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Evaluation of Secondary Metabolites Contents in different explants of Galbanum Medicinal plant in vitro culture

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ABSTRACT: Galbanum (*Ferula gummosa*) is a perninal plant belonging to apiaceae family and one of the most valuable medical plants containing secondary metabolites that are widely used in food, pharmacy and cosmetic industries. Its essential oil contains terpenoid hydrocarbons such as α-pinene, β-pinene, *Δ*-3karen, myrceneandsesquiterpene alcohols. The objectives of the present study were the optimization of galbanumtissue culture to produce α-pinene, investigating the secondary metabolites in the embryos and callus of different explants, and comparing these compounds with secondary metabolites of plants from natural pastures. After disinfection of seeds and embryos culture in the 1/4MS medium with BA hormone, explants were prepared from different parts of the plant. Then, explants were placed in MS mediums with different levels of BA (2 mg/l) and NAA (10 mg/l). Finally, secondary metabolites were analyzed by gas chromatograph connected to a mass spectrometer (GC/MS) to assess the amount and type of compounds in seedlings from embryo, petiole, cutting the embryo, hypocotyl and root callus. Results of secondary metabolites analysis showed that root and hypocotyl callus had the most amounts of the components. Hypocotyl and root callus had the highest amount of α-pinene (53.64%) and 3- carene (10.59). Root callus had the highest amount of α-thujone (4.37%), thujone (7.51%) and camphor (24.57%). Petiole callus had the highest amount of α-pinene (7.35%) and seedlings from embryos had the highest amount of decamethylcyclopentasiloxane (11.53%) and dodecamethylcyclohexasiloxane (10.43%).

Keywords: Galbanum. Embryo culture, Secondary metabolites, terpenoids, GC/MS.

INTRODUCTION

Despite advances in the field of chemical artifacts, we still require biological resources to produce a number of secondary metabolites like pharmaceuticals (Pezzuto, 1995). Plants, as an important source of drugs, plays a key role in the health of the world people and a lot of valuable medicinal compounds are among the secondary metabolites of plants. In some cases, the artificial synthesize of these materials are difficult or synthetic production is not economically affordable. On the other hand, plant tissue culture can provide an appropriate technique to preserve endangered species and mother genotypes as valuable sources of germplasm (Farshadfar and Bakhshi Khaniki, 2010). Increased use of plant cell and organ culture techniques has led to large-scale production of plant metabolites. Research advances in molecular biology have created new aspects in vitro culture such as increasing yield and new products from transgenic plants (Ramachandra, 2002). Biotechnology provides an opportunity to obtain the desired compound using cultured cells, tissues or organs in vitro. Micropropagation techniques development for the production of some medicinal plants as well as the necessity of its use in many cases have been reported (Naik, 1998). Galbanum (*F. gummosa*) is one of the valuable herbal plant containing secondary compounds.

This perennial herb belongs to the Apiaceae family, Angiosperms phylum and Dicotyledonous class (Azadbakht, 1999). Medicinal importance of galbanum in the world is increasing, so that villagers used it for treating stomach worms, stomach pains, toothache and snake bites in the past and recent technology advances have opened a new window for its chemical analysis. Analysis by gas chromatography have indicated that its juice contains more than 95 valuable chemical and pharmaceutical compounds. Each of these compounds have different applications range from strengthening the heart to controlling human pathogenic bacteria and other animal and herbal consumptions (Ahmadi *et al.*, 1991). Talebi *et al.* (2008) confirmed that α-pinene and α-pinene are two main compounds in Iranian galbanum essential oil. Galbanum is a very valuable medicinal, industrial and aromatic endemic plant of Iran. Nevertheless, it has faced extinction danger due to long period of dormancy, being monocarpic and improper harvesting of natural pastures (Zargari, 1989). Therefore, the present study was aimed to investigate in vitro micropropagation of this plant to developed biotechnological studies to help preventing the extinction, evaluating its secondary metabolites and developing an applied method for mass production.

