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Qualitative and Quantitative Evaluation of Active Constituents in Callus of *Lavandula angustifolia* plant in Vitro

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Abstract:

This study was conducted to describe a protocol for the callus establishing culture of *Lavandula angustifolia* plant and estimating their content of volatile oil. The quantity of volatile oil callus tissues was compared with that of leaves production. Callus was induced from leaf explants on Murashige and Skoog medium (MS) supplemented with Naphthalene acetic acid (NAA) and Benzyl adenine (BA) in different concentrations. Maximum callus fresh weight was obtained in the combination of 10 mg/L BA and 3 mg/L NAA which reached 18 g after four weeks. The results of this work showed that the quantity of volatile oil from the highest fresh weight callus was 6 ml compared with quantity of 18g of leaves which gave 0.5 ml. Volatile oil of leaf and callus extracts were analyzed using gas chromatography mass spectrometry method (GC-MS) which showed linoleic acid (56.61%) and oleic acid (57.93%) as main components.

Key words: Callus induction, Essential oil, GC-mass, *Lavandula angustifolia*, MS medium.

Introduction:

Lavender (*Lavandula* spp.) is flowering plant of the mint family (Lamiaceae) that is endemic in the Mediterranean region, the Arabian Peninsula, the Canary Island and India. There are 39 species, many of which have aromatic and medicinal properties that are highly valued in the fragrance, pharmaceutical, food and flavor industries (1).

Lavender essential oil is produced in glandular trichomes found in surface of leaves and flowers. Essential (Volatile) oil is a mixture of organic compounds that give characteristic odor and flavor to plant and produce their own needs other than nutrition. Lavender oil contains more than 60 chemical compounds, the main components of leaves are 48.49% 2,4-dimethyl-7-ethyl-6,8-dioxabicyclo-3-octene, 12.45% triacontane, 9.44% camphor, 9.1% docosane and 8.1% 1,8-cineole. Other components can be found in minor quantities such as pinene, camphene, limonene and cryptone (2, 3).

The oil composition may vary depending on the geographical origin and environmental factors, such as climate, stage of plant growth and seasonal variations. The extraction and detection methods also effect on oil composition (3).

Oil is generally used in skin care for its anti-inflammatory, analgesic, antiseptic, antispasmodic, cardio protective, bactericidal and fungicidal properties and used in the treatment of burns and insect bites (4,5).

Lavender essential oil is widely used in food industry because of its biological properties against growth of microorganisms, particularly *Candida albicans*, *Streptococcus aureus* and *Escherichia coli* (6)

Production of plant metabolites depend on many environmental factors that effect on the quality and quantity of pharmaceuticals. Plant tissue cultures have been used as efficient alternative methods that cause an increase in secondary metabolites. Among the in vitro techniques callus cultures have been successfully established to produce secondary metabolites. The induction of callus growth is accomplished by differential application of growth regulators and control of conditions in the culture medium (7, 8)

Lavender is one of the most important medicinal plant that production of its secondary metabolites especially essential oil. The advantages of producing valuable secondary metabolites in tissue cultures include absence of seasonal constraints, predictability of production, the rapid and efficient isolation of target compounds compare to extraction from whole plant (9). Effect of different combination of plant growth regulators on

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Lavender plantlets focus on growth and secretory gland activity. Quantity and secretion of essential oil increased 150% in presence of BA (2, 10). The present study was carried out to optimize callus induction in order to provide useful offer for increasing efficiency of essential oil production.

Material and Methods:

The Lavender seeds obtained from nursery of Istanbul/ Turkey, after surface sterilization with 70% ethanol for 30 seconds and emerged in sodium hypochlorite for 10 min, seeds washed off with sterile distilled water and cultured on MS (11) free of growth regulators. Culture subjected to photoperiod 16/8 hours (light/ dark) in growth chamber, temperature was set at 25 °C. Germination was measured after 14 days. After germination, leaf explants of *Lavandula* were cut at the end into sections approximately 0.5 cm in length under aseptic conditions and cultured on MS medium supplemented with NAA (0, 1, 2 or 3) mg/l and BA (0, 5, 10 or 15) mg/l.

Extraction of essential oil

A Clevenger apparatus was used for hydrodistillation extraction of leaves and callus of *Lavendulla*, 18 g of leaf and (18,16.5,16 or 13) g of callus placed in round bottom flask with 120 ml distilled water. Then the flask was left for 12 hours to ensure that the extraction of essential oil was completed, the oil distillates were collected with small dark glass bottle, sealed and stored in refrigerator at a temperature 4 °C until further analysis (12).

