Abstract:

In an attempt to increase the production of some secondary metabolites in tissue cultures of *Salvia officinalis* compared to the intact plant, several experiments were carried out. Callus was induced and maintained on MS medium supplemented with 0.5 mg/l kinetin and 0.05 mg/l 2,4-D from leaf and stem explants. NaCl was added to the culture medium at concentrations 50 or 100 mM as a stress agent for elicitation.

Gas chromatography technique was used to identify and quantify the compounds. Results showed that α-pinene increased more than four folds in callus cultures initiated from leaf and grown on a medium containing 100mM NaCl compared with the same explant excised from the intact plant. The above mentioned medium also increased apigenin and linalool production more than three folds. Rutin increased up to 2.5 times in cell suspension cultures initiated form stem explants. Other compounds such as geraniol, quercetin and coumarin increased at different ratios using tissue culture systems. Alkaloids and steroids were not detected neither in intact plant nor tissue cultures.

Water and ethanolic extracts produced from samples that gave the highest level of secondary metabolites were investigated for their antimicrobial activity.

Ethanolic extract of callus initiated from leaf explants and grown on a medium supplemented with 100mM NaCl, revealed the highest

antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* and to a lesser extent to *Pseudomonas aeriginosa* and *Bacillus ceries.*

الملخص:

في محاولة لزيادة انتاجية المركبات الثانوية في المزارع النسيجيه لنبات السالفيا *Salvia officinalis* تم تنفيذ عدد من التجارب لهذا الغرض.

استحث الكالس و أديم على وسط MS المجهز ب ٠,٥ ملغم/لتر من الكاينيتين و ٠,٠٥ ملغم/لتر من 2,4–D باستعمال اجزاء الورقة أو الساق كمصادر لنشوء الكالس.

أضيف ملح كلوريد الصوديوم NaCl الى الوسط الغذائي كعامل اجهاد بتركيز ٥٠ أو ١٠٠ ملمولر.

استخدمت تقنية كروموتوغرافيا الغاز في تشخيص و تقدير كميات المركبات الثانوية. أوضحت النتائج زيادة مركب α-pinene الى اكثر من أربعة اضعاف في أنسجة الكالس الناشئ من نسيج الورقه و المنمى على وسط غذائي حاو على ١٠٠ملمولر من ملح كلوريد الصوديوم مقارنة بنفس الجزء النباتي المستأصل من النبات الكامل. كما لوحض و باستعمال الوسط اعلاه زيادة مركبي linalool و apigenin الى اكثر من ثلاثة اضعاف. أما مركب Rutin فقد ازداد الى أكثر من ضعفين و نصف في مزارع المعلقات الخلويه الناشئه اصلا من أجزاء الساق. و ازدادت المركبات الاخرى المدروسه، coumarin, quercetin, geraniol بنسب مختلفه في الأجزاء المكثره نسيجيا. بينما لم يكشف عن وجود القلويدات و الستيرويدات في النبات الكامل وانسجته المزروعه خارج الجسم الحي.

اجري الاستخلاص المائي و الكحولي من عينات الزراعه النسيجيه التي اعطت اعلى انتاجيه من المركبات الثانويه و اختبرت فعالية المستخلص ضد بعض انواع البكتيريا.

كما وأوضحت النتائج بأن المستخلص الكحولي لانسجة الكالس الناشئ من اجزاء الورقه المنمى على وسط MS المجهز ب ١٠٠ ملمولر من ملح كلوريد الصوديوم قد أعطى أعلى فعاليه تثبيطيه في نمو أنواع البكتيريا *Staphylococcus aureus* و*Escherichia coli* و بنسبة تثبيط أقل لل *Pseudomonas aeriginosa* و *Bacillus ceries* .

وزارة التعليم العالي و البحث العلمي
جامعة النهرين
كلية العلوم

دراسة بعض نواتج الايض الثانوي خارج وداخل الجسم الحي في نبات السالفيا *Salvia officinalis* و تأثيرها في تثبيط نمو بعض انواع البكتيريا

رسالة

مقدمة الى كلية العلوم في جامعة النهرين

و هي جزء من متطلبات نيل درجة الماجستير علوم

في علوم التقانة الاحيائية

من قبل

اسراء ازهر سلمان الجبوري

بكالوريوس تقانة احيائية-كلية العلوم-٢٠٠٣

جامعة النهرين

تشرين الأول 2006

رمضان ١٤٢٧

Committee certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

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Name:

Member/ Supervisor

I hereby certify upon the decision of the examining committee

Signature:

Name:

Title:

Address: Dean of the College of Science

Date:

Supervisor Certification

I, certify that this thesis was prepared under my supervision in Al-Nahrain University/ College of Science/ Department of Biotechnology as a partial requirement for the degree of Master of Science in Biotechnology.

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In review of the available recommendations, I forward this thesis for debate by the examining committee.

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Date:

1.2-Herbal medicine:

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

Herbs natural compounds have practical advantages with regard to availability, suitability for oral application, approval of efficacy and mechanism of action. The other advantage of the treatment with plant drug is the reduction of the side effects that often occur with the synthesized medicine. This may be due to the lower concentrations of the active compounds found in the plants that the human body would need(Tsuda *et al*., 2004).

1.3-Sage (*Salvia officinalis*):

Sage's Latin name *Salvia* is derived from the old Roman verb salvar which means save, cure, and *officinalis* also means medicinal. Latin words in the sage's name refer to cure and medicine. The plant is known and shows the appreciation since Roman times (Amr and Dordevic, 2000).

Salvia officinalis common names are: Sage, Garden Sage, Salvia, Meadow Sage, Salvia Virgen, True Sage, and Dalmatian Sage. The Sage belongs to the genus *Salvia* of labiatae family comprising about 220

genus and 4000 species most of them are important in medicinal and economic terms (Jamzad *et al*., 2003).

Sage is a large group of perennials and annuals. Plants grow (30-90) cm high and form branched clumps. Their leaves are ovate (2-6) cm long. The flowers form on spikes that grow above the foliage. Flowers may be white, blue, red, purple, pink, rose or violet (Retal, 1994).

Labiatae family is distributed in the Mediterranean and Irano-Turanian regions (Feinbrun, 1978). Geographically, the plant is distributed in Palestine, Lebanon, Syria, Crete, Cyprus, Turkey, Greece, Sicily, Iran and the South of Italy (Mouterde, 1970).

Robbers and Tyler (1999) considered this pant indigenous to Western Asia and Eastern Mediterranean region. The plants also found in South America, Southern Europe, Northern Africa and North America.

1.4-Sage History and Popular Uses:

In the first century, Greek physician (Dioscorides) reported that aqueous decoction of Sage stopped bleeding of wounds and cleaned ulcers and sores. He also recommended Sage juice in warm water for hoarseness, curing cough and for enhancing memory functions. Its use in traditional Greek medicine spread to India, where the dried leaf and fluid extract are used in traditional Indian and Unani medicines (Blumenthal *et al*., 2000).

In Chinese medicine, it was used in treating yin conditions such as weakness of the stomach, nerves and digestive system (Tyler, 1999).

In Germany, Sage is used as a standard medicinal tea to treat gastrointestinal catarrh and night sweats. The tea is also applied topically as a rinse or gargle for inflammations. Sage fluid extracts that contain essential oils are used in prepared medicines for mouth and throat and as gastrointestinal remedies (Leung, 1996).

In United States, Sage is used as a component of dietary supplement products for similar conditions, usually in aqueous infusion or alcoholic tincture dosage forms. Dry herb or dry extract is used as capsules and tablets. Sage was formerly official in the United State Pharmacopoeia from 1840 to 1900 as a gargle in inflamed sore throat (Boyle, 1991). Medicinal saying confirms the importance of the plant "Why should a man die while sage grows in his garden" (Chevallier, 1996). Folklore also said that sage would make women fertile. It was employed in ancient Egypt to increase the fertility of women (Schauenberg and Paris, 1990).

1.5-Chemical Composition:

Santos and Fernandes (2003) considered sage as a rich source of volatile oils, terpenes, tannins, flavonoids, glycosides and saponins. *Salvia* leaf contains tannins (catchin), phenolic acids including (rosmarinic acid, hydroxycinnamic acid, caffeic acid, chorogin acid,

ferulic acid and gallic acid), phenylpropanoids (e.g. coumarins), flavonols and flavons (e.g. apigenin, rutin, quercetin and luteolin derivatives) and saponins that have a wide treatment activity (Blumenthal *et al*., 2000; Ody, 1993).

The sage volatile oil components are: monoterpenes (e.g. α-pinene, β-pinene, camphene, linalool, limonene, geraniol, thujone and many other biologically important compounds) (Miladinovic, 2001), sesqueterpenes (e.g. α-humulene, β-pinene, caryophyllene and borneol) and diterpenes (e.g. manool) (Moujir *et al*., 1993, Anonymous, 1997).

Tzakou *et al*., (2001) isolated seventy-five compounds from the volatile oils of sage with 1, 8-cineole and alpha-pinene as the major constituent. El-Sayed *et al*., (2001) detected and identified twelve hydrocarbons, three triterpenes, nineteen fatty acids, two phenolic acids and five flavonoids of medicinal importance in sage; also sage contains vitamins like (Vit. C and Vit. E), rich with calcium and potassium elements.

It was found that *S. officinalis* roots have royleanon and its derivatives in addition to cryptotanshinone (Brieskorn and Buchberger, 1973). Masterova *et al*., (1996) found that sage roots have diterpene quinones. Al-Rahowi (2004), measured the protein content of fresh *Salvia* leaves which was 0.14%. Chemical investigation of sage natural products showed no alkaloids or steroids (Al-Khaphagi, 2000). The sagerinic acid, salvianolic acid and rosmarinic acid were isolated by Lu

and Foo (1999). They isolated three structurally related caffeic acid trimmers in the methanolic extract of sage.

1.6-Biological Activity:

In current herbal medicine, sage is used to treat indigestion, inflammation of the throat and effective in improvement of patients suffering from anxiety disorders (Barnes *et al*., 2002).

Salvia species are used to relieve gastric disorders, abdominal pain, gum and tooth pains, nervous conditions and diabetes (Abu-Ramaileh and Afifi, 2000; Ali-Shtayeh *et al*., 2000; Salah and Jager, 2005).

Sage high antioxidant activity was reported by Partico and Delanty, (2000); Pizzule *et al*., (2002); Ozcan (2003).

The extract of sage exerts antimutagen effects (Gimsey, 1996). *S. officinalis* water extract reduces DNA damage caused by H_2O_2 (Al-Rahowi, 2004).

It was suggested that oil extracted from sage plant has potent suppressive activities against tumor promotion in mouse skin and thus could be an effective chemo-preventive agent against skin cancer (Gali-Muhtasib and Affara, 2000).

Sage is considered today to be relevant to the treatment of Al-zheimer's disease (AD) that is characterized by loss of short term memory (Perry *et al*., 2001). Also used for treating hyperthyroidism (Eric and Kathy, 2006). Radtke *et al*., (2003) examined a series of sage phenolics for activity against Leishmania parasites. Sage can be applied

for pains in joints, dislocation, typhoid fever (Mabez, 1988). Perspiration reduction, ulceration and in the treatment of skin abrasion, germs, intestine worms and rheumatism (Reverned, 1998). In the form of tea, it is effective in diarrhea and flatulence, kidney disturbances, lungs and stomach haemorrhagia (Dobrynin *et al*., 1976).

Sage tea also used as analgesic (Golshani *et al*., 2004). Ceres (1984) reported that sage has been used for wasp stings and insect bites.

Sage used for worts on legs. The use of sage also it has been recommended for varicose veins and leg ulcer (Buchman, 1987). Sage also used as a lotion for wounds, easing muscular pain, sciatica and for loosing stiff and painful joints (Back, 1987). Sage also effective against fever (Reverned, 1998), asthma and trouble in blood circulation (Sadraei *et al*., 2003), release of excessive mucus from respiratory system (Velickovic *et al*., 2002).

Garden sage has value as a spasmolytic, antiseptic and is used in a variety of complains, the most relevant being inflammation of the mouth, tongue and throat, as a gargle or mouth wash, also has been recommended for use in bad breath (Buchmann, 1987).

S. officinalis shows a wide range of antimicrobial activity (Baricevic *et al*., 2001; Creaven *et al*., 2004). The sage oil showed a very strong activity against G-ve and G+ve. bacteria except *Pseudomonas aeruginosa* (Velickovic *et al*., 2001). Also a significant activity against some tested fungi such as C*andida albicans, Candida voginalis and Torulopsis glabrate* (Tzakou *et al*., 2001).

It was discovered that aerial plant parts have effect on both G+ve and G-ve bacteria except *Pseudomonas aeruginosa*, hypotensive properties, central nervous system depressant action and antispasmdic activity (Newall *et al*., 1996). Monoterpene exhibits virocidal activity against the herps simplex virus (Armaka *et al*., 1999), also against herps labialis (Saller *et al*., 2001). Geraniol and other terpenes are responsible for antimicrobial activity of essential oils (Kalinkina *et al*., 1998). Linalool is therapeutically effective since it acts as a sedative. Diterpenes also exhibit antiviral activity (Tada *et al*., 1994) and wide antibacterial effect (Ulubelen *et al*., 2001).

The antimicrobial properties of sage tannins made it of benefit in reduction the plaque growth, the inhibition of gingival inflammation and have positive effect on prophylaxis patients (Willershausen *et al*., 1991).

Ethanolic extracts of *S. officinalis* showed activity against *Candida albicans* (Al-Suhaily, 2002). Tada *et al*., (1994) Reported antiviral activity of two diterpenoids, isolated from the aerial parts of *Salvia officinalis*.

The glycosidic compounds especially (caffeic acid) have antioxidant effect and anti-free radicals (Wang *et al*., 2000). Rosmarinic acid has antioxidative effect (Lamaison *et al*., 1991). The apigenin, anticarcinogenic flavonoid, showed its interference with cell proliferation and survival (Czyz *et al*., 2005). Rutin is another flavonoid type has anti-oxidant activity, anti-inflammatory agent, decreases capillary fragility and eczema treatment (Tadera *et al*., 2003). Quercetin

displays a variety of biological activities, including cardiovascular protection, anti-cancer activity and anti-inflammation (Bronner, 1985; Kim, 1998).

1.7-Plant Secondary Metabolites:

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

Primary metabolites (proteins, carbohydrates and fats) are important in plant physiological process such as growth and development (Mann, 1987).

Synthesis of various classes of secondary metabolites from primary metabolites is presented in fig. (1). Acetyl co-enzyme and mevalonic acid play a key role in the synthesis of various terpenoids (Ramawat, 2004), Phenolic compounds are formed via the shikimic acid pathway or malonic acid pathway. The nitrogen-containing secondary products such as alkaloids are biosynthesized primarily from amino acids (Taiz and Zeiger, 2002).

The importance of secondary metabolites in plants is: firstly to protect the plants against being eaten by herbivores and against being infected by microbial pathogens. Secondly, they serve as attractants for pollinators. and thirdly, as agents of plant plant competition.

Secondary metabolites are divided into three chemically distinct

groups: Terpenes, Phenolics, and Nitrogen-containing compounds (Oomah, 2003).

1.7.1-Phenolic Compounds:

In (2002), Taiz and Zeiger reported that the term phenolic compounds includes a wide range of plant substances which posses in common an aromatic ring bearing one or more hydroxyl substituents. Some phenolic substances tend to be water-soluble carboxylic acids and glycosides, some are soluble only in organic solvents and others are large insoluble polymers, since they most frequently occur in combination with sugar as glycosides. Among natural phenolic compounds, the flavonoids form the largest group monocyclic phenols, phenylpropanoids and phenolic quinones.

The major group of phenolic compounds we deal with in this study is: Flavonols and Flavones.Flavonols are very widely distributed in plants with a considerable range of flavonol glycosides. More than a hundred different glycosides of quercetin have been described. The most common flavones are apigenin and luteolin (Harborne, 1973).