MATERIALS AND METHODS

A. Sample preparation

Galbanum seeds obtained from cooperative company of Pounezar (Fereydunshahr, Iran). Two Murashige and Skoog medium (MS) and a quarter of MS (for embryo) were used. At first, seeds were placed under running water for 24 hours in order to soften the endosperm tissue surrounding the embryo. Then, the seeds were washed by liquid soap and then with distilled water to clean up the pollutions and potential inhibitors in the seed coat. Other steps to disinfect the surface of seeds were performed in chamber with sterile air flow (laminar flow). To this purpose, the seed were repeatedly disinfected using 70% ethanol for 1 min and 2.5% sodium hypochlorite for 25 min. Then, the seeds were thoroughly washed with double strile distill water for three times. After sterilization, the embryonic axes were removed from the seed coats and embryos were placed inside glass jars containing 1/4 MS medium and 2 mg/lit BA hormone. After the establishment of the embryos in vitro, they were transferred to a chamber with a temperature of 20°C and light intensity of 3000 lux for 16 h. To investigate the secondary metabolites found in various parts of callus and plants produced in vitro in comparison with compounds from natural areas, samples were air-dried at room temperature and weighed in proper amounts. The secondary metabolites present in each sample were analyzed by gas chromatograph connected to a mass spectrometer (GC/MS).

Studied samples were as follows:

Seedling obtained from embryos cultured in the quarter-MS.

Petiole callus cultured in 2 mg/l BA and 10 mg/l NAA.

Cutting the embryo callus cultured in 2 mg/l BA and 10 mg/l NAA.

Hypocotyl callus cultured in 2 mg/l BA and 10 mg/l NAA.

Root callus cultured in 2 mg/l BA and 10 mg/l NAA.

Fig. 1. Different explant of Galbanum in vitro. (A: Seedling obtained from embryos cultured in the quarter-MS. B: Petiole callus cultured in 2 mg/l BA and 10 mg/l NAA. C: Cutting the embryo callus cultured in 2 mg/l BA and 10 mg/l NAA. D: Hypocotyl callus cultured in 2 mg/l BA and 10 mg/l NAA. E: Root callus cultured in 2 mg/l BA and 10 mg/l NAA).

B. Gas chromatograph connected to a mass spectrometer (GC/MS) analysis

Gas chromatograph connected to a mass spectrometer (GC/MS) using mass detector with electron ionization source 5975 Agilent (EI) coupled with chromatograph 5975 Agilent and HP-5MS column, length 30 m, internal diameter 0.25 mm and a film thickness of 0.25 mm was used. Intel temperature was 280°C and intel ionization temperature was 150 °C.

Kudarpel temperature was 230°C. The interface temperature between the GC and MS was 280°C. Oven temperature was set at 40°C for 5 min and then increased to 270°C with a constant speed of 5°C/min, and finally fixed at 280 °C for 5 min. To measure the essential oils, Headspace sampling system was used as the sample was placed at 120 °C for 30 min.

RESULTS

A. Compounds in seedlings of fetal growth (control seedlings)

Fig. 1 shows the compounds found in seedlings derived from emberyos. The results showed that the seedlings grown in quarter-MS medium contained heptanal, α-pinene, α-pinene, 2-pentyfuran, 3-carene, 1-limonene, decamethylcyclopentasiloxane and dodecamethylcyclohexasiloxane.

Regarding the results shown in Table 1, it was revealed that compounds in the seedling according to the exit time were as follow; heptanal (1.10%), α-pinene (48.71%), α-pinene (5.31%), 2-pentyfuran (3.42%), 3-carene (5.05%), 1-limonene (2.87%), decamethylcyclopentasiloxane (11.53%) and dodecamethylcyclohexasiloxane (10.43%) .

B. Compounds in petiole callus

The results obtained from the graph of compounds derived from petiole callus indicated that its compounds according to the exit time were as follow; heptanal (0.74%), α-pinene (49.45%), α-pinene (7.35%), 2-pentyfuran (3.55%), 3-carene (5.24%), decamethylcyclopentasiloxane (9.81%) and dodecamethylcyclohexasiloxane (8.51%).