Gas chromatography/ Mass spectrometry analysis (GC-MS)

Quantitative and qualitative analysis of *Lavendula* extracts were performed using GC-MS (Model QP 2010, Shimadzu, Japan) Inert Cap Pure Cap Wax capillary column was used (30m x 0.25 mm x 0.25 µm film thickness) with helium as carrier gas at a flow rate of 1.53 ml/ min. The source was operated in positive ionization mode (electron impact energy: 70eV) and the detection was performed in full-scan mode. The inlet and the transfer line temperature was maintained at 170°C, while the ion source was kept at 220°C. Samples were injected in split or split less mode (2:1) and separated using temperature gradient program as follows: 70°C for 3 min, to 120°C at 15°C/ min and then maintained 120°C for 2min; then to 200°C for further 8 min. GC-MS spectra were evaluated by

Postrun software and searched in National Institute of Standards and Technology (NIST) MS Search V2.0 browsers (13).

Statistical Analysis

The statistical analysis system- SAS (2012) program was used to effect of difference factors in study parameters. Least significant difference-LSD test was used to significantly compare between means in this study (14).

Results and Discussion:

Seed germination

The sterilized seeds of *L.angustifolia* were grown on MS free medium. Seedlings were fully germinated in range 3-4 weeks (Fig 1).



Figure 1. Seedling of *Lavandula* grown on MS hormone free medium after 20 days

Callus induction

Callus induction from leaf explants were studied by using MS medium supplemented with different concentrations of NAA, BA and their combinations. Table (1) shows that adding BA exhibited positive effect on *Lavendula* callus growth at concentrations 5 mg/l in combination with 2 mg/l NAA. Inclusion of BA at concentration 10 mg/l with 2 mg/l or 3 mg/l NAA gave significantly higher callus fresh weight (16) g and (18) g respectively. On the other hand, the lowest fresh weight (2) g was obtained in medium with 5 mg/l BA.

The results are in agreement with (15) who proved lavender callus grown on MS medium containing BA and NAA, while (16) found the best medium for callus induction was MS with 0.1 mg/l IAA, 0.002 mg/l BA and 2,4-D at 0.2 mg/l recorded complete callus formation after 6 weeks incubation. (17) Mentioned that suitable treatment for callus induction from leaf explant of Lavender was MS with 2 mg/l 2,4-D and 2 mg/l BA.

Table 1. The effect of NAA and BA and their combinations on fresh weight (g) of callus initiation from leaf explants after 30 days of culture on MS medium.

BA(mg/l)	NAA(mg/l)				Mean
	0	1	2	3	
0	0	2.5	6	3.8	3.07
5	2	2.2	13	8.4	6.40
10	3	4	16.5	18	10.37
15	5	8.4	4.2	16	8.40
Mean	2.50	4.28	9.93	11.55	---

LSD: NAA: 2.944 *, BA: 2.944 *, NAA*BA: 5.371 *.

Induced callus of Lavender in this study were different in color (brown, brownish cream, cream and white), so the explant treated with BA produced a cream or white while brownish callus

was produced on medium containing NAA. These results indicate that treatment of explants with various hormones had different results in color of callus production as shown in Fig. 2.