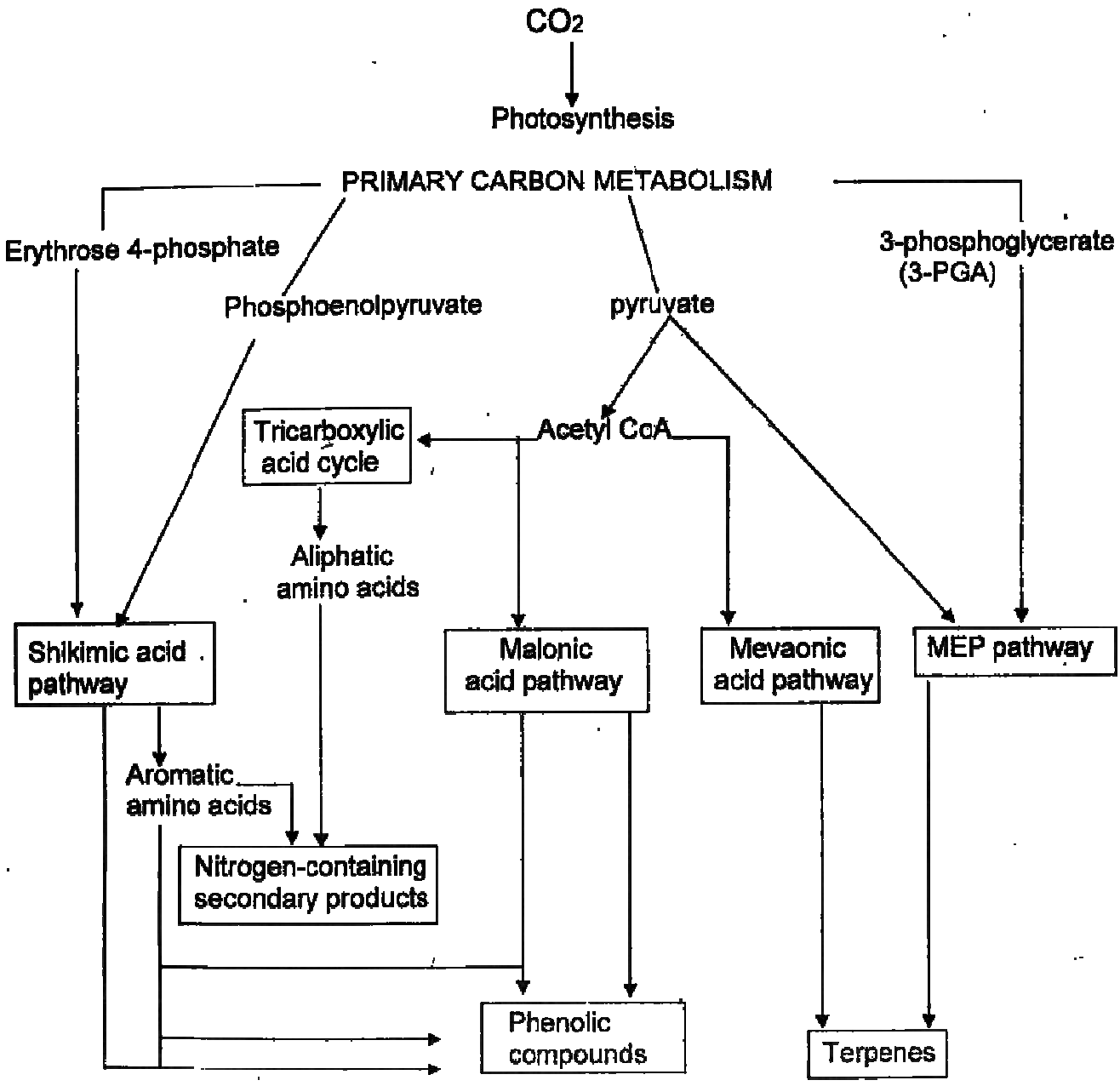


Fig. (1): Pathway of the major secondary metabolite classes. (Taiz and Zeiger, 2002).

1.7.2-Terpenoids and Essential Oils:

Terpenoids are based on the isoprene molecule and built from the union of two or more of C_5 units. This forms a skeleton on which side groups are attached to give rise to the many different volatile oil compounds. The properties of the volatile oil are determined by the number of isoprene units and by the different side groupings that may be attached (Cabrera, 2001).

Terpenoids are classified according to whether they contain two (C_{10}), three (C_{15}), four (C_{20}), six (C_{30}) or eight (C_{40}) units. They range from the essential oil components, the volatile oil monoterpenes and sesqueterpene (C_{10} and C_{15}), through the less volatile diterpenes (C_{20}) to the involatile triterpenoids and sterols (C_{30}) and carotenoid pigments (C_{40}). Some terpenes occur in the glycosidal (sugar-linked) form (Trease and Evans, 1989).

Chemically, the terpene essential oil can be divided into two classes, the mono- and sesquiterpenes, C_{10} and C_{15} isoprenoids (Harborne, 1984), thus monoterpenoids are hydrocarbons consisting of two isoprene units.

They are the most common type of terpene in volatile oils and may be either cyclic or acyclic (e.g. geraniol, linalool).

Cyclic monoterpenes can be mono-cyclic (limonene) or bi-cyclic (α- and β-pinen) and various side chains may be added to form the alcohols, ketones, aldehydes, esters and so on (Price and Price, 1995). Stumpf and Conn (1981) reported the most natural ‘terpenoide’ has cyclic structure with one or more functional group (e.g. hydroxyl, carbonyl).

The functional group of sage volatile oil is ketone, which is characterized by a side group formed from oxygen double bonded carbon (Cabrera, 2001).

1.7.3-Nitrogen Compounds:

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

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

1.8- *In vitro* Approaches For Production of Secondary Metabolites:

The production of secondary metabolites *in vitro* is possible through plant tissue culture (Barnum, 2003). Karam and his colleagues (2003) studied the accumulation of rosmarinic acid (RA) in *S. fruticosa* callus, cell suspension and root cultures.

Large scale production of phytochemicals with plant cell and tissue culture technologies has the following advantages: controlled environmental factors (climate, pests), no geographical and seasonal constraints, defined production systems and when required, more

consistent product quality and yield, continuous and homogenous supply of plant material in a uniform physiological state, use of recombinant DNA technologies for yield improvement, production of novel compounds *in vitro*, which are absent in the parent plant material and the ability for large-scale cultivation of cells and organs in bioreactors for easier and higher product recoveries (Zafar and Datta, 1992).

1.8.1-Callus Cultures:

Callus cultures consist of an undifferentiated, proliferating mass of cells usually arising on wounds of differentiated tissues. Generally, plants which accumulate relatively high yields of specific secondary metabolites, give rise to tissue cultures producing high levels of secondary metabolites and visa verse (Lindsey and Yeoman, 1985). Table (1) represents some medicinal plants that produce secondary metabolites at high levels by plant cell cultures.

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

There are two phases during callus formation, “wound response” this phase is characterized by a rapid increase in metabolic activity but

does not lead to callus formation, and "growth response" this phase results in cell division and dependent upon exogenous supply of cytokinin and may be auxin.

Sateesh (2003) divided callus growth to:

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

1.8.2-Suspension Cultures

When callus clumps are dispersed in moving liquid media, this is known as cell suspension cultures that are characterized by faster and uniform growth rates coupled with secondary metabolite production (Parr, 1989).

This dispersed culture grows in a form of curve (Street, 1977) and goes through a number of phases such as lag phase (absence or very low rate of cell division, increase of protein synthesis and low level of secondary metabolites), log phase (a short period of exponential cell division, increase in average cell size, increase in nucleic acid content,

Table (1): Secondary products found in callus and cell suspension cultures in concentrations more than intact plants (combined from Endress, 1994; Stockigt *et al*., 1995).

Plant species	Metabolites	*In vitro* Culture (%dry wt.)	Intact Plant (%dry wt.)	Culture Type
Cassia tora	Anthraquinones	0.33	0.21	c
Catharanthus roseus	Ajmalicine	2.2	0.30	c
	Serpentine	1.8	0.5	c
Coleus blumei	Rosmarinic acid	27.0	3.0	c
Coleus blumei	Rosmarinic acid	15.0	3.0	s
Coptis japonica	Berberine	10.0	3.0	s
Lithospermum erythrorhizon	Shikonin	20.0	1.5	s
Morinda citrifolia	Anthraquinones	18.0	0.3	s
Panax ginseng	Ginsenosides	27.0	4.5	c
Trypterygium wilfordii	Triptolide	0.05	0.001	c

c = callus, s = suspension culture

increase in primary metabolites which are diverted into secondary metabolism) and stationary phase (period of decelerating cell division due to exhaustion of an essential nutrients, cessation of cell division and hence absence of growth, decrease in respiration and protein synthesis).

There are many types of suspension cultures that can be used for the production of secondary metabolites (Sateesh, 2003). These include:

1. Batch Culture:

Where the cell suspension culture is grown in a fixed volume of nutrient culture medium. This system also known as closed culture vessel within a single batch of medium. During the incubation, cell suspension increases in biomass by cell division, and cell growth continues until a factor in the culture environment (nutrients or oxygen availability) becomes limiting and then growth ceases.

2. Continuous Culture:

In this culture a continuous supply with nutrients by the addition of fresh medium but the culture volume remains constant. Here cells are continuously in the exponential growth phase and are also known as open system culture.

The continuous culture system may be of two types, chemostat and turbidostat.

A-Chemostat: In this system, growth rate and cell density are held constant by a fixed rate of input of a growth nutrient medium. In such a medium, all the constituents are present at a concentration required to

maintain the desired rate of cell growth. The growth limiting substance is so adjusted that it increases or decreases the growth rate of cells.

B- Turbidostat: In this system fresh medium flows in response to increase in the turbidity so as to maintain the culture at a fixed optical density of suspension. A preselected biomass density is maintained by the washout of cells.

1.8.3-Differentiated Cultures:

Secondary metabolites are also synthesized or stored in organized structures such as roots, shoots, stigmas, embryos, transformed roots and transformed shoots. These cultures can easily be exploited in bioreactors (Parr, 1989; Hahn *et al*., 2003).

A. Multiple shoot cultures. The synthetic capacity of dedifferentiated tissue often differs substantially from that of fully differentiated tissues, both qualitatively and quantitatively. The differing synthetic capacities are usually a direct result of differences in enzyme profiles which reflect the organ-specific expression of biosynthetic genes (Endress, 1994). The differentiated cultures often show biochemical (Flores and Filner, 1985) and genetic (Aird *et al*., 1988) stability and hence offer a predictable and high-productivity system which does not require extensive optimization.

B. Hairy root cultures. The *Agrobacterium rhizogenes* bacteria was shown to cause 'hairy root phenotype' in infected plants (White *et al.*, 1982). It inserts the root inducing plasmid into wounded tissue, causing the growth of very fine adventitious roots, so called "hairy-roots".

Hairy roots are characterized by highly branching growth on hormone-free media, morphological and chromosomal stability, productivity of secondary metabolites and spontaneous and frequent regeneration into entire plants (Christey, 1997; Tanaka, 1997). Because of these reasons they are favored over suspension cell cultures for secondary metabolite production.

The genetic transformation of *Pueraria phaseoloides* with *Agarobacterium rhizogenes* is a good example for the production of puerarin in hairy roots (Shi and Kintzioz, 2003).

1.8.4-Immobilized Cell Cultures:

Plant cells have been successfully immobilized in number of matrices either by entrapment in a polymeric network or by growth into performed structures. Cells are now commonly immobilized by entrapment in calcium alginate, potassium carrageenan or in agarose beads.

The main reason for the use of immobilized plant cells as an alternative to mass cell culture for the production of secondary products is the ability to reuse biomass, which is expensive to produce, by retention of the cells in the formation and recovery of product from the

medium, or to separate physically the cells from the medium and hence from products.

1.9-Strategies Adopted to Increase the Synthesis of Secondary Metabolites:

Cultured plant cells often produce reasonable quantities and different profiles of secondary metabolites when compared with the intact plant. The poor product expression in some cases attributed to the lack of differentiation in cultures (Whitaker, 1986). On the other hand, there are some cases that cultures over produce metabolites compared with the whole plant (Kurz and Constabel, 1998).

The choice of original plant material having high-yields of the desired phytochemical may be important in establishing high-yielding cultures (Deus and Zenk, 1982). Different strategies have been employed to increase secondary metabolite production, of these that have yielded the best results are:

1.9.1-Culture Medium.

Media components can play a vital role in stimulating the secondary metabolite production, e.g. many plant cells that grown in limited amount of nitrogen or phosphate gave enhanced yields of secondary metabolites.

Sucrose is the main carbon source of the most media. Increasing the

sucrose concentration caused an increase in metabolites (Zenk, 1977).

Cell suspension cultures of *Coleus blumei* produce maximum yield of rosmarinic acid (3.5g/l) when 5% of sucrose was added to the culture media but a sharp reduction up to (0.7g/l) occurred in media containing 3% sucrose only (Zenk *et al*., 1977).

1.9.2-Plant Growth Regulators.

Another important component of the culture media is the growth regulator (phytohormone). Auxins and cytokinins have shown the most remarkable effects on growth and productivity of plant metabolites. In general, an increase in auxin levels, such as 2,4-D led to decrease in the level of secondary metabolites (Kuang and Cheng, 1981). Kinetin is one of the most popular cytokinins, it was reported to stimulate production of an anti-tumor compound like tripdiolide (Misawa, 1985). While Lui and Staba, (1982) reported that GA_3 was also effective on plant cell cultures, the growth of callus of digoxin-producing plant, *Digitalis lanata*, was promoted by the addition of GA_3 into the media.

The suspension cultures of *Catharanthus roseus* initiated from stem and leaf explants on a medium containing NAA and kinetin has been established to increase secondary products formation by Zhao *et al*., (2001).

1.9.3-Physical Factors.

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

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

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

A temperature of (17-25) °C is normally used for induction of callus tissues and growth of cultured cells. But, each plant species may favor a different temperature. It was found that lowering the cultivation temperature to 10°C cause increased phospholipids in *Rauwolfia serpentine* (Yamada *et al*., 1980).

1.9.4-Addition of Precursors.

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

1.9.5-Elicitation.

The scientists reviewed the possible correlations between stress and secondary metabolism in cultured cells (Smith *et al*., 1987; Ravishankar, 1988; Zhao, 2001a and Zhao, 2001b). Microbial infection of plant cultures as well as intact plants often elicits the synthesis of specific secondary metabolites. Fungal pathogens are potential inducers of the secondary metabolites (DiCosmo and Tallevi, 1985).

Recent developments in phytochemical elicitation have shown that simple inorganic and organic molecules can induce product accumulation. Smith, (1987), reported substances stimulate secondary product accumulation including sodium chloride, potassium chloride, and sorbitol as well as abscisic acid. Processes such as these, employing

simple and cheap elicitors have much promise in industrial scale plant cell cultures.

Although the mechanism by which elicitors increase the productivity of secondary plant metabolites has not been elucidated, their stimulating activity is quite significant if an appropriate elicitor is chosen to stimulate synthesis of a particular product. However, the use of microbial elicitors may not be economical since an elicitor-producing microorganism should be cultivated in a fermentor separate from cocultivation of plant cells using another fermentor. The fermentation cost for an elicitor-producing microorganism is not always inexpensive. In this sense, simple and cheap compounds should be employed as elicitors.

2.1-Materials:

2.1.1- Apparatus and equipments:

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

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

2.1.2-Chemicals:

Chemicals	Company
Ethanol	BDH
Lead-acetate	BDH
Ferric chloride	BDH
Petrolium ether	BDH
Chloroform	BDH
Acetic anhydride	BDH
Sulphuric acid	BDH
Ammonia	BDH
Ammonium nitrate	Mall
Potassium nitrate	BDH
Sodium chloride	Fluka
Calcium chloride anhydrate	Fluka
Magnesium sulphate anhydrate	Fluka
Potassium phosphate monobasic	Fluka
Boric acid	Merk
Potassium iodide	Tetanal
Manganese sulphate.$4H_2O$	BDH
Zinc sulphate.$7H_2O$	BDH
Molybdic acid (sodium salt).$2H_2O$	BDH
Cupric sulphate.$5H_2O$	BDH

Chemicals	Company
Cobalt chloride.$6H_2O$	BDH
Sodium ethylene diamine tetraacetate	Fluka
Ferrous sulfate.7 H_2O	BDH
Thiamine.HCl	BDH
Nicotinic acid(free acid)	Kochligh
Pyrodoxine.HCl	BDH
Glycine	BDH
kinetin	BDH
2,4-diclorophenoxyacetic acid	BDH

2.1.3- Culture media:

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

2.2-Methods:

This study was carried out in the plant tissue culture laboratory, Biotechnology Department/ College of Science/ Al-Nahrain University during the period 1/10/2004 to 1/7/2006.

2.2.1-Plant Material:

Sage plants, *Salvia officinalis* (Labiatae) were purchased from local nurseries in 12cm clay pots. Some plants are grown directly in the soil (picture 1) for continuous supply of explants for tissue culture work and as a source for vegetative parts for intact plant studies.

