nunez, D. and Obon de Gastro, C. (1992).Palaeoethnobotany and archaebotany of the Labiatae in Europe and Near East. Ibvn Harley, R. M. and Reynolds, T. Advances in Labiatae Science. Royal Botanical Gardens, Kew, London, pp: 437-454.

Offord, E.; Mace, K.; Ruffieux, C.; Malone, A. and Peiter, A. (1995). Rosemary components inhibit benzo-α-pyrene-induced genotoxicity in human bronchial cells.Carcinogenesis, 16(9): 2057-2062.

Okamura, N.; Fujimoto, Y.; Kuwabara, S. and Yagi, A. (1994). High performance liquid chromatographic determination of carnosic acid and carnosol in *R. officinalis* and *Salvia Officinalis*. J. Chromatog., 679: 381-386.

Oyedemi, S. O.; Pirochenra, G.; Mabinya, L. V.; Bradely, G. and Afolayan, A. J. (2008). Composition and comparisons of antimicrobial potencies of some essential oils and antibiotics against selected bacteria. African J. of Biotechnol., 7(22): 4140-4146.

Ozlem, Y.; Pinar, N.; Aynur, G.; Erdal, B. and Fazilet,V. (2007). Determination of phenolic content and antioxidant activity of extracts obtained from *Rosmarinus officinalis* calli. Ege University Sci. and Technol. Cent., Bornova-Izmir, Tyrkey.

Pamham, M. J. and Kesseiring, K. (1985). Rosmarinic acid. Drugs of the future. J. of Herb Drugs, 10: 756-757.

Panizzi, L. (1993). Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae. J. of Ethnopharm., 39: 167-170.

Parr, A. J. (1989). The production of secondary metabolites by plant cell cultures. J. Biotechnol., 10: 1-26.

Pattanaik, S.; Subramanyam, V. and Kole, C. (2002). Antibacterial and antifungal activity of the essential oils *in vitro*. Microbiol., 86(349): 237-248.

Pauli, A. (2006). Anticandidal low molecular compounds from higher plants with special reference to compounds from essential oils. Med. Res. Rev., 26: 223-268.

Pavlov, A. and Hieva, M. (1999).The influence of phenylalanine on accumulation of rosmarinic and caffeic acids by *Lavandula vera* MM. cell culture. World J. Microbiol. Biotechnol., 15: 397-399.

Pecora, J.; Sousa-Neto, M. and Estrela, C. (1999). Solucoses irrigadoras auxiliaries do prepare do canal radicular. In: Endodontia-Principios Biologicos Mecanicos. Estrela C, Figueiredo JAP.Eds. Sao Paulo: Artes Medicas, 552-569. Cited from Mechanism of Action of Sodium Hypochlorite.

Peng, Y. (2005). Determination of active components in rosemary by capillary electrophoresis with electrochemical detection. J. of Pharmaceut. and Biomed. Anal., 39: 341-437.

Petersen, M. and Simmonds, M. S. (2003). The biosynthesis of rosmarinic acid in suspension cultures of *Coleus blumei*.Plant Cell Tissue Org, Cult.,38: 171-179.

Pierik, R. L. M. (1987). *In vitro* Culture of Higher Plants. 3rd ed. Marinis Nijhoff Publishers, Dordrecht, the Netherlands.

Phillips, R. and Rix, M. (1998). Conservatory and Indoor Plant and 2-Pan Books, London.

Porte, A.; Godoy, R. L.; Koketsu, M.; Goncalves, S. L. and Torquilho, H. S. (2000). Essential oils. J. of Essential oil Res., 12(5): 577-580.

Proestos, C.; Sereli, D. and Koaitis, M. (2006). Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. Food Chem. J., 17: 44-52.

Radmab, R.; Saez, T.; Bucke, C. and Keshararz, T. (2003). Elicitation of plant and microbial cell systems. Biotechnol. App.. Biochem., 37: 91-102.