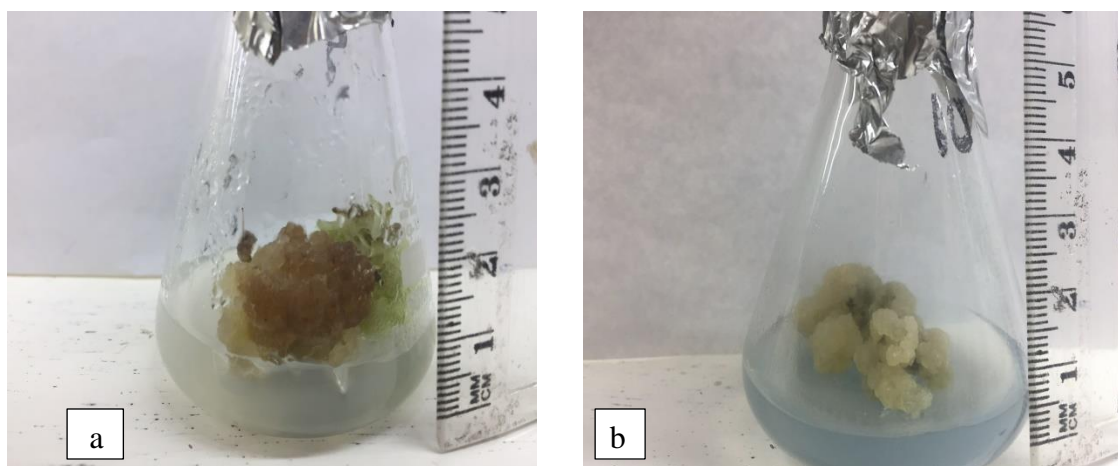


Figure 2. Callus induction from leaf explant of *L. angustifolia* plant
a. On medium containing NAA b. On medium containing BA

(17) Mentioned that the type of explant did not have an effect on callus color but the type of hormones had different results in the color of callus production.

GC-Mass analysis of leaf extract

The results of GC-mass analysis of *Lavandula* leaf extract show that the essential oil of leaves contain 12 components (Table2, Fig.3) and the linoleic acid has higher concentration than other compounds reaches to 47.63% follow by methyl palmitate 12.92%, octadecyl acrylate 3.14 and tricosanoic acid 2.87.

Table 2. Essentials oil components concentrations of *Lavandula* leaf extract

N	Name of compound	%
1	Heptyl acetate	0.40
2	Trimethyl silyl	0.56
3	Lauric acid	0.51
4	Butyl acetate	0.03
5	Methyl palmitate	12.92
6	Linoleic acid	47.63
7	Isopropyl palmitate	0.49
8	Octadecyl acrylate	3.14
9	Henicosanoate	1.74
10	Tricosanoic acid	2.87
11	Henicosane	2.06
12	Stearic acid	0.43

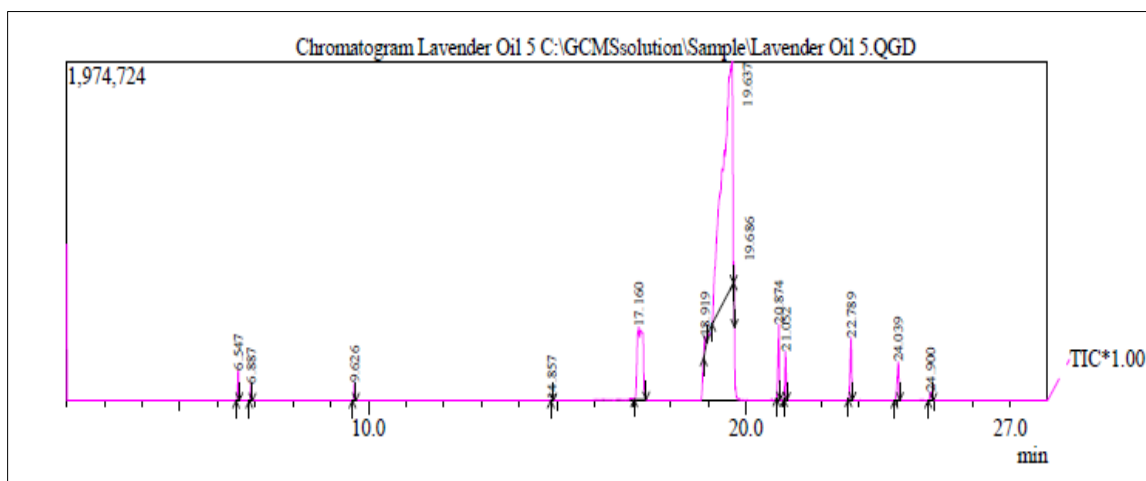


Figure 3. GC-mass analysis of essential oil of *Lavandula* leaf extract

These results did not agree with (3), wherein they stated the major characteristic components of *Lavandula* essential oil represented by cineole, borneol and camphor. This disagreement could be due to genome of plant, environment conditions and extraction methods. Thus, these changes led to huge effect in quantity and quality of essential oil and its components. (3)

GC-mass analysis of *L. angustifolia* callus extracts

The data indicate that 16-20 components were identified in callus extracts of *Lavandula*. The major constituents being, linoleic acid 57.84, oleic acid 57.93, methyl palmitate 18.29 and pentadecanoic acid 16.18 as shown in Tables (3, 4, 5, 6) and Figs. (4, 5, 6, 7).