Picture 1: Sage (*Salvia officinalis*) plant grown in a soil garden to maturity as a source for plant material used in this experimental work.

2.2.2-Sterilization of Explants:

Stem explants (1.5) cm long and (1.5) cm in diameter for leaf discs were excised, rinsed with tap water for 10 min. then transferred to laminar air flow-cabinet where submerged in 70% ethanol for 1 min., washed with sterilized DDH_2O, then rinsed with sodium hypochlorite at different concentrations (0, 0.6, 1.2 or 3)% for 3 or 5 min. Explants then rinsed with sterilized DDH_2O for three times. For each concentration 12 explants were used and the ends of each explant were cut to remove tissues affected by sterilization solution. The final length of stems was 1 cm and the final diameter of the leaf discs were 1 cm using a cork borer (Pierik, 1987).

2.2.3-Preparation of Culture Medium:

MS (Murashige and Skoog, 1962) medium was prepared and used (table 2). Sucrose 30000 mg/L, Myoinositol 100 mg/L and the plant growth regulators (2,4-D and kinetin) at different concentrations were added.

The pH was adjusted to 5.8 using NaOH or HCl (1N), then 7g/l of the agar type (Agar-Agar) was added to the medium, placed on a hot plate magnetic stirrer till boiling, then aliquots of 10 ml were despensed into (8 ×2.5) cm culture vesseles. The medium was left at room tempreture to cool and become ready to culture explants.

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

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

2.2.4-Plant Growth Regulators:

Different concentrations of the auxin 2, 4-D (0, 0.05, 0.1, 0.5 or 1.0) mg/l and the cytokinin kinetin (0.0, 0.1, 0.5 or 1.0) mg/l were prepared and added to the culture media as required before autoclaving.

2.2.5-Media and Instruments Sterilization

Culture media were sterilized by autoclaving at 121°C under (1.04 Kg/cm^2) pressure, for 15 min. while glassware and other instruments either by autoclaving or using electric oven (180-200) °C for 2 hrs (Cappuecino and Sherman, 1987).

2.2.6-Incubation of Cultures:

Surface sterilized explants (1) cm long were inoculated into the culture vesseles under aseptic conditions, placed in the incubator (Sanyo Electric Co., Ltd.) at 25°C for 16/8 hrs. light/dark photoperiod using day light inflorecents and light intensity of 1000 lux was used.

2.2.7-Initiation of Callus Cultures:

Different combinations of plant growth regulators were examined to determine the most effective one for callus induction. Explants were placed onto MS medium containing 2, 4-D and kinetin as in 2.2.4. Cultures were placed in the incubator. The response of these explants to auxin and cytokinin combinations was evaluated after 21 days in culture to determine the proper combination for callus induction.

2.2.8-Maintenance of Callus Cultures:

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

2.2.9-Cell Suspension Cultures:

Cell suspension cultures were initiated by placing 5 g callus pieces from stem or leaf explants origin into 100 ml of maintenance medium in 250 cm^3 flasks which placed on a rotary shaker at 100 rpm/min for 21 days. Suspension cultures were filtered and the pellet was oven dried at 40°C for 24 hrs. to be ready for use in GC work.

2.2.10-Callus Cultures with Elicitor:

Ten callus pieces (400 mg each) were placed on the surface of callus maintenance medium supplemented with (50 or 100) mM NaCl for 21 days, then dried at 40°C for 24 hrs. for extraction and analysis for the presence of secondary metabolites and for antimicrobial studies.

2.3-Collection of Samples:

Samples harvested from intact plant taken from stems and leaves, from callus and cell suspension cultures initiated from both stems and leaves, placed on a medium supplemented with or without elicitor (NaCl 50 or 100)mM. All these samples were oven dried at 40°C for 24 hrs.

(Bos, 1997), ground into a powder using a pestle and mortar, then subjected to ethanolic extraction.

2.3.1-Gas Chromatography Specifications:

Gas chromatography method was used for identification of compounds. The analysis was performed on GC-9A Shimadzu column supele Co wax 10 (15 ft × ⅛ 1n) stainless steel, internal diameter 2.1 mm, oven tempreture was programmed on 50°C for 2 min., then increased to 200°C with a rate of 2°C/min., helium flow rate 50 cm/sec., with flame ionization detector as specified by the manufacturer.

2.3.1.1-Standards Source:

Standards of terpenes and flavonoides were obtained from Aldrich Co. U.S.A., as pure stadards.

2.3.1.2-Separation and Identification by Gas Chromatography Method:

A sample of 0.1 mg of each dry weight was mixed throughly with 0.5 ml ethanol. The supernatant was separated and 10 µl was injected into the GC column and the retention time was determined. The area for each peak was calculated and compared with the known concentration of the prepared samples(Greenham, 2003). The concentration of each compound was calculated using the equation (Illiana and Alfermann, 1989):

$$\text{Conc. (mg/g)} = \frac{\text{area of sample}}{\text{area of standard}} \times \text{conc. of standard}$$

2.4-Preparation of Plant Extracts for Antimicrobial Activity:

The water and ethanolic extracts of callus that originally initiated from leaf grown on a medium supplemented with 100mM NaCl as elicitor (the sample that gave the highest quantity of secondary metabolites) were prepared for its antimicrobial activity as below:

2.4.1-Water Extraction:

The method used for intact plant extraction was used for callus extraction. The dried samples (50 g) were soaked with 250 ml DDH_2O. The mixture was left in a shaker incubator for 24 hrs. then filtered through a filter paper (Whatman no. 1). The filterate was concentrated using rotary evaporator at 40°C untill dryness.

2.4.2-Ethanolic Extraction:

A quantity of 50 g of callus powder was mixed with 250 ml ethanol by soxhlet apparatus for 8 hrs. at (40-60) °C. The solution then evaporated to dryness in an oven at 40°C (Harborne, 1973).

2.5-Detection of Some Active Compounds:

2.5.1-Detection of Tannins:

A quantity of (10) g of the plant powder was mixed with 50 ml DDH_2O using a magnatic stirrer. The mixture was left boiling in a water bath for few minutes, then filtered, and the filterate was treated with a few drops of 1% lead-acetate solution, the development of greenish-blue precipitate was an indication of the presence of tannins (Shihata, 1951).

2.5.2-Detection of Saponins:

A liquots of 5 ml plant extract was added to (1-3) ml of ferric chloride solution. A white precipitate was developed indicating the presence of saponins (Stahl, 1969).

2.5.3-Detection of Flavonoids:

Ethanolic extracts of the plant material was partitioned with petrolium ether; the aqueos layer was mixed with the ammonia solution. The appearance of dark color was an indication for the presence of flavonoids (Harborne, 1973).

2.5.4-Detection of Glycosides:

Equal amounts of the water extract was mixed with Fehling's reagent in a test tube, then boiled in a water bath for 10 min. The formation of red precipitate indicated the presence of glycosides (Shihata, 1951).

2.5.5-Detection of Terpenes and Steroids :

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

The appearance of dark blue color after few minutes indicated the presence of steroids in the extracts (Al-Abid, 1985).

2.5.6-Detection of Alkaloids:

A liquot of 0.2 ml of the water extract was throughly added to 5 ml of 1% HCl in a steam bath, then 1 ml of the filterate was treated with Mayer's reagent. The appearance of white precipitate was an evidence for the presence of alkaloids (Treas and Evans, 1987).

2.5.7-Detection of Coumarins:

A little amount of the dried water extract was dissolved in some drops of alcohol in a test-tube, covered with filter paper, sprayed with NaOH, then placed in a water bath till boiling, then the filter paper was placed under UV light spectrum. The appearance of greenish-yellow color indicated the presence of coumarins (Harborne, 1984).

2.6-Study of The Antimicrobial Activity:

The following microorganisms were isolated in the Dept. of Biotechnology, Al-Nahrain University and used in this study:

Staphylococcus aureus, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus ceries.*

2.7-Preparation of Bacterial Culture Media:

The bacteria were cultured on nutrient agar by mixing 20 g of agar with 8 g of the nutrient broth in a conical flask, then the volume was completed to (1) liter with DDH_2O (Tilton *et al.*, 1992).

2.8-Preparation of Antibacterial Samples:

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

The medium was mixed well and poured in petri-dishes. Five wholes with 5 mm in diameter were made using cork borer, then the plant extracts were added (100 µl/whole) at different concentrations. The inoculated plates were incubated at 37°C for 24 hrs. (NCCLs, 1993).

2.9: Statistical Analysis:

A completely randomized design (CRD) was used with 12 replicates. Least significant differences (LSD) were obtained to compare means at probability of 0.05%. In secondary metabolite quantification, means were calculated and standard errors were computed for three sample replicates (Gomes and Gomes, 1984).

3.1-Sterilization of Explants:

NaOCl was used for explants sterilization (stems and leaves) of *Salvia officinalis.* Fig.(2) shows that the most effective concentration of NaOCl was 3% for 3 min. That gave the highest percent (100%) of survival, increasing time to 5 min. caused damage to plant tissues, whereas lowering the concentration of NaOCl led to high rate of contamination. Most concentrations of NaOCl used for 5 min. reduced survival rate.

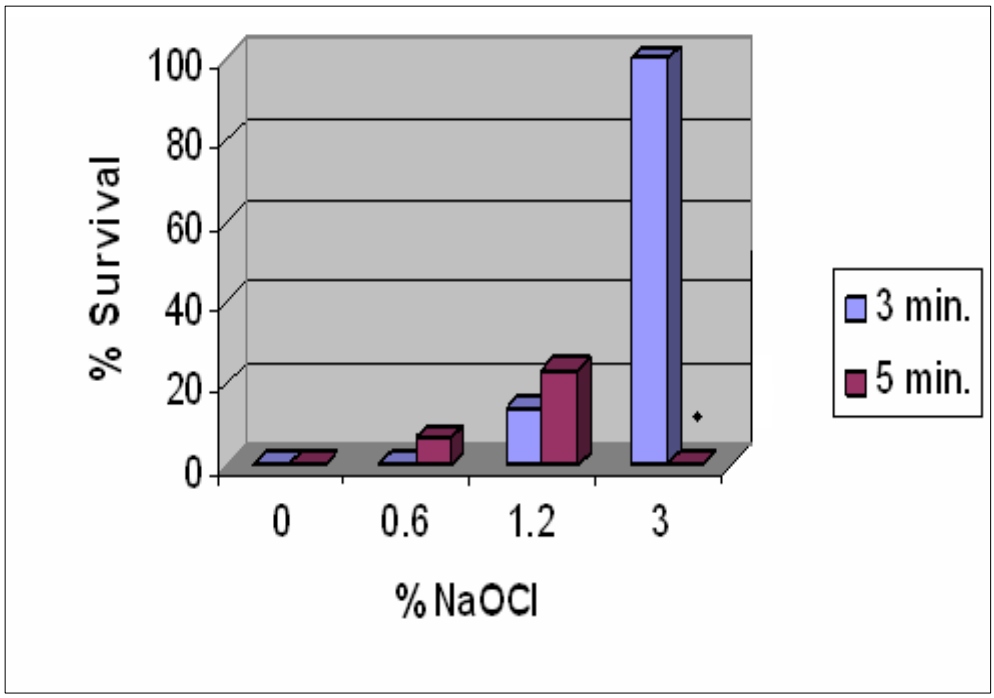


Fig. (2): Effect of different concentrations of NaOCl on explants survival at sterilization periods of 3 or 5 min. n= 12.
*** explants were seriously damaged by NaOCl.**

Using NaOCl was important to eliminate the contaminants. It is used widely for tissue sterilization. The selection of sterilizing material depends on the source of explants, roughness of its surface and other

factors. The sterilization material should be easy to remove from explants when washed with sterilized DDH_2O (Yeoman and Macleod, 1977; Sateesh, 2003).

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

3.2-Induction of Callus Cultures:

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

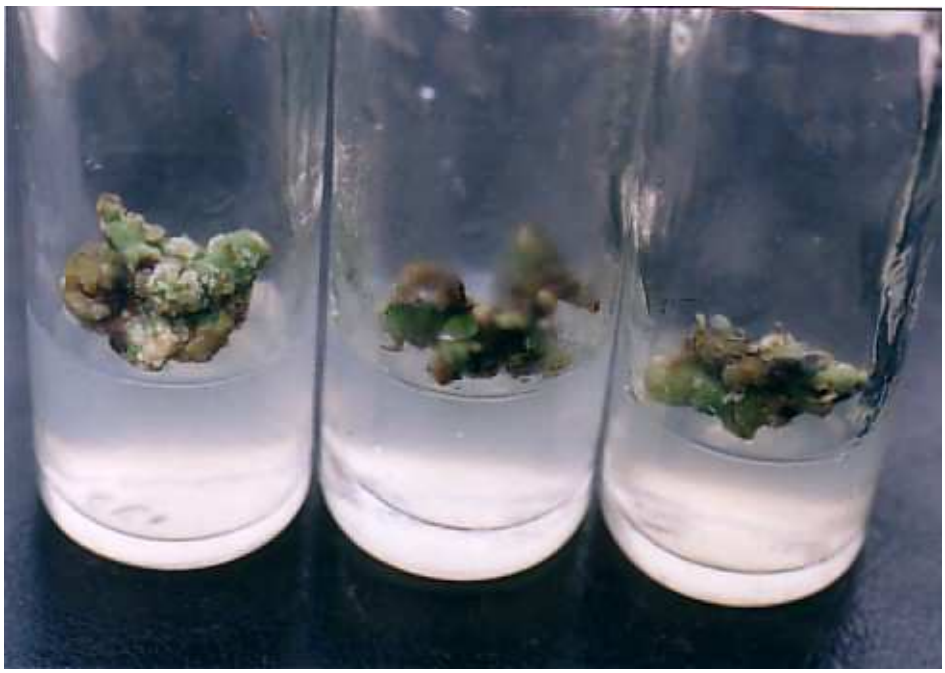
Table (3): Effect of different concentrations of 2,4-D and kinetin on the response (%) of callus induction on *S. officinalis* leaf explants (n= 12).

2,4-D (mg/l)	Kinetin (mg/l)				Mean
	0.0	0.1	0.5	1.0	
0.0	0.0	41.7	50.0	33.3	31.3
0.05	25.0	41.7	100.0	91.7	64.6
0.1	16.7	25.0	66.7	58.3	41.7
0.5	8.3	33.3	33.3	41.7	29.2
1.0	16.7	16.7	8.3	0.0	10.4
Mean	13.3	31.7	51.7	45.0	
LSD 0.05	kin. = 5.2 2,4-D= 6.1 kin. ×2,4-D =13.22				

The highest response to callus induction using kinetin only was achieved on explants treated with 0.5 mg/l (51.7%), followed by 45% in leaf explants treated with 1.0 mg/l kin. Lowest response was recorded on explants treated with 0.1 and control ones reached 31.7 and 13.3 respectively. All responses were significantly different ($p \leq 0.05$) from each other.

Maximum response percentage in 2,4-D treated explants (64.6%) appeared on explants treated with 0.05 mg/l of 2,4-D, followed by 0.1, 0.0, 0.5 or 1.0 mg/l, giving 41.7, 31.3, 29.2 and 10.4 % respectively.
All response were significantly different except between those treated with 0.0 and 0.5 mg/l of 2,4-D.

The interaction between the two growth regulators achieved 100% response in a combination of 0.5 mg/l kin. and 0.05 mg/l 2,4-D (Picture 2). While no callus induction was reported on untreated leaf explants.
The effect of different concentrations of kin. and 2,4-D on the percentage of stem explants responded to callus induction is shown in table (4) and picture (3).



Picture 2: Callus induction on leaf explants grown on MS medium containing a combination of 0.5 mg/l kin and 0.05 mg/l 2,4-D, 21 days after culture.

Picture 3: Callus induction on stem explants grown on MS medium containing a combination of 0.5 mg/l kin and 0.05 mg/l 2,4-D, 21 days after culture.

Table (4): Effect of different concentrations of 2,4-D and kinetin on the response (%) of callus induction on *S. officinalis* stem explants (n= 12).