Ramawat, K. G. (2008). Plant Biotechnology. S. Chand and Company LTD, Ram Nagar, New Delhi.

Rico, M. C. and Rios, J. L. (1989). A review of some antibacterial compounds isolated from medicinal plants. Phytother. Res., 3: 116-117.

Rice-Evans, C. A.; Miller, N. J. and Paganga, G. (1997). Antioxidant properties of phenolic compounds. London, Trends in Plant Sci., 71: 152-159.

Robert, M. F. (1988). Medicinal Products Through Plant Biotechnology. In: Rj, Rhodes MJC.(eds). Manipulating Secondary Metabolism in Culture. Cambridge University Press, 201-216.

Robert, A. (1993). R. E. D. FACTS. Thymol, United State Environmental Protection Agency. 93-110.

Robins, R. L. (1999). Secondary Products from Cultured Cells and Organs: Molecular and cellular approaches. In: Dixon, R. A., Gonzates, R. A. (eds). Plant Cell Culture. Oxford; IRL, Press.

Roland, R.; Davin, C. and Zryd, J. (1992). Betalain production cell cultures of *Beta vulgaris* L. Var. Bikores monogerm (Red beet). *In vitro* Cell. Dev. Biol., 28: 39-45.

Salamone, A.; Zizzo, G. V. and Scarito, G. (2007). The antimicrobial activity of water extracts from Labiatae. (ww.actahort.org)

Salman, M. A. (1988). Fundamental of Plant Cell and Tissue Culture. Ministry of Higher Education and Scientific Research, University of Baghdad, Iraq. (In Arabic)

Santoyo, S.; Cavero, S.; Jaime, L.; Ibanez, E.; Senorans, F. and Reglero, G. (2005). Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. obtained via supercritical fluid extraction. J. Food Port., 68(4): 790-795.

SAS (2001). SAS/STAT User's Guide for Personal Computers. Release 6.12, SAS Inst. Inc. NC. USA.

Sateech, M. K, (2003). Biotechnology-5. New Age International Publishers.

Sasse, F.; Heckenberg, U. and Berlin, J. (1980). Accumulation of S-carboline alkaloids and serotonin by cell cultures of *Peganum harmala* L. Plant Physiol., (69): 400-404.

Scarpati, M. L. and Oriente, G. (1958). Isolamento ecostituzione dell'acido rasmarinco dar *Rosmarinus off*icinalis. Rec. Sci., 28: 2329-2333.

Schwarz, K. and Ternes, W. (1992). Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis* I. Z. Lenensm. Unters. Forsch., 195: 95-98.

Scragg, A. H. (1992). Bioreactors for Mass Cultivation of Plant Cells. In; Fowler, M. W.; Warren, G. S.(ed.), Plant Biotechnol. Oxford; Peragmon Press.

Sergi, M. B.; Leonor, A. and Karin, S. (2000). The formation of phenolic diterpenes in *Rosmarinus officinalis* L. under Mediterranean climate.Eur. Food Res. Technol., 210: 263-267.

Sergi, M. and Leonor, A. (2001). Subcellular compartmentation of the diterpene carnosic acid and its derivatives in the leaves of rosemary. Plant Physiol., 125: 1094-1102.

Shah, A. (2004). Identification of the antibacterial component of an ethanolic extract of the Australian medicinal plants. Eremophila duttonii.Phytother. Res., 18: 614-615.

Shanjani, P. S. (2003). Nitrogen effect on callus induction and plant regeneration of *Juniperus excelsa*. J. Agri. Biol., 4: 419-422.

Shain-Dow, K. and Shang-FA, Y. (1999). Discoveries in Plant Biology. 1st Edition. World Sci., 2:10-11.

Sharma, J. R.; Lal, R. K.; Misra, H. O.; Gupta, M. M. and Ram, R. S. (1989). Potential of gamma radiation enhancing the biosynthesis of tropane alkaloids in black henbane (*Hyoscyamus niger* L.). Central institute of Medicinal and aromatic plants, Lucknow, India. Euphytica J., 40: 253-258.