Table 3. Essentials oil components concentrations in callus initiation from leaf explant on MS medium with 3 mg/l NAA and 10 mg/l BA

N	Name of compound	%
1	Hexanoic acid	0.18
2	Formyl glycol	0.14
3	Pentadecanoic acid	16.18
4	Linoleic acid	57.84
5	Dibromododecane	0.30
6	Tridecane	0.99
7	Tritetracontane	0.62
8	Hendecenal	0.49
9	Dibromotetr apenta contane	5.59
10	Undecanoic acid	0.72
11	Isopropyl octadecyl sufite	1.11
12	Bromotriacontane	0.90
13	Tridecyl acetylene	0.21
14	Tridecanol	2.83
15	Eicosenoic acid	4.81
16	Stearic acid	3.89
17	Methylene decane	0.89
18	Tetratetra decane	1.01
19	Octyl hexyl ether	0.54
20	Stearic hydrazide	0.77

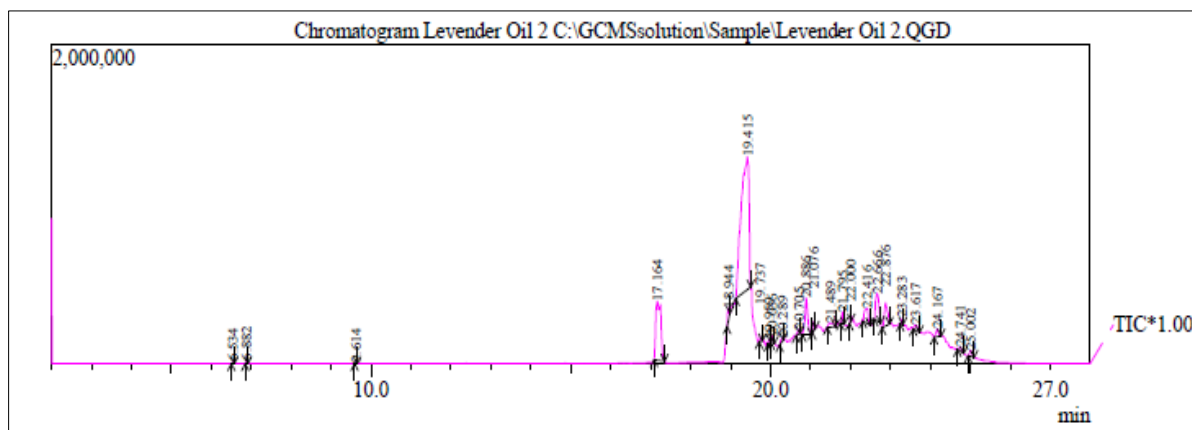


Figure 4. GC-mass analysis of essential oil of callus initiation from leaf explants on MS medium with 3 mg/l NAA and 10 mg/l BA

Table 4. Essentials oil components concentrations in callus initiation from leaf explant on MS medium with 2 mg/l NAA and 10 mg/l BA

N	Name of compound	%
1	Citronellyl propionate	0.65
2	Methyl palmitate	18.29
3	Vitamin E	1.05
4	Vitamin E acetate	16.16
5	Tridecane	1.69
6	Linoleic acid	43.86
7	Myristic acid	2.91
8	Dibromotetrapentacontate	2.03
9	Nano hexacontanoic acid	1.09
10	Tridecane	0.78
11	Octacosane	1.19
12	Penta triacontanol	0.69
13	Leucyl glycyglycine	0.39
14	Octadecyl pentafluoropropionatac	2.89
15	Hexacosane	2.23
16	Sulfonic acid	3.25
17	Diphenyl pentacene	1.05