2,4-D (mg/l)	Kinetin (mg/l)				Mean
	0.00	0.1	0.5	1.0	
0.0	0.00	41.70	41.70	16.70	25.03
0.05	25.00	25.00	100.00	83.30	58.33
0.1	25.00	16.70	41.70	50.00	33.35
0.5	8.30	25.00	25.00	50.00	27.08
1.0	8.30	16.70	0.00	0.00	6.25
Mean	13.32	25.02	41.68	40.00	
LSD 0.05	kin. =6.3 2,4-D =6.7 kin. ×2,4-D =14.3				

There was a significant increase in the (%) response with increasing Kin. concentrations up to 0.5 mg/l. Maximum response reached 41.68% with explants treated with 0.5 mg/l, which not significantly different from the level 1.0 mg/l. Minimum response percentage induction was recorded in a medium deficient of Kin. (13.32 %).

Response to callus induction fluctuated with the inclusion of 2,4-D in the nutrient medium. The level 0.05 mg/l exhibited the highest mean value (58.33%) which differed significantly ($p \leq 0.05$) from other treatments.

The lowest response appeared at 1.0 mg/l 2,4-D giving a mean value of 6.25%. The interaction between Kin. (0.5 mg/l) and 2,4-D (0.05 mg/l) showed a maximum response reached 100%. Other interaction values ranged between this value and no response at all.

Results in tables 2 and 3 show that the best callus induction response for both explants occurred at a combination of 0.5 mg/l Kin. and 0.05 mg/l 2,4-D. Callus induction requires a balanced ratio from auxin(s) and cytokinin(s) as stated by Skoog and Miller (1957). In a number of plant species callus induction favours higher auxins than cytokinins (Ramawat, 2004).

S. officinalis stem and leaf explants may contain some levels of endogenous auxins that made a balanced ratio with the exogenous auxin and may be even cytokinin.

Establishment of a callus from the explants was illustrated by Dodds and Roberts (1998) who divided the process into three developmental stages: induction, cell division and differentiation. The length of these phases depends mainly on the physiological status of the explant's cells as well as the cultural conditions including the appropriate combination of plant growth regulators.

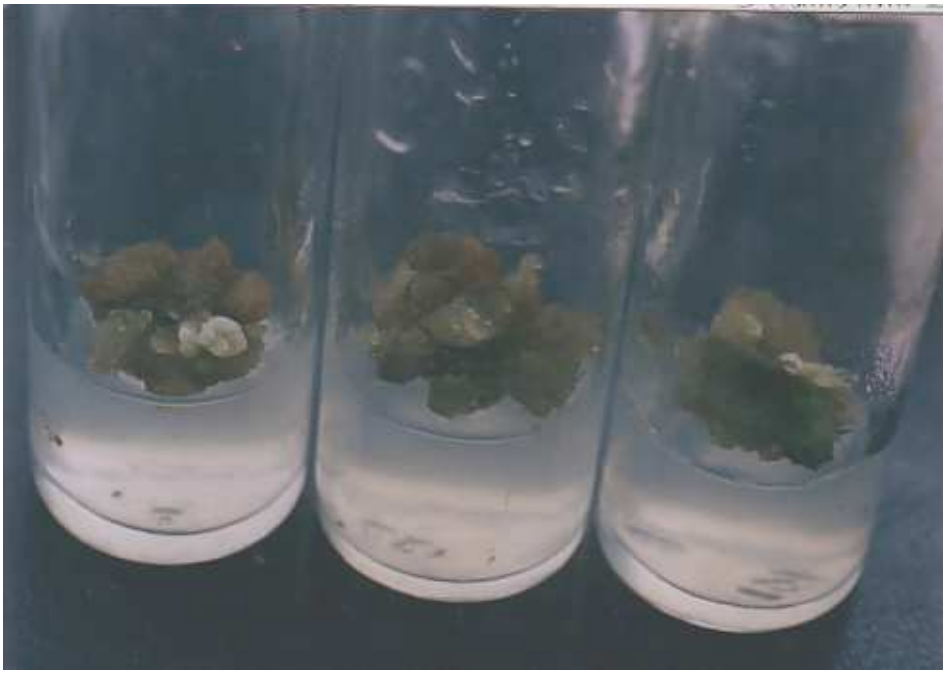
3.3-Maintenance of Callus Cultures:

Callus cultures induced on leaf explants from the best combination of kin. and 2,4-D (0.5 and 0.05 mg/l) respectively, were inoculated into the same combinations of plant growth regulators used for callus induction to determine the appropriate concentration for callus maintenance (Table 5).

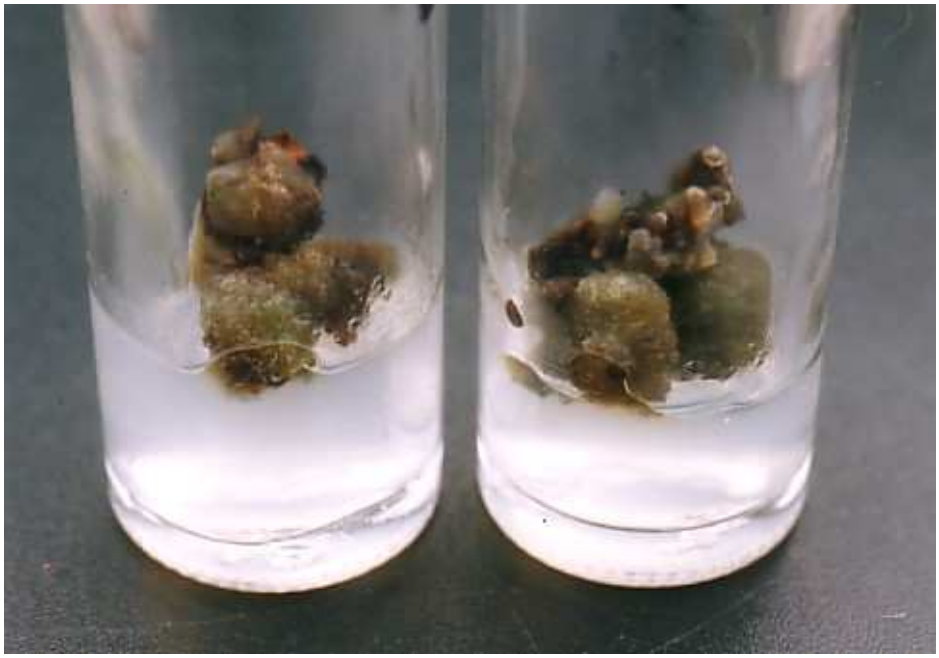
Inclusion of kin. at the concentration of 0.5 mg/l gave significantly higher callus fresh weight (324.58 mg) than other concentrations, while the lowest was in the treatment where no kin. was added to the culture medium. The highest callus fresh weight obtained in 2,4-D treated callus cultures (464.84 mg) was at the concentration 0.05 mg/l (Picture 4). This fresh weight was significantly higher than other treatments.

Table (5): Effect of different concentrations of 2,4-D and kinetin on callus fresh weight (mg) initiated on leaf explants of *S. officinalis* grown on a maintenance medium. Initial weight was 50 mg. (n= 12).

2,4-D (mg/l)	Kinetin (mg/l)				Mean
	0.0	0.1	0.5	1.0	
0.0	0.00	514.62	335.83	234.17	271.16
0.05	151.19	171.25	915.07	621.85	464.84
0.1	132.70	180.15	117.50	245.22	168.89
0.5	115.13	160.02	148.57	301.20	181.23
1.0	103.95	112.22	105.93	0.00	80.53
Mean	100.59	227.65	324.58	280.49	
LSD 0.05	Kin. =40.6 2,4-D= 36.8 kin.× 2,4-D= 74.7				



Picture 4: Callus cultures originated from leaf explants grown on maintenance medium containing 0.5 mg/l kin. and 0.05 mg/l 2,4-D. Cultures were continuously cultured on fresh medium at 21 days intervals.



Picture 5: Callus cultures originated from stem explants grown on maintenance medium containing 0.5 mg/l kin. and 0.05 mg/l 2,4-D. Cultures were continuously cultured on fresh medium at 21 days intervals.

The interaction between the two growth regulators resulted in maximum callus production reached (915.07 mg) at the levels of 0.5 and 0.05 mg/l kin. and 2,4-D respectively.

This combination was significantly higher than all other interactions. Callus tissues showed a reduced growth when inoculated into media lacking or containing 1.0 mg/l kin. and 1.0 mg/l 2,4-D.

Table (6) indicates that the trend was similar in callus cultures initiated on stem explants since the highest fresh weight (836.67 mg) was obtained from the combination of 0.5 mg/l kin. and 0.05 mg/l 2,4-D (Picture 5). This fresh weight was significantly higher than other combinations.

Table (6): Effect of different concentrations of 2,4-D and kinetin on callus fresh weight (mg) initiated on stem explants of *S. officinalis* grown on maintenance medium. Initial weight was 50 mg. (n= 12).

2,4-D (mg/l)	Kinetin (mg/l)				Mean
	0.0	0.1	0.5	1.0	
0.0	0.00	392.33	218.28	180.48	197.77
0.05	123.08	114.48	836.67	552.05	406.57
0.1	133.20	124.25	90.32	209.87	139.41
0.5	97.18	118.77	130.72	199.65	136.58
1.0	96.43	102.17	0.00	0.00	49.649
Mean	89.98	170.40	255.20	228.41	
LSD 0.05	Kin.= 37.2 2,4-D= 42.6 kin.× 2,4-D= 78.7				

Dry weights of callus cultures initiated from both leaf and stem explants are shown in tables 7 and 8 respectively.

Table (7): Effect of different concentrations of 2,4-D and kinetin on callus dry weight (mg) initiated on leaf explants of *S. officinalis* and grown on maintenance medium (n= 12).

2,4-D (mg/l)	Kinetin (mg/l)				Mean
	0.0	0.1	0.5	1.0	
0.0	0.00	82.86	71.09	28.72	45.66
0.05	53.63	21.00	107.95	92.80	68.85
0.1	16.63	24.59	15.33	35.88	23.11
0.5	12.80	25.96	23.83	39.63	25.56
1.0	10.64	15.25	13.97	0.00	9.97
Mean	18.74	33.93	46.43	39.41	
LSD 0.05	Kin.= 6.3 2,4-D= 6.8 kin.× 2,4-D= 10.6				

Table (8): Effect of different concentrations of 2,4-D and kinetin on callus dry weight (mg) initiated on stem explants of *S. officinalis* and grown on maintenance medium (n= 12).

2,4-D (mg/l)	Kinetin (mg/l)				Mean
	0.0	0.1	0.5	1.0	
0.0	0.00	49.33	32.58	24.22	26.53
0.05	15.28	17.92	115.67	73.61	55.62
0.1	18.17	17.97	14.91	30.90	20.49
0.5	14.11	17.14	18.90	25.61	18.94
1.0	12.54	13.36	0.00	0.00	6.48
Mean	12.02	23.14	36.41	30.87	
LSD 0.05	Kin.= 5.4 2,4-D= 8.3 kin.× 2,4-D= 12.3				

The combination of 0.5 mg/l kin. and 0.05 2,4-D showed the highest dry weights in both types of explants. These weights (107.95 mg) and (115.67 mg) were significantly higher than all other treatments.

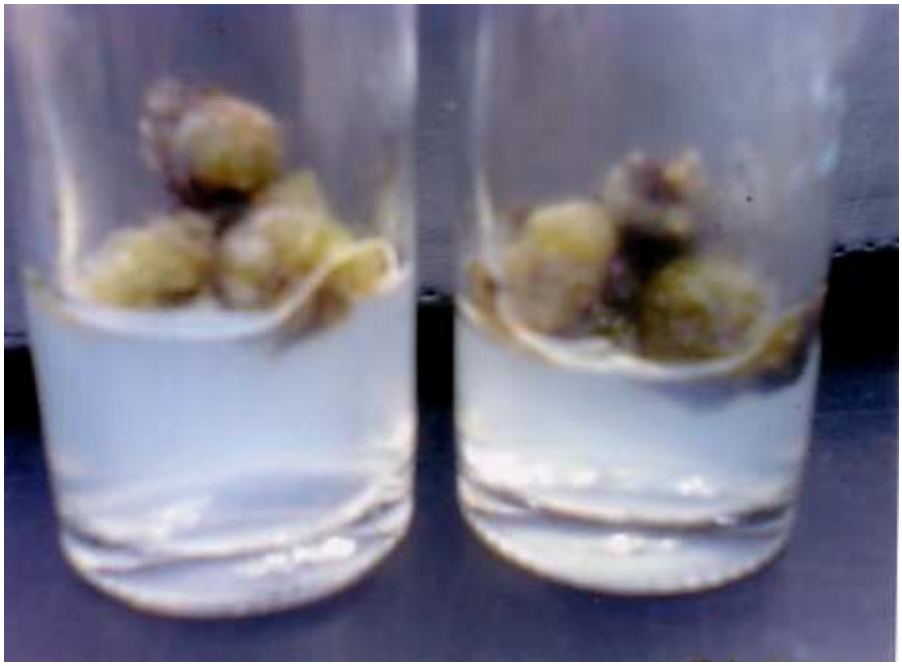
According to the results stated above, callus was induced on leaf and stem explants then maintained for many subcultures on MS medium containing 0.5 mg/l kin. and 0.05 mg/l 2,4-D for subsequent experiments. Induction and maintenance of callus cultures in *S. officinalis* seem to favor low levels of 2,4-D and rather higher levels of kin. Increasing the levels of the two plant growth regulators suppressed callus growth. The increase of callus mass is important for the production of secondary metabolites since they are proportionally related (Ramawat, 2004). It would be convenient from the practical point of view to induce and maintain callus on the same growth nutrients and plant growth regulators requirements. Induction of callus on both types of explants using the same medium components is an additional advantage for plant biotechnologists.

Regeneration in callus cultures was noticed when some callus cultures left on maintenance medium for 40 days without subculture (Picture 6). Shoot proliferation may due to the continuous depletion of nutrients and may be growth regulators over time. This may led to a new balanced ratio suitable for plant regeneration. The process of regeneration is important in such work since it offers a continuous sterile source of plant material under controlled conditions to be used for the production of secondary metabolites especially from differentiated cultures.

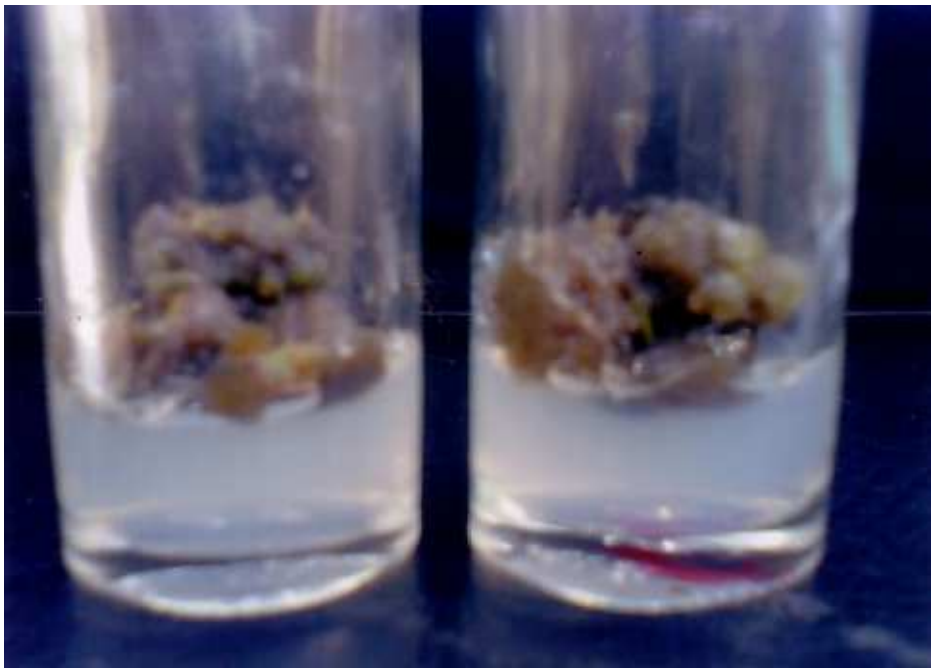
Picture 6: Shoot proliferation in callus cultures after prolonged culture on maintenance medium. Shoot regeneration occurred after 40 days.

3.4-Addition of Elicitor to Callus Cultures:

The addition of 100mM NaCl to MS medium caused browning to callus culture, while callus weight was not much affected for both concentrations (50 and 100) mM of NaCl. The callus browning may relate to the increase in secondary products secretion especially the phenolic compounds. Callus growth initiated from leaf explants and grown on the maintenance medium containing 100mM NaCl was more clumpy, not friable and tended to be yellowish (Picture 7). While callus cultures initiated from stem explants tended to be friable and brown (Picture 8).



Picture 7: Appearance of callus cultures initiated originally on leaf explants and maintained on maintenance medium containing 100mM NaCl as elicitor.