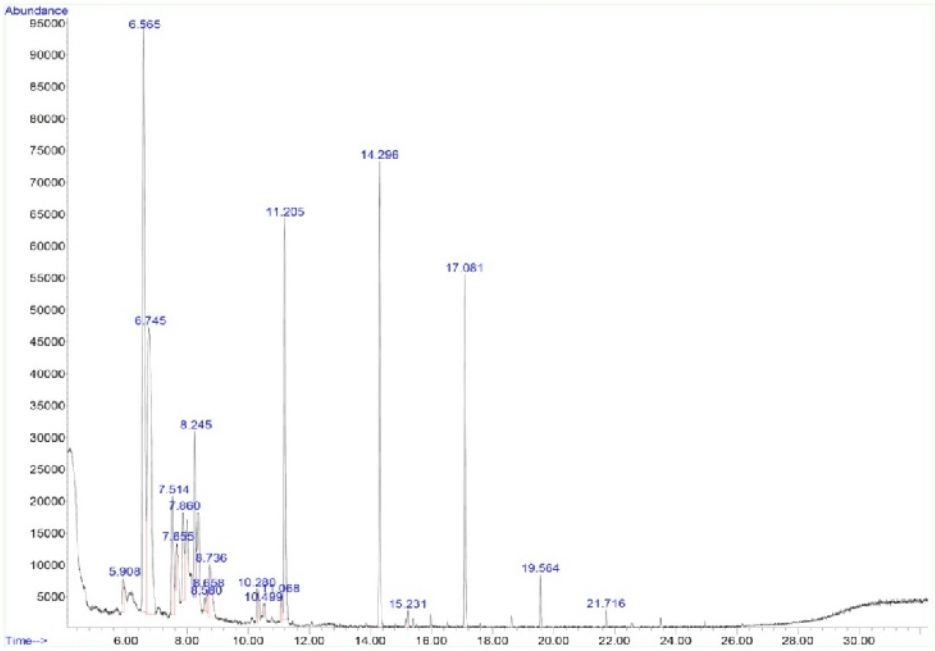

Fig. 2. Compounds in seedlings derived from embryos (control).

Table 1: Compounds in seedlings derived from embryos.

Compounds	Molecular formula	RT	KI	Surface under the pick (%)
Heptanal	$C_7H_{14}O$	5.90	906	1.10
Alpha-Pinene	$C_{10}H_{16}$	6.56	935	26.30
Alpha-Pinene	$C_{10}H_{16}$	6.74	934	22.40
Beta-Pinene	$C_{10}H_{16}$	7.51	978	4.19
Beta-Pinene	$C_{10}H_{16}$	7.65	984	1.11
2-Pentylfuran	$C_9H_{14}O$	7.86	994	3.42
3-Carene	$C_{10}H_{16}$	8.24	1012	5.051
Limonene	$C_{10}H_{16}$	8.73	1035	2.87
Decamethylcyclopentasiloxane	$C_{10}H_{30}O_5Si_5$	11.20	1157	11.53
Dodecamethylcyclohexasiloxane	$C_{12}H_{36}O_6Si_6$	14.29	1327	10.43

Table 2: Compounds in petiole callus.

Compounds	Molecular formula	RT	KI	Surface under the pick (%)
Heptanal	$C_7H_{14}O$	5.90	905	0.774
Alpha-Pinene	$C_{10}H_{16}$	6.57	935	26.59
Alpha-Pinene	$C_{10}H_{16}$	6.74	934	22.85
Beta-Pinene	$C_{10}H_{16}$	7.51	978	4.04
Beta-Pinene	$C_{10}H_{16}$	7.65	984	3.31
2-Pentylfuran	$C_9H_{14}O$	7.86	994	3.55
3-Carene	$C_{10}H_{16}$	8.24	1012	5.24
Decamethylcyclopentasiloxane	$C_{10}H_{30}O_5Si_5$	11.20	1053	9.81
Dodecamethylcyclohexasiloxane	$C_{12}H_{36}O_6Si_6$	14.29	1327	8.51