Shelef, L. A.; Naglik, O. A. and Bogen, B. W. (1980). Sensitivity of some common food borne bacteria to the spices sage, rosemary and all spice. J. of Food Sci., 45: 1042-1044.

Shetty, K. and Labbe, R. G. (2001). Elite herb extracts containing high rosmarinic acid and inhibition of *Listeria monocytogenes*. Dept. Food Science. University of Massaxchusetts, Amherst.

Shetty, K.; Carpenter, T. L.; Kwok, D.; Curtis, O. T. and Potter, T. L. (1996). Selection of high phenolic containing clones of thyme (*Thymus vulgaris* L.) using *Pseudomonas* sp. J. Agric. Food Chem., 44: 3408-3411.

Shibamato, T. (1987). Retention Indices in Essential Oils Analysis. In Sandra, P. and Bicchi, C. (eds). Capillary Chromato. In essential oil Analysis. Dr. Alfred Heuthing Veriag, Heidelberg, 259-274.

Shilpa, R. and Jayabaskaran, C. (2007). UV-B-induced signaling events leading to enhanced-production of catharanthine in *Catharanthus roseus* cell suspension cultures. BMC Plant Biol., 7(61): 9-10.

Simon, J. E. (1990). Essential Oils Culinary Herbs. In: J. Janick and Simon (eds.), Advance in new crops. Timber Press. Portland, 472-483.

Singh, P. (1996). Essential of Plant Breeding. 1ed. Kalyani Publisher, New Delhi, India.

Skoog, F. and Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissue cultured *in vitro*. Symp. Exp. Biol., 11: 118-130.

Smita, S. and Ashok, K. S. (2007). Hairy root culture for mass-production of high-value secondary metabolites. Critical reviews in Biotechnol., 27: 29-43.

Smith, M.; Perry, G. and Pryor, W. (2002). Causes and consequences of oxidative stress in Alzheimer's disease(1, 2). Free radic. Biol. Med., 32: 1048-1049.

Suhr, K. I. and Nielsen, P. V. (2003). Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. Biocentrum-DTU. Technical University of Denmark, Kgs. Lyngby, Denmark.

Su, W. W. and Humphrey, A. P. (1995). Production of rosmarinic acid in high density perfusion cultures of *Anchusa officinalis* using high sugar medium. Biotechnol. Lett., 12: 793-798.

Svoboda, P. and Hampson, J. (1999). Bioactivity of essential oils of selected temperate aromatic plants: antibacterial, antioxidant, anti-inflammatory and other related pharmaceutical activities. Plant Biology Department. SAC Auchincruire, Ayr, Scotland.UK.

Swagata, R. and Jha, S. (2001). Production of withaferin A in shoot cultures of *Withania somnifera.*Planta Med., 67: 432-436.

Takeda, R. and Katoh, K. (1981). Growth and sesquiterpenoid production by *Calypogeia granulata* Inouse cells in suspension culture. Plant., 151: 525-530.

Tatiana, O.; Ecaterina, T. and Doina, A. (1999). Somatic embryogenesis in lavender tissue culture: Influence of medium composition and gamma irradiation on embryo development. J. of Herbs, Spice and Medicinal, Plants. India. 6(3): 89-99.

Thimmaraju, R.; Bhagygyalakshmi, N.; Venkatachalam, L.; Sreeghar, R. and Ravishanker, G. (2006). Elicitation of peroxidase activity in genetically transformed root cultures of *Beta vulgaris* L. Electronic J. of Biotechnol., 9(5): 0717-3458.

Thorsen, M. A. and Hildebrandt, K. S. (2003). Quantitative activity and phenolic compounds in selected herbs. J. Agric Food Chem., 49: 5165-5170.

