www.ThePharmaJournal.com

The Pharma Innovation

ISSN: 2277- 7695
TPI 2016; 5(1): 74-80

www.thepharmajournal.com
Received: 09-11-2015
Accepted: 13-12-2015

Leila Razeghi
Horticulture Department, Ferdowsi University of Mashhad, Mashhad, Iran.

Majid Azizi
Horticulture Department, Ferdowsi University of Mashhad, Mashhad, Iran.

Seyed Mahdi Ziaratnia
Research Institute of Food Science and Technology (RIFST), Mashhad, Iran.

Abdol Reza Bagheri
Department of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran.

Seyed Hossein Nemati
Horticulture Department, Ferdowsi University of Mashhad, Mashhad, Iran.

Correspondence:
Leila Razeghi
Horticulture Department, Ferdowsi University of Mashhad, Mashhad, Iran.

Evaluation *in vitro* culture of *Kelussia odoratissima* Mozaff and secondary metabolites production through suspension cultures

Leila Razeghi, Majid Azizi, Seyed Mahdi Ziaratnia, Abdol Reza Bagheri, Seyed Hossein Nemati

Abstract
Kelussia odoratissima Mozaff. an endangered endemic medicinal plant in Iran, is widely used for the treatment of several diseases. The wide range of curative properties like anti-inflammatory effects, cardiovascular and cerebrovascular properties are due to the presence of bioactive compounds which found in high amount in different parts of the plant. In this study, the effect of hormone combination (2, 4-D (0.5, 1 and 2 mgL^{-1}) + (Kin (0, 0.5 and 1 mgL^{-1}) and (NAA (0.5, 1 and 2 mgL^{-1}) + BA (0, 0.5 and 1 mgL^{-1})) on callus induction (percentage, weight and size) on the MS basal medium was investigated. Combinations of 2, 4-D+Kin and NAA+BA were chosen as the most appropriate hormone combinations. Then suspended cells were grown on the MS liquid media supplemented with chosen hormone combinations with 3 levels of antioxidant (PVP, PVPP and PVP+PVPP) and wet and dry cell weights were measured. Finally, derived freeze dried cells were analyzed by GC/MS to determine and identify bioactive compounds that are produced. Results revealed that cells cultured on the medium containing combination of NAA+BA yielded more dried weight of cells (0.1048 g/20 mL) compared to the cell grown on the medium containing 2,4-D+ Kin combination (0.0787 g/20 mL). The effect of antioxidants on dry weight was significant so that PVP had produced the highest dry weight (0.1016 g/20 mL) than two other treatments while in terms of fresh weight, the combination of PVP+PVPP produced the highest biomass. Different compounds were identified in both cell's types that some like Ergost-5-en-3-ol, (3.beta.)-, Stigmasterol, beta.-Sitosterol, Hexane, 2, 2, 3-trimethyl-, trans-anethole, Cyclohexasiloxane, dodecamethyl-, Heptasiloxane and 1, 2-Benzenedicarboxylic acid, diisooctyl ester have pharmaceutical properties. We concluded that cell suspension culture of this plant possess potential for biosynthesis of valuable compounds.

Keywords: bioactive compounds production, callus induction, cell suspension culture, *Kelussia odoratissima* Mozaff.

Abbreviations

MS	Murashige and Skoog
2, 4-D	2, 4-Dichlorophenoxyacetic acid
NAA	α-Naphthaleneacetic acid
BA	6-Benzylaminopurine
PVP	Polyvinylpyrrolidone
PVVP	Polyvinylpolypyrrolidone
GC/MS	Gas chromatography coupled to Mass Spectrometry

1. Introduction
Kelussia odoratissima Mozaff belongs to the Apiaceae family, it is also called Kelus or wild celery. Kelus is one of the rare endemic medicinal plant species in Iran with economical and ecological values of Zagros Mountains. This perennial aromatic herb is found in restricted area on Western mountains of Iran at heights of 2500 m with 120 days of freezing temperature (Askari-Khorasgani *et al.* 2013) [4]. Overexploitation of the vegetative organs and the long duration of plant establishment and seed production have caused *K. odoratissima* to be among the plants in danger of extinction.
This plant is traditionally used as a medicinal herb to treat stomachache, hypertension, inflammation, ulcers and cardiovascular diseases (Ahmadi *et al.* 2007) [2]. This curative properties are attributed to the presence of Z-Ligustilide, a typical phthalide of many Apiaceae plants like *Angelica sinensis* (Mao *et al.* 1979) [16], *Ligusticum chaungxiong* (Miao *et al.* 2010) [17] and *Cnidium officinale* (Beck & Chou 2007). New researches showed that essential oil