Picture 8: Appearance of callus cultures initiated originally on stem explants and maintained on maintenance medium containing 100mM NaCl as elicitor.

3.5-Identification of Secondary Metabolites:

GC methods were used for detection and quantification analysis of (rutin, α-pinene, linalool, geraniol, apigenin, quercetin and coumarin).

Quantities of the investigated secondary metabolites are presented in table (20). The quantities varied depending on the type of plant tissue and the type of culture.

Table (20): Quantification of secondary metabolites (mg/g) detected in intact plant and different cultures initiated *in vitro*.

Source	Explant	Rutin	α-pinene	Linalool	Geraniol	Apigenin	Quercetin	Coumarin
Intact plant	Leaf	0.338 ±0.019	0.298 ±0.009	0.329 ±0.018	0.309 ±0.007	0.225 ±0.013	0.420 ±0.006	0.411 ±0.009
	Stem	0.162 ±0.017	1.347 ±0.067	0.755 ±0.008	0.153 ±0.016	0.098 ±0.001	0.138 ±0.002	0.298 ±0.022
Callus culture	Leaf	0.410 ±0.024	0.307 ±0.032	0.906 ±0.003	0.110 ±0.003	0.146 ±0.002	0.269 ±0.131	0.313 ±0.066
	Stem	0.357 ±0.049	0.330 ±0.052	0.000	0.093 ±0.020	0.050 ±0.001	0.163 ±0.015	0.330 ±0.040
Cell suspension culture	Leaf	0.390 ±0.012	0.414 ±0.008	0.707 ±0.014	0.524 ±0.007	0.669 ±0.026	0.000	0.134 ±0.013
	Stem	0.418 ±0.023	0.141 ±0.017	0.531 ±0.011	0.155 ±0.003	0.758 ±0.056	0.304 ±0.045	0.000
Callus culture + 50mM NaCl	Leaf	0.287 ±0.006	0.000	0.230 ±0.012	0.171 ±0.13	0.128 ±0.012	0.091 ±0.005	0.143 ±0.002
	Stem	0.166 ±0.005	0.000	0.507 ±0.023	0.233 ±0.024	0.153 ±0.005	0.004 ±0.001	0.311 ±0.031
Callus culture +100 mM NaCl	Leaf	0.448 ±0.002	1.390 ±0.075	1.170 ±0.003	0.547 ±0.033	0.825 ±0.003	0.768 ±0.002	0.590 ±0.015
	Stem	0.286 ±0.001	0.000	1.137 ±0.005	0.076 ±0.034	0.109 ±0.006	0.167 ±0.026	0.440 ±0.046

Rutin showed the highest concentration in callus culture that initiated from leaf and grown on a medium supplemented with 100mM NaCl (0.448 mg/g) as elicitor (fig. 12) followed by cell suspension culture initiated from stem explants (0.418 mg/g) as shown in fig. 9, while the lowest value was in stem explants of the intact plant (fig. 4) and in the sample of callus culture initiated from stem explants and grown on a medium supplemented with 50mM NaCl(0.162 and 0.166 mg/g) respectively (fig. 13).

α-pinene showed the highest concentrations (1.390 mg/g) in callus cultures initiated from leaf and grown on a medium supplemented with 100mM NaCl (fig. 12). The lowest concentration of α-pinene was (0.141 mg/g) found in the sample of cell suspension culture initiated from stem (fig. 9) followed by (0.298 mg/g) of intact plant sample from leaf source (fig 3). α-pinene was not detected in callus cultures that initiated from leaf and stem explants and grown on a medium supplemented with 50mM NaCl (fig. 10, 11) and in the sample of callus culture initiated from stem and grown on a medium supplemented with 100mM NaCl as elicitor (fig. 13).

Maximum concentration of linalool appeared in callus cultures initiated from leaf and stem explants grown on a medium supplemented with 100mM NaCl (1.170 and 1.137 mg/g) respectively (fig. 12, 13). The lowest concentrations were (0.230 mg/g) found in callus samples initiated from leaf and grown on a medium supplemented with 50mM NaCl (fig. 10) followed by leaf explants of the intact plant (fig. 3) with a

value of (0.33 mg/g). Linalool was not detected in callus cultures initiated from stem explants (fig. 6).

Geraniol was detected in all samples achieving the highest level in callus cultures initiated from leaf and grown on a medium supplemented with 100mM NaCl (0.547 mg/g) as shown in (fig. 12) and (0.524 mg/g) in cell suspension cultures initiated from leaf explants (fig.8). The lowest concentration was (0.076 mg/g) detected in callus cultures initiated from stem and grown on a medium supplemented with 100mM NaCl (fig. 13) and (0.093 mg/g) in callus cultures initiated from stem (fig. 6).

Apigenin was found at high concentration (0.825 mg/g) in callus cultures initiated from leaf and grown on a medium supplemented with 100mM NaCl (fig. 12) followed by (0.758 mg/g) in cell suspension cultures of stem explants (fig. 9). Callus cultures initiated from stem explants recorded the lowest concentration (0.050 mg/g) as in (fig. 6) also the sample in (fig. 4) of the intact plant from stem recorded low apigenin content (0.098 mg/g).

Quercetin recorded the maximum concentration (0.768 mg/g) in the sample of callus cultures initiated from leaf explants and grown on a medium supplemented with 100mM NaCl (fig. 12), whereas the lowest level was detected in callus cultures initiated from stems and grown on a medium supplemented with 50mM NaCl (fig. 11). Quercetin was not detected in cell suspension cultures initiated from leaf explants (fig. 8).

Coumarin highest level (0.590 mg/g) was found in callus cultures initiated from leaf and grown on a medium supplemented with 100mM

NaCl (fig. 12) followed by (0.440 mg/g) in callus cultures initiated from stem and grown on a medium supplemented with 100mM NaCl (fig. 13). Coumarin was not detected in cell suspension cultures initiated from stem explants (fig. 9).

It is clear that α-pinene was abundant at high concentration compared with other studied compounds. The table also shows that among all the samples under investigation, the callus culture initiated from leaf and grown on a medium supplemented with 100mM NaCl as elicitor gave the highest concentrations for all tested essential oils and phenolic compounds (fig. 12).

Fried and Sherma (1982) and Culea *et al*., (2003) reported that the separation and purification of secondary products are usually carried out using gas chromatography and TLC methods.

Exposure of cultured plant cells to an elicitor result in some genes expression that leads to the formation of the secondary metabolites which are found in the entire plant. Additionally, inclusion of NaCl in culture medium may induce the synthesis of new proteins (Al-ubaidy, 2006). This may lead to the synthesis of secondary metabolites that plant tissue cultures are utilize for stress tolerance.

Although the undifferentiated cells of plant tissue cultures are generally totipotent, many genes including those involved in secondary metabolism are repressed with the consequence that the yields of desired compounds in cultures are low. However, it is becoming increasingly apparent that a large number of secondary metabolites belong to a class of substances termed phytoalexins. These are stress- related compounds

produced in the normal plant as a result of damaging stimuli from physical, chemical or microbiological factors. When cell cultures are subjected to such elicitors, some genes are depressed, resulting in the formation of the secondary metabolites which are found in the entire plant.

The results agree with Taniguchi (2002) who stated that terpenoid synthesis can be induced by elicitors and Phatak (2002) who reported that the hydrocarbons (terpenes) can be detected in tissue cultures. Wide variety of chemicals can be produced from plant tissue cultures, some produce medicines, essential oils and various other biochemicals.

Stojakowska (1999) reported that various types of phenolics and polyphenols are constituents of plant tissue cultures.

In (2004), Vanisree referred to the major advantages of a cell culture system over the conventional cultivation of whole plants which are:

(1) Useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions.
(2) Cultured cells would be free of microbes and insects.
(3) Cells of, tropical or alpine, plants could easily be multiplied to yield specific metabolites.
(4) Automated control of cell growth and rational regulation of metabolite processes would reduce labor cost and improve productivity.
(5) Organic substances are extractable from callus cultures easily.

Samples of standard, intact plant, callus cultures, cell suspension cultures, and sample of callus cultures grown on a medium supplemented with an elicitor (50 or 100)mM NaCl originally taken from stem and leaf explants are illustrated in figures (3-13).

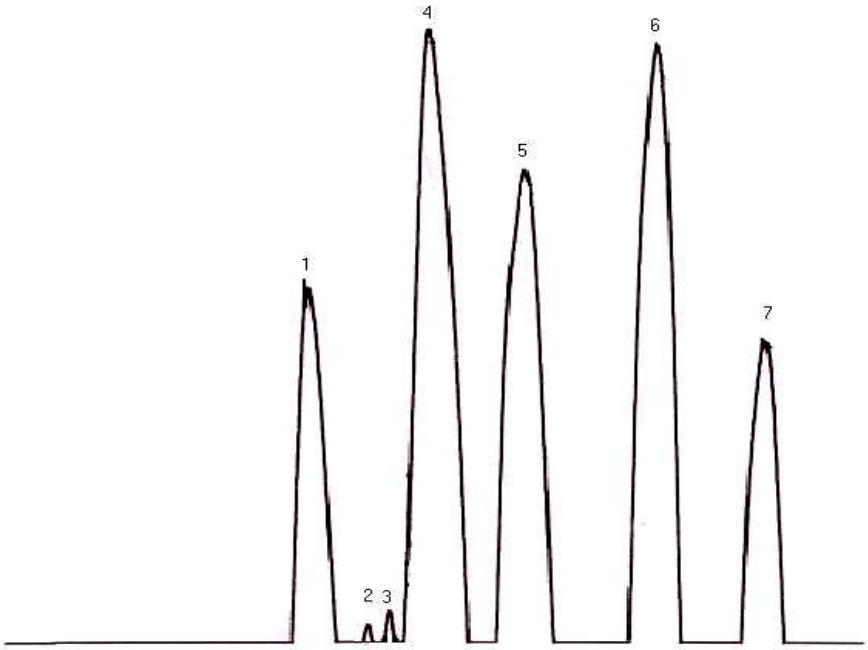


Figure (3): Standard for Gas –chromatography of some essential oils and phenolic compounds at a concentration of 15µg/g. Column supeleCo (wax 10), 15ft×⅛ 1n stainless steel, 2.1mm internal diameter, oven temperature 50°C (2min) then increased to 200°C with a rate of 2°C/min, carrier gas: Helium with flow rate 50 cm/sec., Detection: FID (Flame Ionization Detector).
Key : 1= Rutin ; 2=α -Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin .

Table (9): Rt values for standard used in the experimental work.

Compounds	Rt (min)
Rutin	5.9
α - pinene	7.3
Linalool	7.6
Geraniol	8.4
Apigenin	10.3
Quercetin	12.9
Coumarin	15.1

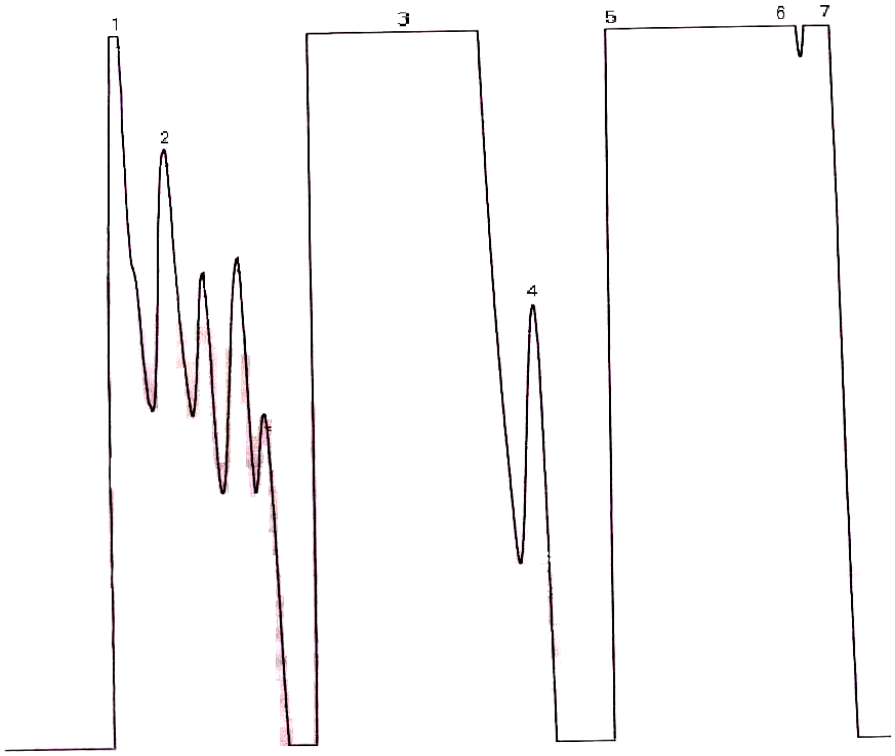


Figure (4): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* leaf explants. Column specifications were as mentioned in fig (3). Key : 1= Rutin ; 2=α-Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (10): Quantification of some terpenes and flavonoids detected in fig. (4).

Compounds	Rt (min)	Sample concentration (mg /g)
Rutin	5.8	0.338 ±0.019
α - pinene	7.1	0.298 ±0.009
Linalool	8.6	0.329 ±0.018
Geraniol	7.9	0.309 ±0.007
Apigenin	10.1	0.225 ±0.013
Quercetin	12.8	0.42 ±0.006
Coumarin	13.8	0.411 ±0.009

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, values are the mean of three sample replicates, all values expressed as mean ± SE.

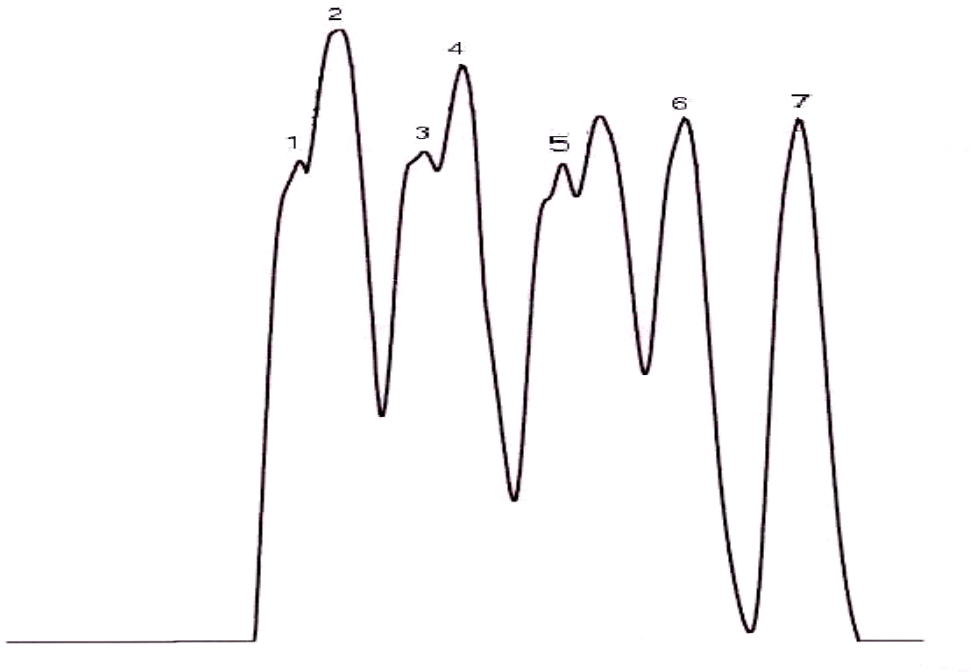


Figure (5): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* stem explants. Column specifications were as mentioned in fig (3). Key : 1= Rutin ; 2=α-Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (11): Quantification of some terpenes and flavonoids detected in fig. (5).

Compounds	Rt (min)	Sample concentration (mg /g)
Rutin	5.5	0.162 ±0.017
α - pinene	6.2	1.347 ±0.067
Linalool	7.8	0.755 ±0.008
Geraniol	8.5	0.153 ±0.016
Apigenin	10.5	0.098 ±0.001
Quercetin	12.8	0.138 ±0.002
Coumarin	15.0	0.298 ±0.022

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, values are the mean of three sample replicates, all values expressed as mean ± SE.