Timothy, L. W. and Nathalie, L. (2006). Plant extracts containing caffeic acid and rosmarinic acid inhibit zoospore germination of *Phytophthora* spp. Pathogenic to Theobroma cacao. Europ. J. of Plant Pathol., 115: 377-388.

Torres, K. C. (1988). Tissue Culture Techniques for Horticultural Crops. 1st Edition. Van Nostrand Reinhold, New York, 73.

Torre, J.; Lorenzo, M. p.; Martinez-Alcazar, M. P. and Barbac, C. (2001). Simple high performance liquid chromatography method for alpha-tocopherol measurement in *Rosmarinus officinalis* leaves. New data on alpha-tocopherol content. J. Chromatog., 15,919(2): 305-311.

Toth, J.; Mrlianova, M.; Tekelora, D. and Korenova, M. (2003). Rosmarinic acid, an important phenolic active compound of lemon balm (M*elissa officinalis* L.). Acta Facultatis Pharmaceuticae. Unversitatis Comenianae. Tomus.

Triataphyllou, K.; Blekas, G. and Boskou, D. (2001). Antioxidative properties of water extracts obtained from herbs of the species Lamiaceae. Int. J. Food. Sci. Nut., (52): 313-317.

Troncoso, N.; Sierra, H.; Carvajal, L.; Delpiano, P. and Gunther, G. (2005). The high performance liquid chromatography and Ultraviolet-Visible quantification of principle phenolic antioxidants in fresh rosemary. J. Chromatogr., 1100(!): 20-25.

Ulbrich, B.; Weisner, W. and Arens, H. (1985). Large-scale Production of Rosmarinic Acid from Plant Cell Cultures of *Coleus blumei* benth. In; Primary and Secondary Metabolism of Plant Cell Cultures (Neumann, K. H.; Barz, W. and reinhard, E., Eds.), Springer, berlin, 293-303.

Ushiyama, K. (1991). Callus Induction and Maintenance. In " Plant Cell Culture in Japan" Komamine, A., M., DiCosmo, F. CMC Co. Tokyo, Japan,: 92-98.

Valero, M. and Salmeron, M. C. (2003). Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. Int. J. of Food Microbiol., 85: 75-81.

Varro, E. T. and Lynn, R. B. (1988). Pharmacognocy. 9th edition. Lea and Febiger. Philadelphia. 104-107.

Withers, L. A. and Anderson, P. G. (1986). Plant Tissue Culture and its Agricultural Applications. London, Butterm. worths.

WHO, (1978). The promotion and development of traditional medicine. Technical report series, 622.

Xu, H.; Kim, Y. K.; Jin, X.; Lee, S. Y. and Park, S. U. (2008). Rosmarinic acid biosynthesis in callus and cell cultures of A*gastache rugosa* Kuntze. J. of Med. plant Res., 2(9): 237-240.

Yamada, Y.; Hara, Y.; Katagi, H. and Senda, M. (1980). Protoplast fusion. Effect of low temperature on the membrane fluidity of cultured cells. Plant Physiol., 65: 1099-1102.

Yang, R.; Potter, T. P.; Curtis, O. F. and Shetty, K. (1997). Tissue culture-based selection of high rosmatrinic acid producing clones of rosemary (*Rosmarinus officinalis* L.) Using *Pseudomonas* strain. Food Biotechnol., 11(1): 73-88.

Yeoman, M. M. and Macleod, A. J. (1977). Tissue Culture Techniques. In: Street H. E. (ed). Plant Tissue and Cell Culture. Blackwell Scientific Publisher. Oxford.

Yujie, F.; YuanGang, Z.; LiYan, C.; XiaoGuang, S.; Zhe, W.; Su, S. and Thomas, E. (2007). Antimicrobial activity of clove and rosemary essential oils alone and in combination. Northeast Foresty University, Harbin. Phytother. Res., 21(10): 989-994.