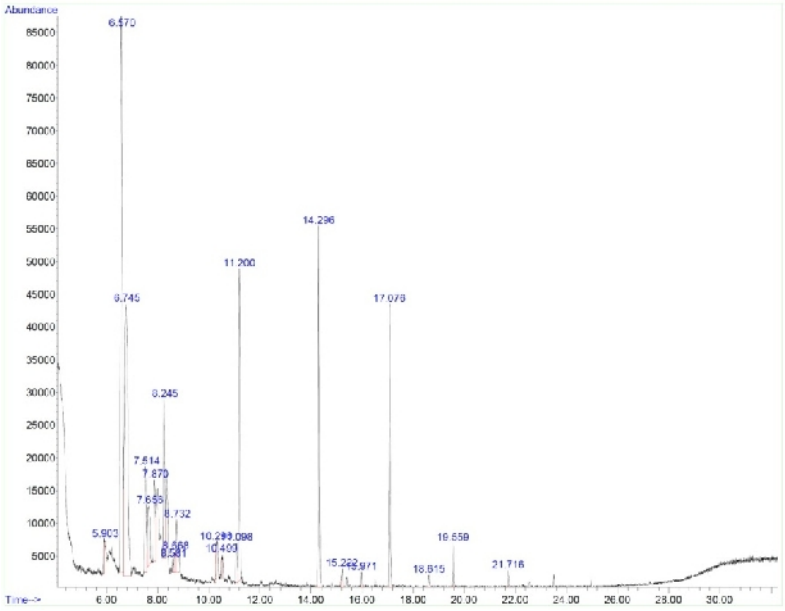

Fig. 3. Compounds in petiole callus.

C. Compounds of cutting the embryo callus

The results obtained from the graph of compounds derived from cutting the embryo callus showed that its compounds according to the exit time were as follow; heptanal (1.68%), α-pinene (40.52%), α-pinene (6.76%), 2-pentyfuran (3.66%), 3-carene (7.00%), eucalyptol (2.39%), α-thujone (0.86%), camphor (1.70%), decamethylcyclopentasiloxane (11.22%) and dodecamethylcyclohexasiloxane (9.19%) .

Table 3: Compounds in cutting the embryo callus.

Compounds	Molecular formula	RT	KI	Surface under the pick (%)
Heptanal	$C_7H_{14}O$	5.90	905	1.68
Alpha-Pinene	$C_{10}H_{16}$	6.57	935	33.58
Alpha-Pinene	$C_{10}H_{16}$	6.74	943	6.93
Beta-Pinene	$C_{10}H_{16}$	7.51	978	5.60
Beta-Pinene	$C_{10}H_{16}$	7.65	984	1.16
2-Pentylfuran	$C_9H_{14}O$	7.86	994	3.66
3-Carene	$C_{10}H_{16}$	8.24	1012	7.00
Eucalyptol	$C_{10}H_{18}O$	8.72	1035	2.39
Alpha-thujone	$C_{10}H_{16}O$	10.50	1121	0.86
Camphor	$C_{10}H_{16}O$	11.08	1151	1.70
Decamethylcyclopentasiloxane	$C_{10}H_{30}O_5Si_5$	11.20	1053	11.22
Dodecamethylcyclohexasiloxane	$C_{12}H_{36}O_6Si_6$	14.29	1327	9.19

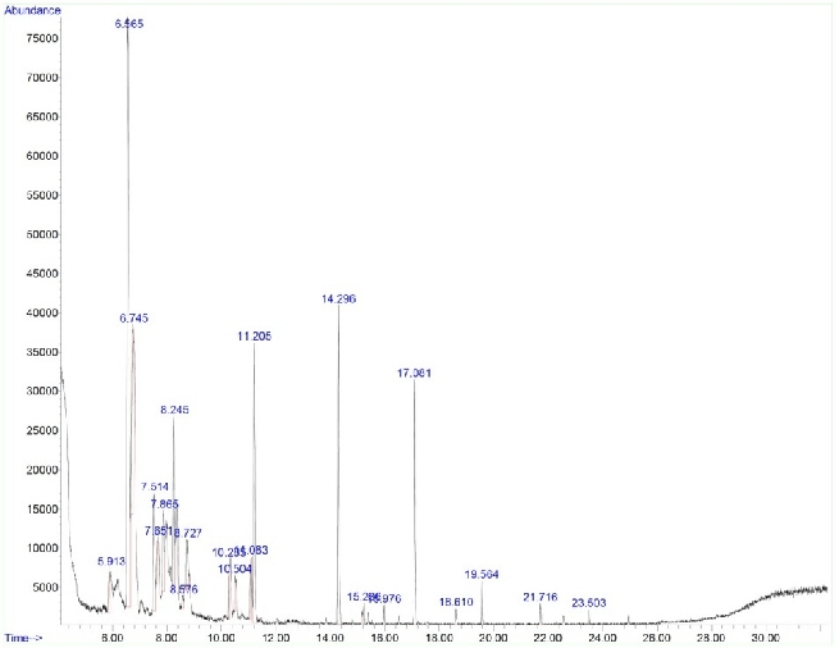

Fig. 4. Compounds of cutting the embryo callus.