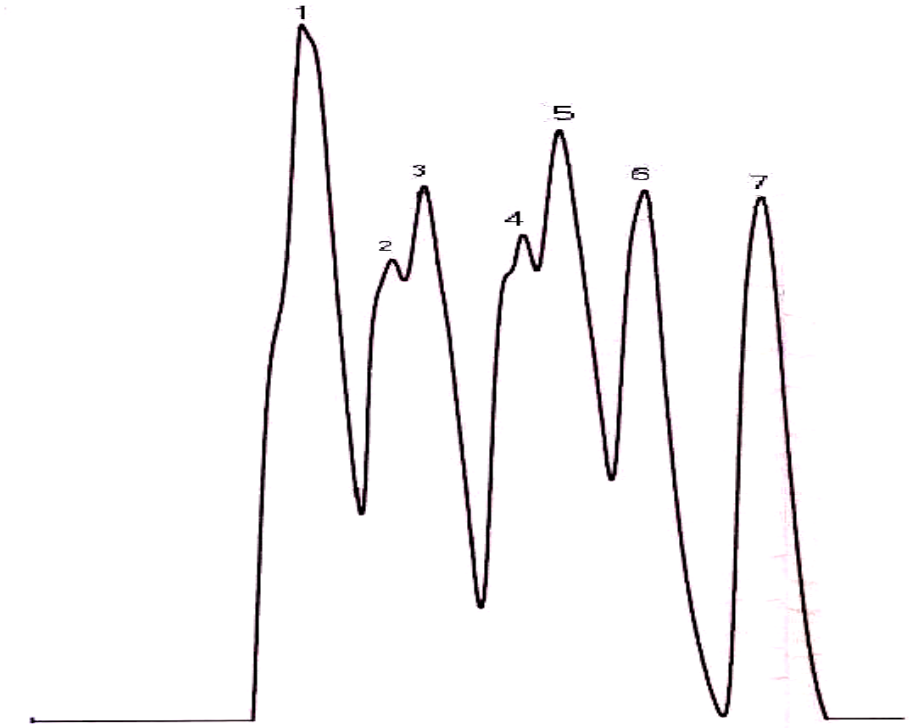


Figure (6): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* callus culture initiated from leaf explants. Column specifications were as mentioned in fig (3).

Key : 1= Rutin ; 2=α-Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (12): Quantification of some terpenes and flavonoids detected in fig. (6).

Compounds	Rt (min)	Sample concentration (mg /g)
Rutin	5.1	0.410 ±0.024
α - pinene	6.02	0.307 ±0.032
Linalool	7.9	0.906 ±0.003
Geraniol	9.8	0.110 ±0.003
Apigenin	10.5	0.146 ±0.002
Quercetin	12.2	0.269 ±0.131
Coumarin	14.5	0.313 ±0.066

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, values are the mean of three sample replicates, all values expressed as mean ± SE.

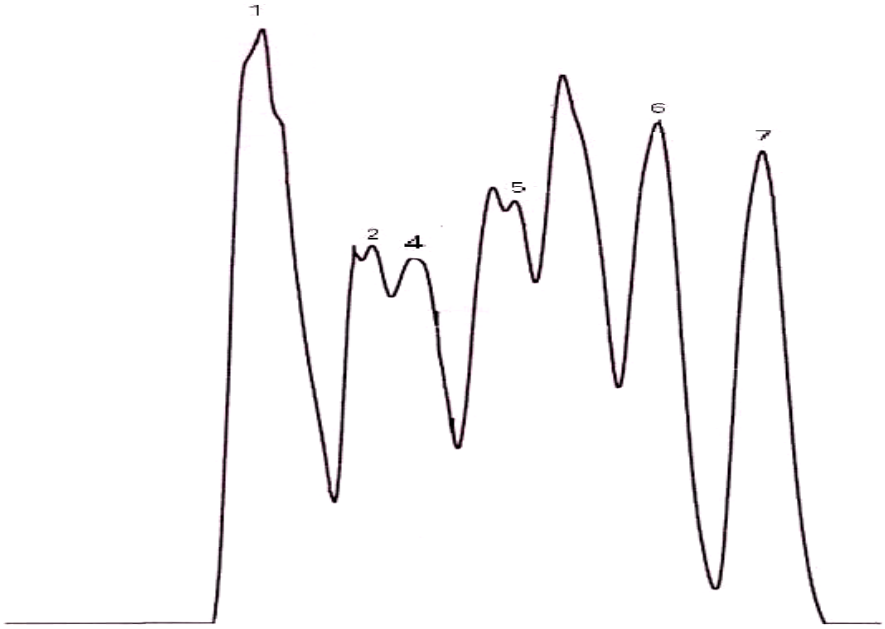


Figure (7): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* callus culture initiated from stem explants. Column specifications were as mentioned in fig (3)

Key : 1= Rutin ; 2= α -Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (13): Quantification of some terpenes and flavonoids detected in fig. (7).

Compounds	Rt (min)	Sample concentration (mg /g)
Rutin	5.4	0.357 ±0.049
α - pinene	7.3	0.33 ±0.052
Linalool	N.D	N.D
Geraniol	8.1	0.093 ±0.020
Apigenin	10.1	0.05 ±0.001
Quercetin	13.0	0.163 ±0.015
Coumarin	15.1	0.33 ±0.040

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, N.D= is not detected, values are the mean of three sample replicates, all values expressed as means ± SE.

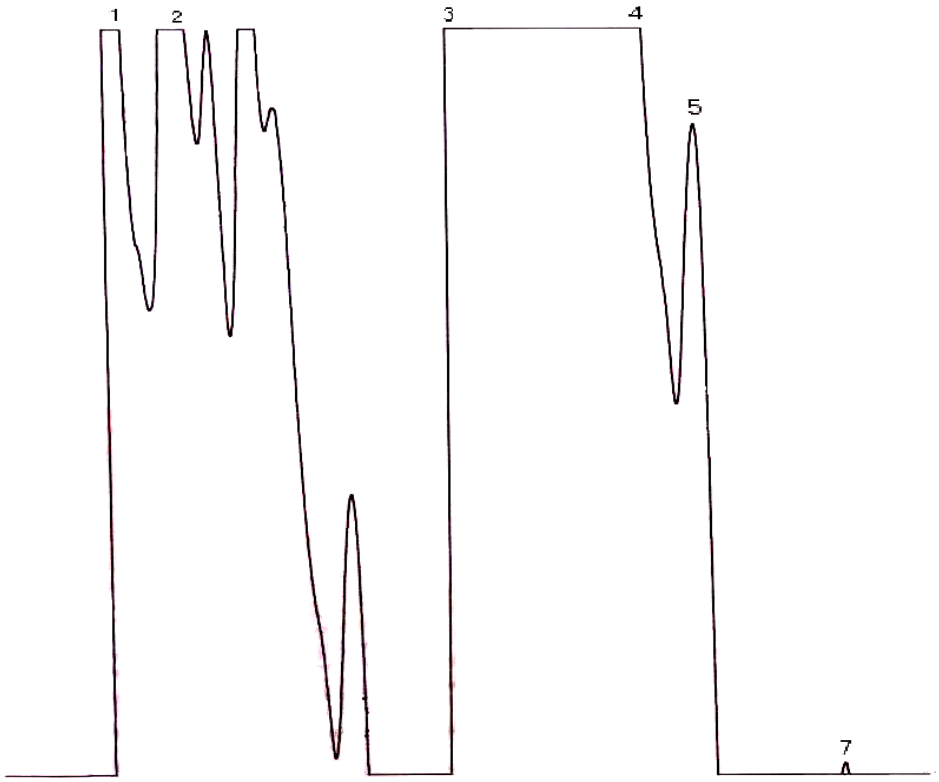


Figure (8): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* cell suspension cultures initiated from leaf explants. Column specifications were as mentioned in fig (3).
Key : 1= Rutin ; 2=α-Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (14): Quantification of some terpenes and flavonoids detected in fig. (8).

Compounds	Rt (min)	Sample concentration (mg /g)
Rutin	6.23	0.390 ±0.012
α - pinene	7.4	0.414 ±0.008
Linalool	8.1	0.707 ±0.014
Geraniol	8.4	0.524 ±0.007
Apigenin	12.9	0.669 ±0.026
Quercetin	N.D	N.D
Coumarin	15.1	0.134 ±0.013

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, N.D= is not detected, values are the mean of three sample replicates, all values expressed as mean ± SE.

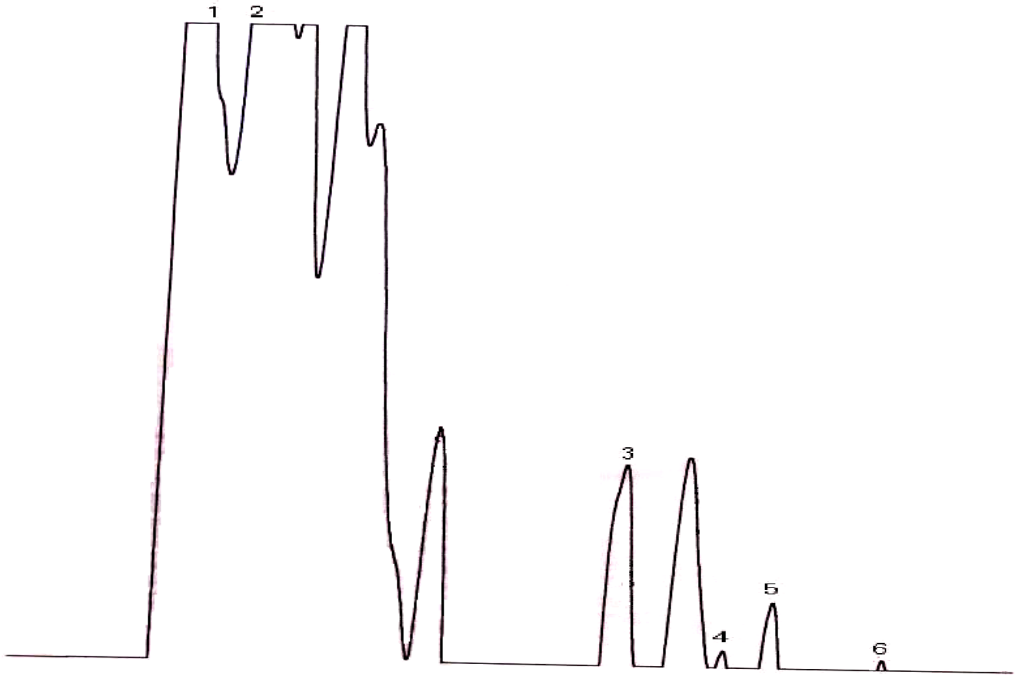


Figure (9): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* cell suspension cultures initiated from stem explants. Column specifications were as mentioned in fig (3).
Key : 1= Rutin ; 2=α-Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (15): Quantification of some terpenes and flavonoids detected in fig. (9).

Compounds	Rt (min)	Sample concentration (mg /g)
Rutin	6.1	0.418 ±0.023
α - pinene	7.4	0.141 ±0.017
Linalool	9.0	0.531 ±0.011
Geraniol	9.6	0.155 ±0.003
Apigenin	12.3	0.758 ±0.056
Quercetin	11.4	0.304 ±0.045
Coumarin	N.D	N.D

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, N.D= is not detected, values are the mean of three sample replicates, all values expressed as mean ± SE.

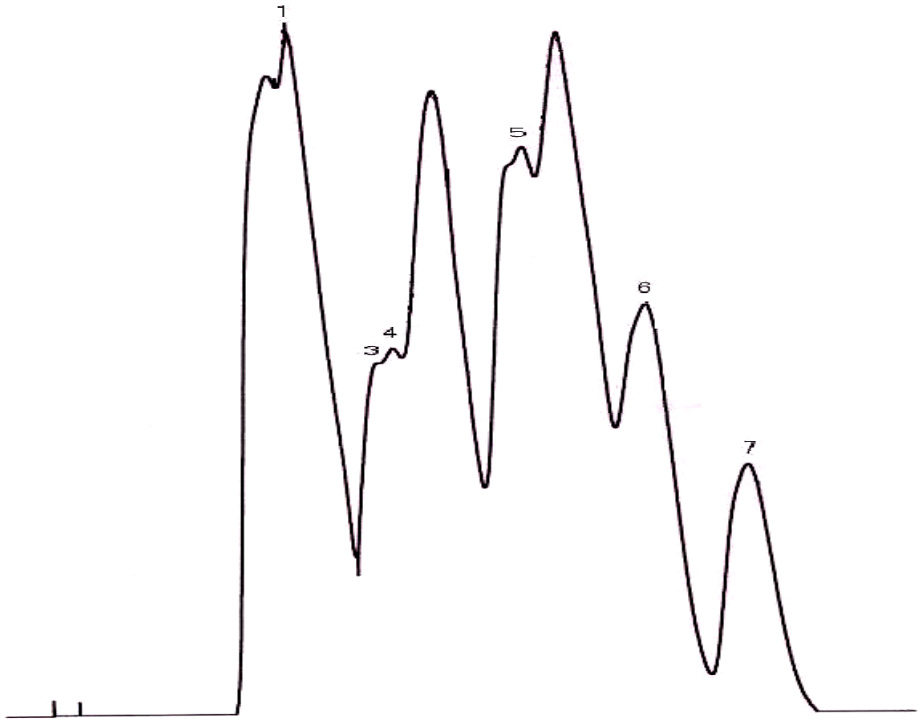


Figure (10): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* callus culture initiated from leaf explants. NaCl was added to the culture medium as an elicitor in a concentration of 50mM. Column specifications were as mentioned in fig (3).
Key : 1= Rutin ; 2=α-Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (16): Quantification of some terpenes and flavonoids detected in fig. (10).

Compounds	Rt (min)	Sample concentration (mg /g)
Rutin	5.9	0.287 ±0.006
α - pinene	N.D	N.D
Linalool	7.7	0.23 ±0.012
Geraniol	8.1	0.171 ±0.13
Apigenin	10.9	0.128 ±0.012
Quercetin	13.4	0.091 ±0.005
Coumarin	15.5	0.143 ±0.002

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, N.D= is not detected, values are the mean of three sample replicates, all values expressed as means ± SE.

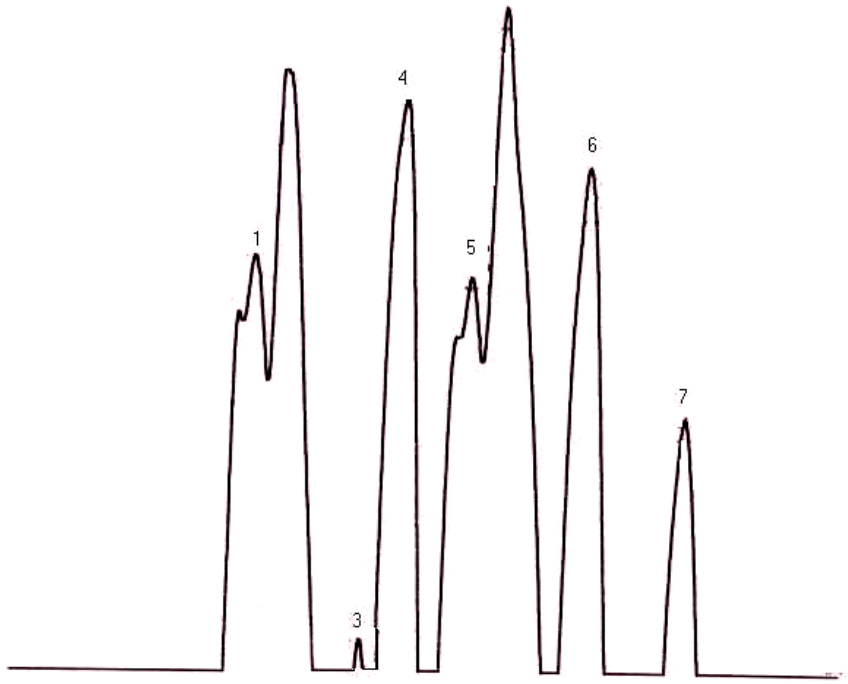


Figure (11): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* callus cultures initiated from stem explants.
NaCl was added to the culture medium as an elicitor in a concentration of 50mM. Column specifications were as mentioned in fig (3).
Key: 1= Rutin; 2= α -Pinene; 3= Linalool; 4= Geraniol; 5= Apigenin; 6= Quercetin; 7= Coumarin.

Table (17): Quantification of some terpenes and flavonoids detected in fig. (11).

Compounds	Rt (min)	Sample concentration (mg/g)
Rutin	5.4	0.166 ±0.005
α - pinene	N.D	N.D
Linalool	7.8	0.507 ±0.023
Geraniol	8.9	0.233 ±0.024
Apigenin	10.4	0.153 ±0.005
Quercetin	13.1	0.004 ±0.001
Coumarin	15.1	0.311 ±0.031

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, N.D= not detected, values are the mean of three samples replicates, all values expressed as mean ± SE.