Zelic, B.; Hadolin, M.; Bauman, D. and Vasic-Racki, D. (2004). Recovery and purification of rosmarinic acid from rosemary using electrodialysis. Acta Chim, Slov., 52: 126-130.

Zeng, H. H.; Wang, H.; Zhou, K.; Tu, P. F.; Wang, B. H. and Lu, J. F. (2002). The State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing.

Zenk, M. H.; El-Shagi, H. and Ulbrich, B. (1977). Production of rosmarinic acid by cell suspension cultures of *Coleus blumei*. Nat. Wissen Schaften, 64: 585-588.

Zenk, M. H. (1978). The Impact of Plant Cell Culture on Industry. In, Thorpe, T. A. (ed). Frontiers of Plant Tissue Culture, University of Galgary. Int. Ass. for PTC, pp: 1-13.

Zhao, J.; Zhu, W. H.; Hu, Q. and Guo, Y. Q. (2001). Compact callus cluster and suspension cultures of *Catharanthus roseus* with enhanced indole alkaloid biosynthesis *in vitro.* Cell Dev. Biol. Plant, 37: 68-72.

الملخص

درست انتاجية نبات اكليل الجبل *Rosmarinus officinalis* L. من الزيوت الاساسية وحامض الروزمارينك. كانت انتاجية نسيج الكالس متميزة مقارنة مع انتاجية النبات الكامل. حفز نسيج الكالس على النشوء من اجزاء الورقة واديم على وسط MS مجهز بتوليفة من داي كلوروفينوكسي اسيتك اسد (2,4-D) وبنزل ادنين (BA) والكاينتين (KIN).

سجلت اعلى نسبة نشوء للكالس (100%) من زراعة اجزاء الورقة عند اضافة توليفة من ٢ ملغم/ لتر 2,4-D و 0.5 ملغم/ لتر BA، تلتها 90% مع التوليفة ٢ ملغم / لتر 2,4-D و 0.1 ملغم/ لتر KIN الى الوسط الغذائي ماراشيج وسكوج (MS).

تم الحصول على اعلى وزن طري للكالس من التوليفة 2 ملغم/لتر 2,4-D و 0.5 ملغم/ لتر BA تحت فترة اضاءة 16/8 ساعة حيث وصل 1780 ملغم، بينما سجلت 1442.5 ملغم من التوليفة 2 ملغم /لتر 2,4-D و 0.1 ملغم / لتر KIN.

عوملت مزارع الكالس بالمؤثرات الحيوية (بكتيرية، فطرية وخلاصة الخميرة) والمؤثرات اللاحيوية (كلوريد الكالسيوم واشعة كاما). اضيفت البادئات (سكروز، فينايل النين و تايروسين) الى مزارع الكالس المنمى على وسط الادامة MS.

اجري التحليل الكمي والنوعي للزيوت الاساسية باستعمال كروماتوكرافيا الغاز GC-MS، كانت المكونات الرئيسية للزيوت الاساسية لاوراق نبات اكليل الجبل: camphor (20.54%)، cineole (13.40 %)، verbenone (12.71%)، bornyl acetate (12.22%)، carene (7.73%)، camphene (4.32%)، α-pinene (2.28%) وβ-pinene (1.77%)، بينما كانت المكونات الاكثر ظهوراً في المستخلص الكحولي للأوراق هي: isocarnosol (5.85%)، camphor (3.88%)، ferruginol (4.53%)، cineole (2.66%)، verbenone (2.50%) و borneol (1.56%). واظهرت نتائج تحليل المستخلص الكحولي للكالس المكونات الرئيسة: cineole (4.44%)، camphor (2.51%)، verbenone (2.30%)، borneol (1.91%)، ferruginol (1.59%)، isocarnosol (1.27%)، bornyl acetate (1,66%)، phytol (9.12%) و quinoline (16.78%).