from K.odoratissima aerial parts includes 23 different types of valuable compounds which Z-LIG is its major components (Saeedi & Omidbaigi 2010) [22]. This means that *K.odoratissima* potentially is a considerable source of Z-LIG. Z-LIG and 3-e-butyl phthalide were also reported by Shojaei *et al.* (2011) [24] as the major compounds of essential oil derived from aerial parts of three different ecotypes of *K.odoratissima.* Plant cell suspension cultures has been known as an alternative method which is potentially able to produce natural compounds at a rate of similar or even superior to the intact plants in quantity point of view. Even in some cases cell suspension culture system has caused to induce new compound which is not present in parental plants (Ziaratnia *et al*. 2009) [27]. Application of plant cell suspension cultures has been accelerated for industrial production of natural products in the last few years.

Nowadays the specific attention has been given to the plant tissue culture methods as these systems are able to scale up for production industrial raw materials in lower costs compared to the intact plants. On the other hand these methods can rescue the endangered valuable medicinal plants. Literature research shows that there are no reports available concerning growth and secondary metabolites formation in *K. odoratissima* cell suspension cultures.

In this study we investigate the production of secondary metabolites and Z-LIG in *K. odoratissima* cell suspension cultures. To achieve this purpose, we studied the influence of different hormonal combinations on callus induction of seedling explants. The GC/MS analysis for identification of secondary metabolites was carried out on extracts derived from liquid culture grown cells.

2. Experimental

2.1. *In Vitro germination*

Seeds of *K. odoratissima* were collected from wild grown plants in July 2011 from Zagros Mountains, Isfahan province, Iran and then identified by voucher specimens of Agriculture and Natural Resources Researches of Isfahan province. The seeds were washed thoroughly with water and few drops of commercial detergent for 15 minutes. They were then immersed in 1% sodium hypochlorite solutions for 20 min followed by pre-disinfected by 70% (v/v) ethanol for 30 seconds in laminar flow cabinet. To remove the detergent residues, the seeds were then rinsed with sterile water for three times. The surface disinfected seeds were incubated in sealed petri dishes added up a little water and refrigerated for 40 days till radicals are emerged. The germinated seeds were allowed to grow in the MS medium without plant growth regulators in growth chamber (16 h light/ 8 h dark photoperiod, at 15 ^{o}C) for further development (6 weeks) until use as plant materials for callus induction.

2.2. *Callus induction*

Several seedlings pieces were cultured on Murashige and Skoog (MS) medium supplemented with 2,4-D (0.5, 1, 2 mgL^{-1}or a_1, a_2, a_3) in combination with Kin (0, 0.5, 1 mgL^{-1} or b_1, b_2, b_3) or NAA (0.5, 1, 2 mgL^{-1} or a_4, a_5, a_6) in combination with BA (0, 0.5, 1 mgL^{-1} or b_4, b_5, b_6), with 30 gL^{-1} sucrose. These 18 hormonal combinations with 5 replications were incubated at 24 ^{o}C under dark condition. After 30 days the treatments were compared based on percentage of callus induction, callus fresh weight and callus size. To avoid callus browning, 1 gL^{-1} PVPP was added to culture medium.

2.3. *Establishment of cell suspension cultures*

The calli derived from the best two treatments from callus induction stage was selected for cell suspension culture. 0.2 g of friable callus was transferred into a 100 ml Erlenmeyer flask containing 20 ml liquid MS medium supplemented with (2,4-D(2 mgL^{-1})+ Kin (0.5 mgL^{-1}) and NAA(2 mgL^{-1})+ BA (0.5 mgL^{-1})) in combination with 3 kinds of antioxidant (PVP, PVPP, PVP+PVPP) at the concentration of 1 gL^{-1}. All the cultures were placed on a rotary shaker at 120 rpm in the dark at 20±1 ^{o}C. The growth was recorded by randomly taking out 2 flasks from each treatment every week. The pattern of cell growth was determined by measuring the fresh and dry weight.

2.3.1. *Production of secondary metabolites and phytochemical analysis*

Phytochemical analysis was performed at Tokyo University of Agriculture and Technology, Laboratory of International Agro-Biological Resources and Allelopathy. Methanolic extracts were prepared from freeze dried cell. 2 samples of dried cells were chosen for extraction (cells grown in 2.4+D+Kin and NAA+BA). 1000 µL methanol was added to 50 mg dried cell centrifuged at 10,000 rpm. One micro liter of filtered supernatant by a 0.45 µm syringe filter was then injected into GC-MS system for identification. The GC-MS QP5050 spectrophotometer was equipped with Shimadtzu GC 17A, EP5MS (5% phenyl methylsilane) capillary column (30 m x 250 µm x 0.25 µm) and helium as gas carrier. The operating conditions of GC/MS were as follows: the GC oven temperature was adjusted from 50 -150 °C with rise of 3 °C/ min, held for 10 min, then raised to 250 °C with rise of 10 °C/ min. The compounds were identified with mass spectra of NIST/NBS.