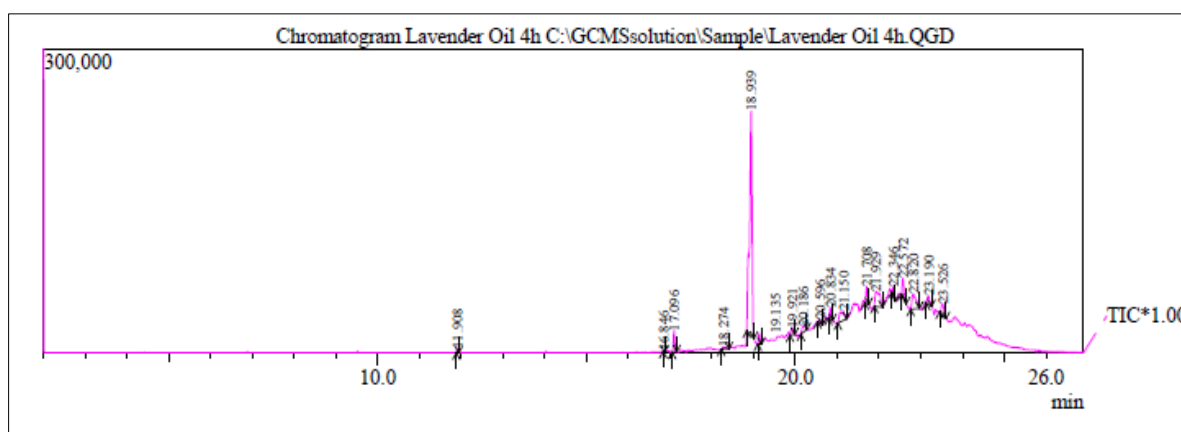


Figure 5. GC-mass analysis of essential oil of callus initiation from leaf explants on MS medium with 2 mg/l NAA and 10 mg/l BA

Table 5. Essentials oil components concentrations in callus initiation from leaf explant on MS medium with 3 mg/l NAA and 15 mg/l BA

N	Name of compound	%
1	Pinacoline	0.07
2	Octanone	0.18
3	Stearic acid	4.89
4	Oleic acid	47.17
5	Palmitic acid	2.11
6	Nona decane	4.49
7	Tritetracontane	8.93
8	Isopropyloctadecyl sulfite	2.60
9	Hexadecane	10.28
10	Icosane	3.17
11	Tetratetracontane	3.80
12	Tridecane	3.26
13	Pentatriacontanol	1.46
14	Dibromotetra pentacontane	2.25
15	Isopropyl	0.97
16	Hexyloctyl ether	1.09

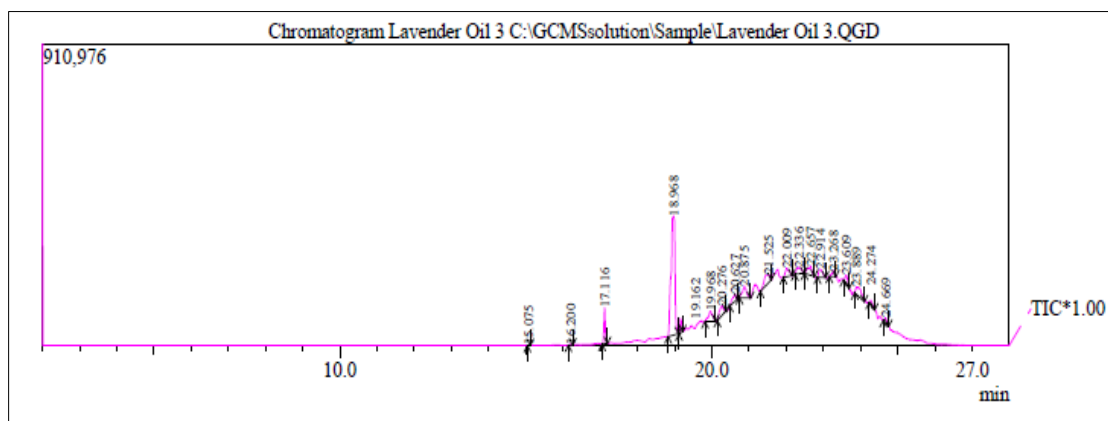


Figure 6. GC-mass analysis of essential oil of callus initiation from leaf explants on MS medium with 3 mg/l NAA and 15 mg/l BA

Table 6. Essentials oil components concentrations in callus initiation from leaf explant on MS medium with 2 mg/l NAA and 5 mg/l BA

N	Name of compound	%
1	Acetylacetamide	0.44
2	Oxalic acid	0.06
3	Heptyl acetate	3.11
4	Butyric anhydride	0.72
5	Oleic acid	57.93
6	Nonanoic acid	1.49
7	Nonene	1.58
8	Tetradecane	1.86
9	Dodecyl iodide	0.57
10	Dodecane	6.6
11	Tritetra contane	2.72
12	Dodecanol	7.72
13	Octanone	1.35
14	Octadecyl sulfonsoure	2.99
15	Dodecyl bromide	6.37
16	Docosanedioic acid	2.09
17	Heptacosene	2.84

Callus extracts also contained vitamin E acetate 16.16, hexadecane 10.28, stearic acid 4.89, sulfonic acid 3.25, and many other components.