كان المؤثر الفطري (*Fusarium oxysporum*) عند التركيز $2x10^4$ مستعمرة/ مل من بين المؤثرات الحيوية واللاحيوية الاكثر تأثيرآ في تحفيز المنتجات الثانوية من كالس نبات اكليل الجبل يليه المؤثر البكتيري (*Pseudomonas aeruginosa*) عند التركيز $1.5x10^5$.

اظهر اضافة السكروز بتركيز ٤٠غم/لتر والفينايل النين (Phe) والتايروسين (Tyr) بتركيز 0.1 ملغم/لتر لكل منهما كبادئات الى وسط زراعة الكالس زيادة في الـ cineole والـ camphor.

التحليل باستعمال HPLC للحوامض الفينولية (حامض الروزمارينك RA، حامض الكافيكCAF) والتربينات الفينولية (حامض الكارنوسك CA، الكارنوسول CAR والروزمانول ROL) لمستخلصات الورقة والكالس اظهر ان انتاج RA، CAF، CA و CAR قد وصل الى 4.5، 2.7، 3.3 و2.8 مايكروغرام/لتر على التوالي عندما اضيف *F. oxysporum* عند التركيز 2 مل/لتر، عدا الروزمانول ROL الذي وجد بمستوى عالي (4.3، 4.2 و 4.6 مايكروغرام/ لتر) فقط في مستخلص الاوراق والكالس غير المعامل والكالس المعامل بكلوريد الكالسيوم بتركيز 0.4 غم/ لتر على التوالي.

فحصت الفعالية البايولوجية لمستخلصات الاوراق الايثانولية ضد ثلاث عزلات بكتيرية (*Escherichia coli*، *P. aeruginosa* و *Bacillus Cereus*) وعزلتان فطرية (*Aspergillus nigar*، *F. oxysporum*). بينت النتائج بأن المستخلص الايثانولي كان مؤثرآ ضد جميع الاحياء المجهرية المختبرة. كانت قيم اقل تركيز مثبط MIC 320 مايكروغرام/مل لـ *E. coli* و640 مايكروغرام/مل لـ *P. aeruginosa* و ١٦٠ مايكروغرام /مل لـ *B. cereus* . كما وجد ان *P. aeruginosa* هي اكثر انواع البكتريا مقاومة.

اما قيم MIC للمستخلص الايثانولي ضد *F. oxysporum* و *A. nigar* فكانت ١٦٠ و320 مايكرغرام/مل على التولي. وان *F. oxysporum* كان اكثر تحسسآ مقارنة مع *A. nigar*.

بسم الله الرحمن الرحيم

اوَاتِ وَالأَرْضِ مَثَلُ نُورِهِ كَمِشْكَاةٍ فِيهَا
بَاحُ فِي زُجَاجَةٍ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ
جَرَةٍ مُّبَارَكَةٍ زَيْتُونِةٍ لاَّ شَرْقِيَّةٍ وَلاَ
يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ نُّورٌ عَلَى
نُورٍيَهْدِي اللَّهُ لِنُورِهِ مَن يَشَاءُ وَيَضْرِبُ اللَّهُ الأَمْثَالَ
لِلنَّاسِ بِكُلِّ شَيْءٍ عَلِيمٌ

سورة النور الآية ٣٥

صدق الله العظيم

جمهـــورية العـــــراق
وزارة التعليم العالي والبحث العلمي
جامـــعة النهـــــــرين
كلية العـــــــــلوم
قسم التقانة الاحيائية

دراسة تاثير العوامل الحيوية واللاحيوية في تحفيز الزيوت الاساسية وحامض الروزمارينك في نبات اكليل الجبل *Rosmarinus officinalis* L. خارج الجسم الحي

اطروحة

مقدمة الى كلية العلوم، جامعة النهرين كجزء من متطلبات نيل درجة دكتوراه علوم في التقانة الاحيائية

من قبل

خليل ابراهـــيم رشـــيد المفرجي

ماجستير تقانة حيوية /٢٠٠٥ /الكلية التقنية المسيب

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آذار 2010

ربيع الاول1431