D. Compounds of hypocotyl callus

The results obtained from the graph of compounds derived from hypocotyl callus indicated that its compounds according to the exit time were as follow; heptanal (0.70%), α-pinene (53.64%), α-pinene (3.56%), 2-pentyfuran (2.29%), 3-carene (10.59%), eucalyptol (3.20%), α-thujone (1.13%), camphor (5.23%), decamethylcyclopentasiloxane (5.72%) and dodecamethylcyclohexasiloxane (4.16%) (Table 4, Fig. 4).

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E. Compounds in root callus

The results obtained from the graph of compounds derived from root callus indicated that its compounds according to the exit time were as follow; α-pinene (30.73%), α-pinene (2.71%), camphene (1.46%), 2-pentyfuran (0.92%), 3-carene (7.74%), 1,8-cineole (9.07%), cis-thujone (7.51%), α-thujone (4.37%), camphor (24.57%), decamethylcyclopentasiloxane (1.57%) (Table 5, Fig. 5).

Table 4: Compounds in hypocotyl callus.

Compounds	Molecular formula	RT	KI	Surface under the pick (%)
Heptanal	$C_7H_{14}O$	5.90	905	0.70
Alpha-Pinene	$C_{10}H_{16}$	6.57	935	25.61
Alpha-Pinene	$C_{10}H_{16}$	6.74	943	23.93
Alpha-Pinene	$C_{10}H_{16}$	7.51	978	3.56
Beta-Pinene	$C_{10}H_{16}$	7.65	984	2.29
2-Pentylfuran	$C_9H_{14}O$	7.86	994	5.59
3-Carene	$C_{10}H_{16}$	8.24	1012	4.99
Eucalyptol	$C_{10}H_{18}O$	8.72	1035	3.20
Alpha-thujone	$C_{10}H_{16}O$	10.50	1121	1.11
Camphor	$C_{10}H_{16}O$	11.08	1151	5.23
Decamethylcyclopentasiloxane	$C_{10}H_{30}O_5Si_5$	11.20	1053	5.72
Dodecamethylcyclohexasiloxane	$C_{12}H_{36}O_6Si_6$	14.29	1327	4.16

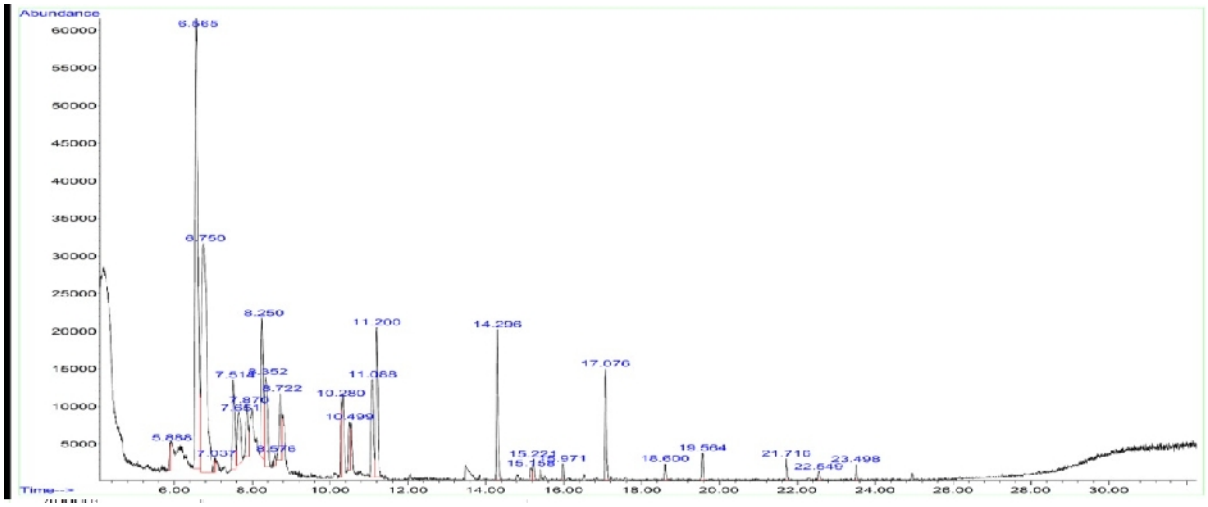

Fig. 5. Compounds in hypocotyl callus.