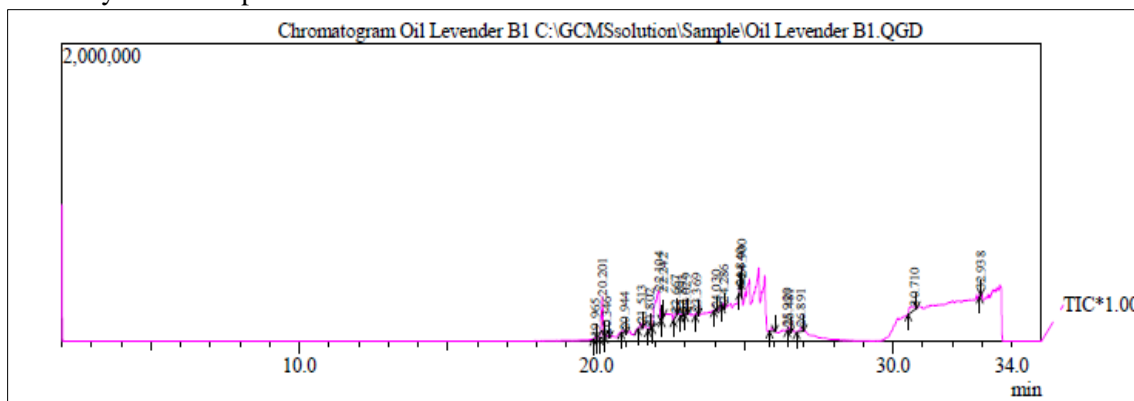


Figure 7. GC-mass analysis of essential oil of callus initiation from leaf explants on MS medium with 2 mg/l NAA and 5 mg/l BA

The data showed that callus extract produced high percentage for some constituents as compared with the same constitute in leaf extract. Linoleic acid percentage reached 57.84% in callus

extract of *Lavandula*, while it was found to be 47.63% in leaf extract. Other constituents such as oleic acid, vitamin E acetate, pentadecanoic acid were found only in callus extracts in different

percentage and absence in *Lavandula* leaf extract. It was clear that callus extracts of *Lavandula* plant contain more components than that found in leaf extract and at higher percentages. The essential oil (is mixture of chemical compounds mainly monoterpenes, diterpenes and sesquiterpenes) production is changed quantitatively and qualitatively by factors such as photoperiod, light quality, nutrition, temperature, growth regulators and storage structure. Thus, it can be concluded that application of auxin and cytokinin enhanced biochemical and physiological parameters which influenced the terpenoid pathway, which improved the quality and quantity of essential oil. Plant growth regulators enhance biomass production which results in increasing biosynthesis of secondary products (18, 19, 20).

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

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التقدير الكمي والنوعي للمركبات الفعالة المستخلصة من كالس نبات اللافندر خارج الجسم الحي

سمية فاضل حمد زينب عمران سلمان بشرى محمد جابر علوش

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الخلاصة:

صممت هذه الدراسة لإنشاء مزرعة لكالس نبات اللافندر وتقدير محتواه من الزيت الطيار وقورنت كمية الزيت المستخلص من الكالس مع كمية الزيت المستخلص من الاوراق. استحث الكالس من اجزاء من اوراق نبات اللافندر على الوسط الغذائي موراشيچ وسكوج MS مع اضافة تراكيز مختلفة من منظّمات النمو وهي نفتالين اسد NAA والبنزل ادنين . BA تم الحصول على اعلى وزن طري للكالس من التوليفة 10 ملغم/لتر NAA و3 ملغم/لتر BA حيث بلغت 18 غم بعد اربع اسابيع من الزرع . وظهرت النتائج بان كمية الزيت الطيار المستخلص من هذا الكالس كانت 6 مل مقارنة مع كمية الزيت المستخلص من 18 غم من الاوراق وكانت 0.5 مل. استخدمت تقنية كروماتوغرافيا الغاز السائل GC-MAS لمعرفة كمية ونوعية المركبات الفعالة الموجودة في الزيت الطيار وبينت النتائج اكثر المركبات تفوقا هي حامض اللينولك وبلغت نسبته 56.61% وحامض الاوليك وكانت نسبته 57.93%.

الكلمات المفتاحية: استحث الكالس، الزيوت الطيارة، GC-mass ، *Lavandula angustifolia* ، وسط MS.