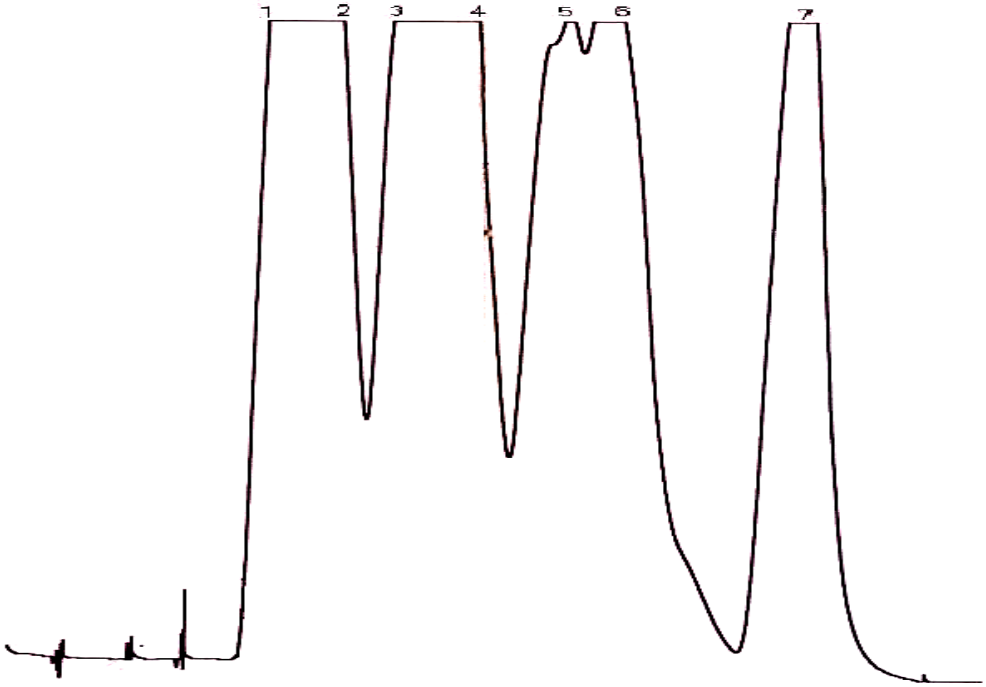


Figure (12): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* callus culture initiated from leaf explants. NaCl was added to the culture medium as an elicitor in a concentration of 100mM. Column specifications were as mentioned in fig (3).
Key : 1= Rutin ; 2=α-Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (18): Quantification of some terpenes and flavonoids detected in fig. (12).

Compounds	Rt (min)	Sample concentration (mg /g)
Rutin	5.5	0.448 ±0.002
α - pinene	6.2	1.39 ±0.075
Linalool	7.9	1.17 ±0.003
Geraniol	8.6	0.547 ±0.033
Apigenin	11.0	0.825 ±0.003
Quercetin	11.8	0.768 ±0.002
Coumarin	15.6	0.59 ±0.015

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, values are the mean of three sample replicates, all values expressed as mean ± SE.

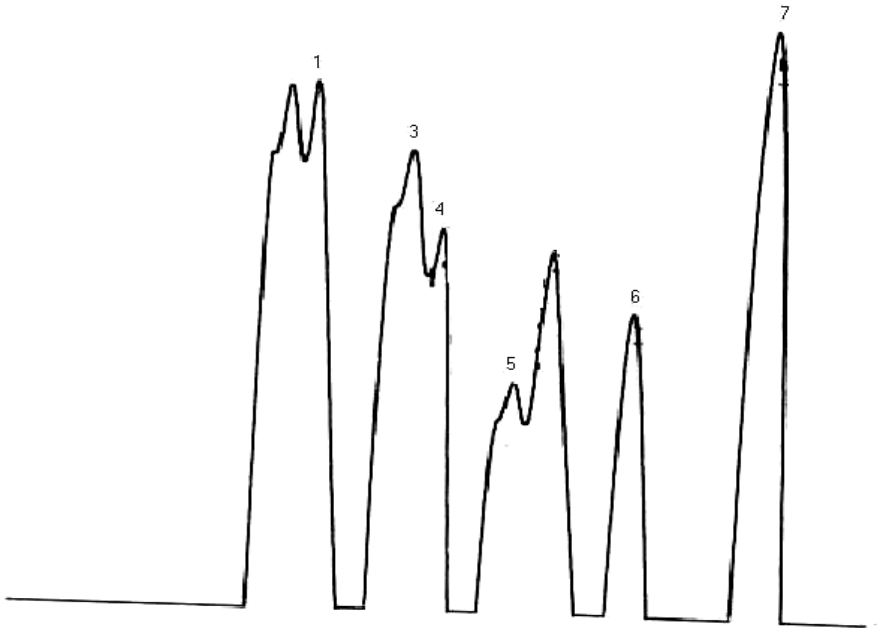


Figure (13): Gas-chromatography for the separation of some essential oils and phenolic compounds extracted from *Salvia officinalis* callus culture initiated from stem explants. NaCl was added to the culture medium as an elicitor in a concentration of 100mM. Column specifications were as mentioned in fig. (3).

Key : 1= Rutin ; 2=α-Pinene ; 3= linalool ; 4= Geraniol ; 5= Apigenin ; 6= Quercetin ; 7= Coumarin.

Table (19): Quantification of some essential oils and phenolic compounds detected in fig. (13).

Compounds	Rt (min)	Sample concentration (mg/g)
Rutin	5.1	0.286 ±0.001
α - pinene	N.D	N.D
Linalool	8.0	1.137 ±0.005
Geraniol	8.6	0.076 ±0.034
Apigenin	10.2	0.109 ±0.006
Quercetin	12.6	0.167 ±0.026
Coumarin	15.3	0.44 ±0.046

Rt is the retention time (min.), the concentration was calculated as mg/g dry weight, N.D= is not detected, values are the mean of three samples replicates, all values expressed as mean ± SE.

Table (21) shows a comparison between tissue culture systems and the intact plant in increased or decreased ratios of the secondary metabolites investigation.

Table (21): Ratios represent the studied secondary metabolites in tissue cultures to the original intact plant.

Source	Explants	Rutin	α-pinene	linalool	Geraniol	Apigenin	Quercetin	Coumarin
Callus culture : Intact plant	Leaf	1.216	1.030	2.750	0.357	0.649	0.640	0.762
	Stem	2.202	0.245	0.000	0.607	0.510	1.184	1.109
Cell suspension culture : Intact plant	Leaf	1.156	1.390	2.147	1.697	2.973	0.000	0.325
	Stem	2.578	0.104	0.703	1.009	7.735	2.200	0.000
Callus culture + 50mM NaCl : Intact plant	Leaf	0.850	0.000	0.698	0.552	0.569	0.217	0.348
	Stem	1.023	0.000	0.671	1.523	1.561	0.029	1.046
Callus culture +100mM NaCl : Intact plant	Leaf	1.326	4.670	3.553	1.769	3.668	1.829	1.435
	Stem	1.763	0.000	1.506	0.496	1.112	1.208	1.477

Rutin showed an increase in all tissue culture systems except when NaCl was added at 50mM to callus cultures initiated from leaf. This increment was more than doubled (2.578) in cell suspension cultures initiated from stem explants.

α-pinene showed an increased ratios in callus cultures initiated from leaf explants (1.030), cell suspension culture initiated from leaf (1.390) and increased to more than four folds (4.670) in callus cultures initiated from leaf and grown on a medium containing 100mM NaCl. Tissue

cultures initiated from stem explants mostly showed either reduced levels of α-pinene or not detected.

Linalool increased to about 3 folds in callus cultures initiated from leaf explants grown on a medium containing 100mM NaCl. It is more than doubled in callus and cell suspension cultures initiated from leaf (2.750 and 2.147) respectively. Additionally callus cultures initiated from stem and grown on a medium containing 100mM NaCl showed an increased ratio reaching (1.506).

High ratios of geraniol were recorded in callus cultures initiated from leaf explants grown on a medium supplemented with 100mM NaCl, cell suspension cultures initiated from leaf explants, callus cultures initiated from stem explants and grown on a medium supplemented with 50mM NaCl and in cell suspension cultures initiated from stem explants, these ratios were (1.769, 1.697, 1.523 and 1.009) respectively.

Apigenin recorded an increase to more than seven folds (7.735) in cell suspension culture initiated from stem explants followed by callus cultures of leaf explants that grown on a medium supplemented with 100mM NaCl that increased to more than 3 folds (3.668), to a lesser extent in cell suspension cultures initiated from leaf (2.973), followed by (1.965) in cell suspension cultures initiated from stem explants.

High ratio of quercetin (2.200) was noticed in cell suspension cultures initiated from stem and callus cultures of leaf explants grown on a medium supplemented with 100mM NaCl (1.829). This followed by (1.208 and 1.184) for the callus cultures initiated from stem explants and

grown on a medium supplemented with 100mM NaCl and callus cultures initiated from stem explants respectively.

Coumarin in the callus cultures initiated from stem and grown on a medium supplemented with 100mM NaCl showed an increased ratio reached to approximately 1.5 fold (1.477). Similar ratio (1.435) in leaf explants of the same sample was recorded. Callus cultures initiated from stem and callus cultures initiated from stem explants and grown on a medium supplemented with 50mM NaCl showed ratios (1.109 and 1.046) respectively.

The amount of the product may vary depending on the amount of specific supplements such as NaCl in the medium. One benefit of using tissue cultured cells is that they may offer higher metabolites concentrations per cell and synthesis rate than whole plants.

Scott and Dougall (1987) reported that the mechanisms by which these compounds increase may be associated with the amounts of essential enzymes in the biosynthesized pathway or it may be associated with supply of enzyme synthesized precursors.

Dix and Pearce (1981), showed the phytochemical effects of nutrient stress in cultured plant cells by the addition of NaCl that caused an increase in secondary metabolites, accumulation of amino acids and other substances in *N. tabacum.*

The results disagree with Scott and Dougall (1987) who indicated that the plant tissue culture system may produce lower secondary metabolites compared with the intact plant, while Jogdand (1993) reported that suspension cultures are ideal for the production of

secondary metabolites which are of therapeutic value. His study also confirmed by (Sateesh, 2003).

Stress affects secondary metabolism of cultured plant cells. One of the features of cultured cells is the activation of genes coding for compounds not usually produced at the whole plant level. This response can occur through the stress mediated induction of specific mRNA.

Alternatively, some compounds that are characteristic of the intact plant may not be synthesized (DiCosmo and Towers, 1984).

Ramawat, (2004) reported that the secondary plant products are genetically controlled phenomenon. However, various biotic and abiotic factors influence secondary metabolites production via gene activation or by stimulating the physiological processes leading to enhanced accumulation of such products. Furthermore, the synthesis of most of the secondary metabolites is a several steps reaction involving several enzymes (several genes) which means that the synthesis may be stimulated at any step to enhance their production.

Table 22 represents the total concentration of the studied essential oils (α-pinene, geraniol and linalool) and phenolic compounds (rutin, apigenin, quercetin and coumarin).

This table shows that *S. officinalis* essential oils concentration increased in most of tissue cultured systems compared with intact plant, The samples recorded higher concentrations than intact plant were: callus culture initiated from leaf gave (1.323 mg/g), cell suspension culture initiated from leaf (1.645 mg/g) and callus cultures initiated from

leaf and stem explants grown on a medium supplemented with 100mM NaCl as an elicitor (3.107 and 1.213 mg/g) respectively.

Phenolic compounds also showed increased concentrations in the *in vitro* systems. They increased in callus cultures initiated from stem (0.900 mg/g), the sample of cell suspension culture initiated from stem explants (1.480 mg/g) and in the sample of callus cultures initiated from both explants and grown on a medium supplemented with 100mM NaCl which recorded 2.631 mg/g for leaf explants and 1.002 mg/g for stem explants.

It was found that in all studied samples, leaf explants showed an increase in essential oils, whereas stem explants showed an increase in phenolic compounds only, except for the sample of callus grown on a medium containing 100mM NaCl which showed increased concentrations of essential oils and phenolic compounds in both stem and leaf explants.

The sample of callus cultures grown on a medium supplemented with 50mM NaCl initiated from both explants showed no increase in essential oils or phenolic compounds.

This table indicates that *S. officinalis* contains active compounds in leaf and stem explants but at different concentrations.

Nakiboglu (2002), Abu-Shanab *et al*., (2004) and Naghibi *et al*., (2005) used leaf explants of *S. officinalis* for extraction of active compounds whereas; Kavvadias *et al*., (2003) stated that the medicinal parts of S. *officinalis* are leaves and stems.

Various stress factors impact on the qualitative and quantitative accumulation of valuable secondary products in nature. Lila, (2005) indicated that elicitors from biotic and abiotic sources can be added to culture media to stimulate secondary metabolites production.

Table (22): Total concentrations of the studied essential oils and phenolic compounds detected in intact plant and different cultures initiated *in vitro*.

Source	Essential oil mg/g		Phenolic compounds mg/g	
	leaf	stem	leaf	stem
Intact plant	0.936	2.255	1.394	0.696
Callus culture	1.323	0.423	1.138	0.900
Cell suspension culture	1.645	0.827	1.186	1.480
Callus culture + 50mM NaCl	0.401	0.740	0.649	0.634
Callus culture + 100mM NaCl	3.107	1.213	2.631	1.002

3.6-Water Extract:

The extract appeared with dark brown color and weighting 10% of the dry material of plant. This extract had a specific odor.

3.7-Ethanolic Extract:

Extraction with ethanol resulted in a gelatinus form weighing 13% of the dry material of plant.

3.8 -Detection of Some Active Compounds:

Different chemical reagents and solutions were used for detection of various active compounds found in the *S. officinalis*, displayed in table (23).

The pH of water extract of *S. officinalis* was 5.98 which indicates the acidity of water extract.

Results obtained by chemical detection indicated the presence of flavonoids, glycosides, saponins and terpenes in plant extracts.

Table (23): Detection of some secondary metabolites in callus cultures initiated from leaf explants. NaCl as an elicitor was added at a concentration of 100mM.

Type of secondary metabolites	Result of detection
Tannins	+ve
Saponins	+ve
Flavonoids	+ve
Glycosides	+ve
Terpenes	+ve
Steroids	-ve
Alkaloids	-ve
Coumarins	+ve

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

3.9-Effect of water and ethanolic extracts on the growth of some Bacteria:

Water and ethanol extracts taken from samples that gave the highest level of compounds were investigated for their antimicrobial activity against (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeriginosa* and *Bacillus cereus).*

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

3.9.1-Effect of Water Extract:

Results displayed in table (24) indicate that only high concentrations of sage water extract (20 and 40mg/ml) have inhibitory effects against Gram-positive bacteria (*S. aureus)* with 13 and 19.3mm inhibition zone diameter respectively. While *B. cereus* gave 10mm in a concentration of 20mg/ml, 16mm at a concentration of 40mg/ml). The inhibitory ability was more pronounced against *S. aureus*, whereas it showed no activity against *E. coli* and *P. aeruginosa* (Pictures 9 and 10).

These results agree with those of Cowan (1999) who regarded Gram-positive bacteria especially *B. subtilis* and *S. aureus* are sensitive to water extracts of *S. officinalis*.

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

In addition, El-Astal *et al*., 2005 referred that *E. coli* had no response against *Salvia officinalis* water extract.

3.9.2-Effect of Ethanolic Extract:

Sage ethanolic extract exhibited antimicrobial action against all tested microorganisms at high concentrations of extract (20 and 40) mg/l. The largest inhibition zone was observed against *S. aureus* recording 25mm in diameter at 40mg/ml whereas, reached 20mg/ml at a concentration of 20 mg/l. At low concentration of extract (5mg/ml) *S. aureus* showed slight inhibition followed by *E. coli* which showed (13 and 19)mm inhibition zone at (20 and 40)mg/ml subsequently. Slight inhibition was observed at a concentration of 10mg/ml. *B. cereus* was inhibited only at high ethanolic extract concentration (40mg/ml). It showed 10mm inhibition zone diameter, while *P. aeruginosa* showed only slight inhibition at 40mg/ml of the ethanolic extract (Pictures 11 and 12).

It is clear from the data presented in table (24) that among the four tested microorganisms, *S. aureus* was the most susceptible microbe to the two extracts. The finding that *S. aureus* is susceptible to a variety of extracts was recorded by several researchers (Okemo *et al.*, 2001; Madamombe and Afolayan, 2003). Furthermore, our results are in agreement with Jaslsenjak *et al.*, (1997) who showed the inhibitory activity of *S. officinalis* extracts against Gram-positive and Gram-negative bacteria at high concentrations.