Table 5: Compounds in root callus.

Compounds	Molecular formula	RT	KI	Surface under the pick (%)
Alpha-Pinene	$C_{10}H_{16}$	6.56	935	19.18
Alpha-Pinene	$C_{10}H_{16}$	6.75	943	11.54
Camphene	$C_{10}H_{16}$	7.51	978	1.46
Beta-Pinene	$C_{10}H_{16}$	7.65	984	2.71
2-Pentylfuran	$C_9H_{14}O$	7.85	994	0.92
3-Carene	$C_{10}H_{16}$	8.25	1012	4.15
3-Carene	$C_{10}H_{16}$	8.36	1017	3.59
1,8-Cineole	$C_{10}H_{18}O$	8.71	1034	9.07
cis-thujone	$C_{10}H_{16}O$	10.28	1109	7.51
Alpha-thujone	$C_{10}H_{16}O$	10.49	1121	4.37
Camphor	$C_{10}H_{16}O$	11.08	1151	24.57
Decamethylcyclopentasiloxane	$C_{10}H_{30}O_5Si_5$	11.20	1053	1.57

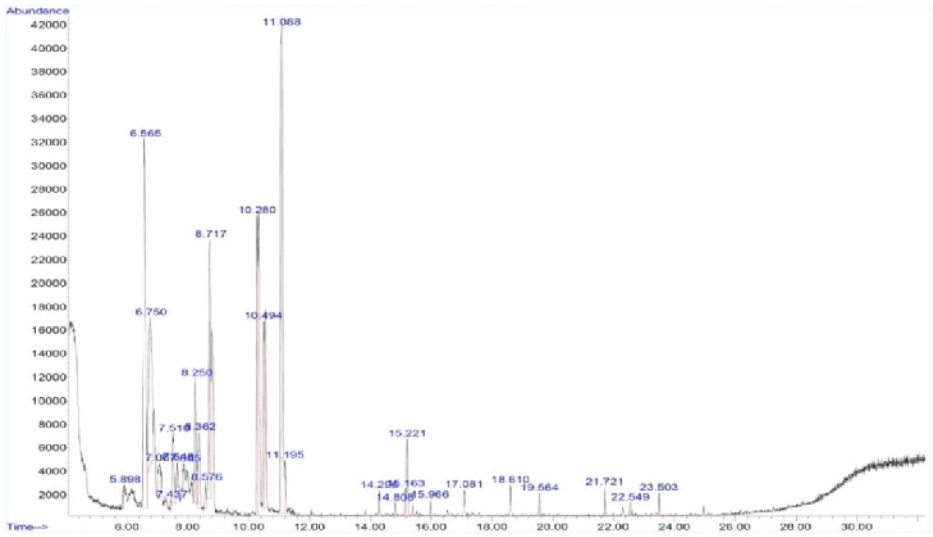

Fig. 6. Compounds in root callus.

F. Comparison of compounds in different explants

Table 6: Comparison of compounds in different explants.

Compounds	Percent of compounds in the samples				
	seedling	**petiole callus**	**cutting the embryo callus**	**Hypocotil callus**	**root callus**
1,8-Cineole	-	-	-	-	9.07
2-Pentylfuran	3.42	3.55	3.66	2.29	0.92
3-Carene	5.05	5.24	7.00	10.59	7.74
Alpha-Pinene	48.71	49.45	40.52	53.64	30.73
alpha-thujone	-	-	0.86	1.13	4.37
Beta-Pinene	5.31	7.35	6.76	3.56	2.71
Camphene	-	-	-	-	1.46
Camphor	-	-	1.70	5.23	24.57
cis-thujone	-	-	-	-	7.51
Eucalyptol	-	-	2.39	3.20	-
Heptanal	1.10	0.74	1.68	0.70	-
Limonene	2.87	-	-	-	-
Decamethylcyclopentasiloxane	11.53	9.81	11.22	5.72	1.57
Dodecamethylcyclohexasiloxane	10.43	8.51	9.19	4.16	-
Sum (%)	88.45	84.66	85.01	90.25	90.68

DISCUSSION

Plant tissue culture techniques that is based on pluripotency (the ability of cells and organisms to create a complete inventory) of live organisms, especially plants is widely used in living organisms. It has numerous applications in agriculture and in vitro plant propagation and a very useful technique for the production of high quality secondary compounds and herbal medicines. Regarding the results of the present study obtained through analysis of compounds derived from different samples by in vitro culture and headspace method, main components of the samples were terpenoids (monoterpene), heptanal and furan. The highest amount of the detected compounds were found in root callus samples with 90.68%, hypocotyl callus with 90.25%, seedlings derived from embryos (control) with 88.45%, cutting the embryo callus with 85.01% and petiole callus with 84.66%, respectively, which indicates that tissue culture and in vitro propagation can increase the volatile compounds (secondary metabolites) galbanum plants.

Results of secondary metabolites analysis showed that root and hypocotyl callus had the most amounts (90%) of the components and the least amount was found in petiole and cutting embryo callus. Hypocotyl had the highest amount of α -pinene (53.64%) and 3- carene (10.59). Root callus had the highest amount of α-thujone (4.37%), thujone (7.51%) and camphor (24.57%). Petiole callus had the highest amount of α -pinene (7.35%) and seedlings from embryos (control) had highest amount of decamethylcyclopentasiloxane (11.53%) and dodecamethylcyclohexasiloxane (10.43%). Meanwhile, the amount of compounds in the root and petiole of newly germinated galbanum seeds analyzed by Rezai *et al* (1381) showed galbanum callus grows well in different hormonal treatments but the combination of essential oils or resin (anti-bacterial compounds) were not appropriate. Results of Shams Ardekani *et al* (1383) also showed that callus obtained from fennel on MS medium with plant hormones could produce volatile compounds including (E, E) 2,4-Decadienal (22.64%) and 1,8 cineole (17.35%). Kikaska and TM (2009) after studying the in vitro culture and secondary metabolites in plant, *Eryngium alpinum* L. stated that the analysis of ethanol extract of the cultivation containing meristem and root cultures by thin-layer chromatography indicated that both cultures were able to produce bioactive compounds including polyphenols, flavonoids and saponins (terpenoids).

In vitro culture showed that the amount of α -pinene in the callus of hypocotyl and petiolewere 53.64 % and 49.45%, respectively, while Sayyah *et al* (2005) by the GC/MS analysis of the oil of the galbanum fruit revealed that α -pinene with 43.78% was the highest compound and after that α -pinene with 27.27% and myrsen with 3.37% were the most important ingredients in the fruit oil.

All previous studies have confirmed that α -pinenenad α -pinene are two two major component of Iraninan galbanum essentila oil. Mortazaeinejad and Sadeghian (2006) also showed that α -pinene with 18.3%, and α-pinene with 50.1 were two major compounds in the galbanum essential oil and form 70% of the oil extracted form the stems and roots. Amidbeigi (1376) reported the amount of leachate essential oil in natural plant about 15 to 26 % that contaied 85% terpenoid hydrocarbons like α-pinene (7 to 21 %), α -pinene (45 to 65 %) and Delta-3-caren (2.5 to 16%).

The pungent odors of the essential oil are related to nitrogenous compounds such as 2- methoxy 3-isobutyl piperazine and 5-butyl, 3-methyl, 2-butyrat, sulfur compounds such as ethyl methyl propyl disulfide that the majority of these compounds were not found in vitro culture. The results of this study showed the highest amount of secondary metabolites in root callus (90.68%), hypocotyl callus (90.25%) respectively, seedlings derived from embryos (control) (88.45%), cutting the embryo callus (85.01%), petiole callus (84.66%), respectively. α-pinene is an expensive and rare compound that is widely used in essence industry while natural α-pinene is not enough available to fulfill the essence industry. Thus, its natural production through tissue culture is very important. In this study, the amount of α-pinene in the hypocotyl and petiole callus were 53.64% and 49.45 %, respectively, which indicates tissue culture and in vitro proliferation can increase volatile oils (secondary metabolites) in galbanum plant.

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