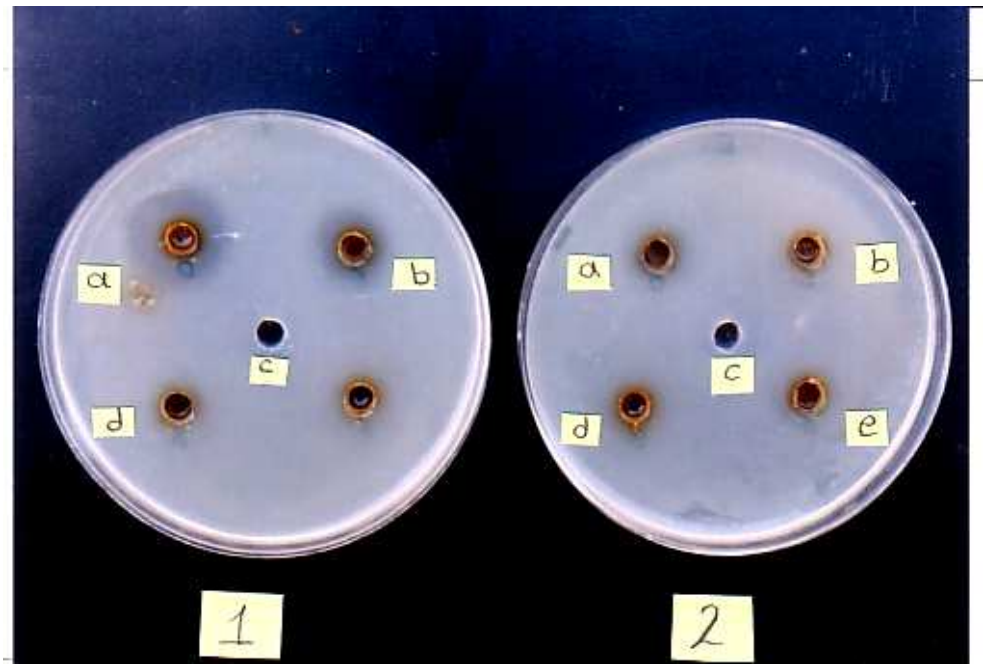
Table (24): Diameter of inhibition zone of *Salvia officinalis* extracts at various concentrations on some bacteria.

Type of Extract	Conc. mg/g	Diameter of inhibition zone (mm)			
		S. aureus	***E. coli***	***B. cereus***	***P. aeriginosa***
Water	40	19.3 ±0.67	-ve	16 ±1	-ve
	20	13 ±0	-ve	10 ±0	-ve
	10	-ve	-ve	-ve	-ve
	5	-ve	-ve	-ve	-ve
Ethanolic	40	25 ±0	19 ±0	17 ±0.58	10 ±0
	20	20.67 ±0.88	13 ±0	10 ±0	slight inhibition
	10	14 ±0.58	slight inhibition	-ve	-ve
	5	slight inhibition	-ve	-ve	-ve

-ve = no activity.
Values = are means of 3 readings ± SE.

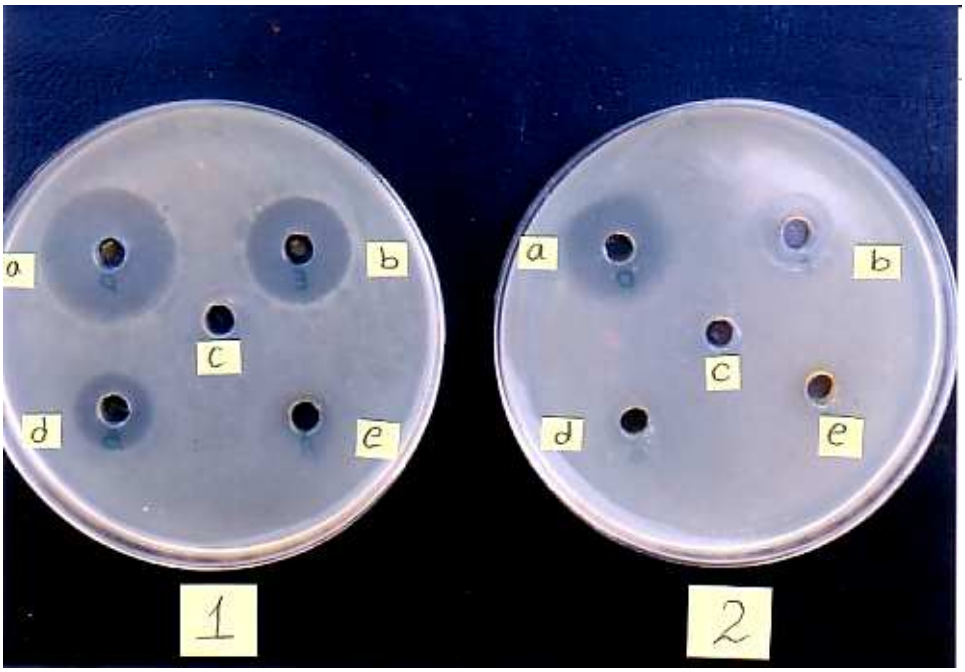
It appears that ethanolic extract is more efficient than water extract, the reason for this may be due to the compounds already extracted by ethanol particularly terpenes as stated by (Cowan, 1999).

Although *S. officinalis* contains many active compounds, most studies attributed the antimicrobial activity in sage to terpene compounds (Lamer *et al*., 1992; Moujir *et al.,* 1993; Tada *et al*., 1994; Masterova *et al*., 1996).

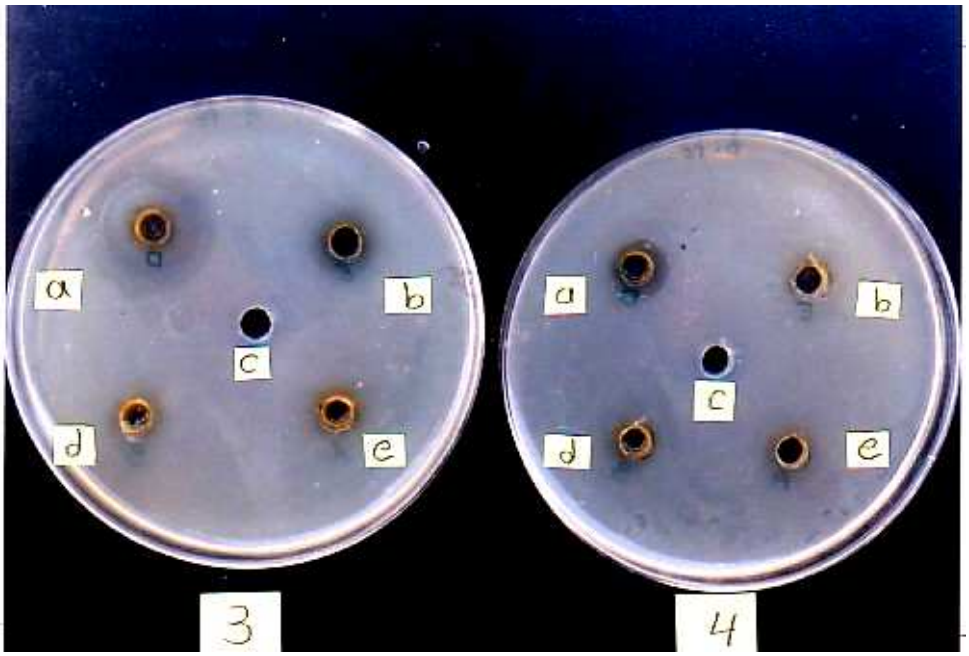


Picture 9: Effect of water extract on the growth of *S. aureus* and *E. coli.* Extract was taken from callus cultures initiated from leaf explants and grown on a medium containing 100mM NaCl.

Picture 10: Effect of water extract on the growth of *B. cereus* and *P. aeruginosa.* Extract was taken from callus cultures initiated from leaf explants and grown on a medium containing 100mM NaCl.
1= *S. aureus*, 2= *E. coli*, 3= *B. cereus*, 4= *P. aeruginosa.*
a= 40 mg/g, b= 20 mg/g, c= control, d= 10 mg/g, e= 5 mg/g.



Picture 11: Effect of ethanolic extract on the growth of *S. aureus* and *E. coli.* Extract was taken from callus cultures initiated from leaf explants and grown on a medium containing 100mM NaCl.



Picture 12: Effect of ethanolic extract on the growth of *B. cereus* and *P. aeruginosa.* Extract was taken from callus cultures initiated from leaf explants and grown on a medium containing 100mM NaCl.
1= *S. aureus*, 2= *E. coli*, 3= *B. cereus*, 4= *P. aeruginosa.*
a= 40 mg/g, b= 20 mg/g, c= control, d= 10 mg/g, e=5 mg/g.

Pitarokili *et al*., (1999) suggested that the activity of *S. officinalis* could be attributed to the existence of α-pinene (monoterpenes) that showed high inhibition activity against *Staphylococcus aureus, Staphylococcus epidermidis, Enterobacter cloacae, Klebsiella pneumonia*, *Escherichia coli, Pseudomonas aeriginosa, Candida albicans, Candida vaginalis* and *Torulopsis glabrata.*

Since that our results indicated the abundance of monoterpenes, which agreed with Velickovic *et al*., (2003) who indicated that *S. officinalis* is a potential source of monoterpenes and therefore it can be used as antimicrobial agent.

Meanwhile, the results disagree with Esteban *et al*., (1996) who reported that water extracts of aerial parts of *S. officinalis* have higher activity than ethanolic ones.

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In vivo and *in vitro* studies on the production of some secondary metabolites from *Salvia officinalis* L. and their antibacterial activity

A thesis

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By

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1.1- Introduction:

Plants have been an important source of medicine for thousands of years and are also the source of many modern medicines (Tripathi and Tripathi, 2003). It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from plant substances (Katzung, 1995). The most popular analgesic, aspirin and some of the most valuable anti-cancer agents such as Paclitaxel and Vinblastine are derived solely from plant sources (Verpoorte and Alfermann, 2000).

Garden sage, *Salvia officinalis*, is a medicinal herb that has long been used in popular medicine (Velickovic *et al*., 2003). The majority of sage is related to its content of an important active constituents mainly volatile oils, that composed of monoterpenes (e.g. linalool, geraniol, pinen and thujone) and sesquiterpenes, phenolic compounds as phenylpropanoids (e.g. coumarins), flavonols and flavons (e.g. quercetin, apigenin, rutin and luteolin), glycosides, tannins, and many other important compounds that have value as pharmaceuticals (Zegorka and Glowniak, 2001; Lu and Foo, 2002).

Sages are well-known for their anti-oxidative properties (Hohmann *et al*., 1999), used in the food processing industry, but applicable also to the area of human health (Pearson *et al*., 1997).

The plant extracts have a wide range of biological activities, such as: antibacterial, fungistatic, virustatic, astringent, eupeptic and anti hydrotic effect (Farag *et al*., 1989).

Sage has antiviral activity therefore, it includes an active ingredient for the treatment of acute and chronic bronchitis (Monolova *et al*., 1995). *Salvia officinalis* extract showed hypotensive properties, central nervous system depressant action and anti-spasmodic activity (Newall *et al*., 1996).

Gas liquid chromotography methods are used for separation, identification and quantification of the extracted compounds (Budhiraja, 2004).

The production of secondary metabolites *in vitro* is possible through plant tissue culture (Braz and Ellis, 1981; Deus and Zenk, 1982). *In vitro* study holds a potential for the production of high-quality plant based medicines (Murch *et al*., 2000). This can be achieved through different methods including micropropagation of cell lines capable of producing high yield of secondary compounds in cell suspension cultures (Zenk, 1978).

The accumulation of secondary products in plant cell cultures depends on many factors including the composition of the culture medium and environmental conditions (Stafford and Morris, 1986). The addition of stress agents to the culture medium may increase the formation of secondary metabolites in cultured cells (Ravishankar and Grewal, 1991).

As a result of the importance of this locally grown plant as a potential source of phytochemicals, this research work was aiming to:

1. Detection and quantification of some essential oil compounds (α-pinene, geraniol and linalool) and phenolic compounds (apigenin, rutin, coumarins and quercetin) which have a medicinal value in intact plant using GC method.
2. Initiation of tissue cultures from the plant, then examination of the cultures for the existance of these metabolites.
3. Inculsion of NaCl to culture medium as a stress stimulus agent in an attempt to increase the production of such secondary metabolites.
4. Comparison between productivity of such compounds between intact plant parts and tissue cultures after and before NaCl addition.
5. Study the inhibitory effect of water and ethanolic extracts on the growth of some microorganisms causing diseases.

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List of Abbreviations

Abbreviation	Full name
AD	Al-zheimer's disease
α	*alpha*
β	beta
°C	degree Celsius
CRD	Completely randomized design
DDH_2O	Double distilled water
EDTA	Ethylene diamine tetraacetate
GA_3	Gibberellic acid
GC	gas chromatography
hrs	hours
Kin	kinetin
LSD	Least significant differences
MEP	2-C-methyl-D-erythritol 4-phosphate/deoxy-xylulose phosphate
MS	Murashige and Skoog medium
n	number of replicates
NAA	1-naphthalene acetic acid
NaOCl	sodium hypochlorite
N. D	not detected
Rt	Retention time
S. E	standard error
UV	Ultraviolet (light)
wt	weight
2,4-D	2,4-diclorophenoxyacetic acid

Conclusions.

1. Callus cultures of *S. officinalis* can be induced and maintained on MS medium supplemented with 0.5 mg/l kin. and 0.05 mg/l 2,4-D using stem and leaf explants.
2. Plant tissue culture techniques are potential source for increasing the production of secondary metabolites. Rutin is increased in all tissue culture systems except when NaCl was added at 50mM to callus cultures initiated from leaf.
3. α-pinene production can be increased to more than four folds (4.670) by callus culture initiated from leaf and grown on a medium containing 100mM NaCl, cell suspension culture initiated from leaf (1.930).
4. Linalool production can be increased to about 3 folds in callus cultures initiated from leaf explants grown on a medium containing 100mM NaCl.
5. Geraniol is increased to (1.769) times using callus cultures initiated from leaf explants grown on a medium supplemented with 100mM NaCl.
6. Apigenin production can be increased to more than seven folds (7.735) using cell suspension cultures of stem explants.
7. Quercetin production is increased by (2.200) times in cell suspension cultures initiated from stem and callus cultures of leaf explants grown on a medium supplemented with 100mM NaCl (1.829).

8. Coumarin production can be increased in approximately 1.5 times in callus cultures initiated from stem and grown on a medium supplemented with 100mM NaCl.
9. Essential oils can be produced at higher concentrations using plant tissue culture systems rather than whole plant.
10. Phenolic compounds can be increased using *in vitro* systems in the callus cultures initiated from leaf and stem explants grown on a medium supplemented with 100mM NaCl.
11. High concentrations of sage water extract (20 and 40mg/ml) have inhibitory effects against Gram-positive bacteria (*S. aureus and B. cereus*) whereas it showed no activity against *E. coli* and *P. aeruginosa.*
12. Ethanolic extract can be used against all tested microorganisms at concentrations (40 and 20)mg/ml. The largest zone of inhibition was observed against *S. aureus* and *E. coli.*

Recommendations.

1. Investigation of other medicinal plants as a source for phytochemicals using tissue culture techniques.
2. Investigating physical elicitors such as high or low pH, different light intensities, different light qualities and different temperature regimes. for increasing the production of secondary metabolites.
3. Investigating other chemical elicitors such as, inclusion or exclusion of specific inorganic elements from the culture medium, addition of specific amino acids or addition of certain enzymes.
4. Using genetic engineering approaches for the manipulation of genes responsible for higher production such as gene transfer techniques and hairy root production using *Agrobacterium rizogenes* as a vector.
5. Application of plant cell bioreactors for mass production of secondary metabolites.
6. Purification of the target secondary metabolite then examination for antimicrobial activity to find out which compound is the most effective.

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الاهداء

أحب أن ابدأ كلماتي هذه ببراقة محبه و إخلاص وشكر، أتمنى أن يصل أعماق قلوبكم مثلما خرج من أعماق قلبي..
اهدي مشروعي هذا كثمره نجاح صغيره لعائلتي التي وقفت إلى جانبي و ساندتني في كل الأوقات..

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