2.4. *Statistical analysis*

Statistical analysis of collected data from the callus experiment was carried out in completely randomized design. The data were subjected to ANOVA according to GLM procedure of SAS institute (2005) software. The means was compared at significant level of $P<0.05$ using a Duncan's multiple range test. The data of liquid medium were also analyzed using repeated measurement design by PROC MIXED of SAS software.

3. Results and discussion

3.1. Influence of PGRs on callus induction:

All plantlet organs were used as explant due to the low survival *in vitro* germinated seedlings of *K. odoratissima*. Callus induction was observed after approximately 30 days. Frequency of callusing was found to be significantly influenced by hormonal combinations. The frequency of callus induction was almost 93.7% when 2, 4-D (2 mgL^{-1}) was combined with Kin (1 mgL^{-1}), while the lowest callusing induction rate (29.8%) was observed on medium supplemented with a combination of NAA (0.5 mgL^{-1}) and BA (0.0 mgL^{-1}) after that the highest were a_1b_1 and a_5b_5 with 30 and 33 percent respectively. Results of this study show that combination of 2, 4-D and Kin caused better callus induction than NAA and BA (fig. 1).

Hormonal combination had significant effect on callus weight ($p\leq0.05$). Treatment of a_5b_6 (NAA, 1 mgL^{-1}+ BA, 0.5 mgL^{-1}) had caused the highest weight of callus (0.14 g) after 30 days while a_1b_1 (2, 4-D, 0.5 mgL^{-1}+ Kin, 0.0 mgL^{-1}) and a_4b_4 (NAA, 0.0 mgL^{-1}+ BA, 0.5 mgL^{-1}) had the lowest callus weight with 0.030 g and 0.038 g respectively. Combinations of 2, 4-D+

Kin and NAA+ BA showed a similar trend in this case (fig. 2). The treatment that gave rise to the highest callus size was a_3b_2 (2, 4-D, 2 mgL^{-1}+ Kin, 0.5 mgL^{-1}) with 6.413 mm. Among different concentrations of auxins applied in callus induction, 2 mgL^{-1} auxin (regardless of auxin type) in combination of 0.5 mgL^{-1} of cytokinin induced maximum size. This experiment also showed that diameter growth in a_1b_1 induced the lowest callus size (3.361 mm) and there was no significant difference between this treatment and a_4b_4 in terms of callus size.

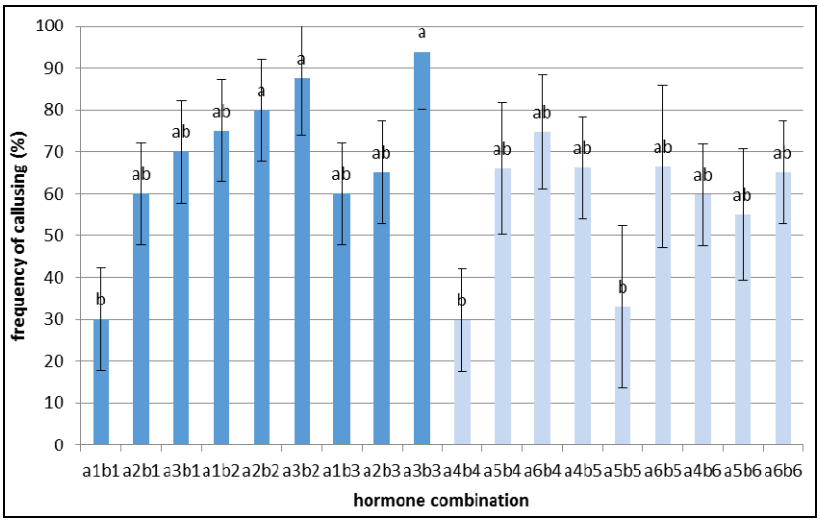

Fig 1: Effect of various hormonal combinations on callus frequency after 30 days of incubation. MS medium supplemented with 2,4-D (0.5, 1, 2 mg L^{-1}or a_1, a_2, a_3) and with Kin (0, 0.5, 1 mg L^{-1} or b_1, b_2, b_3) or NAA (0.5, 1, 2 mg L^{-1} or a_4, a_5, a_6) and with BA (0, 0.5, 1 mg L^{-1} or b_4, b_5, b_6).

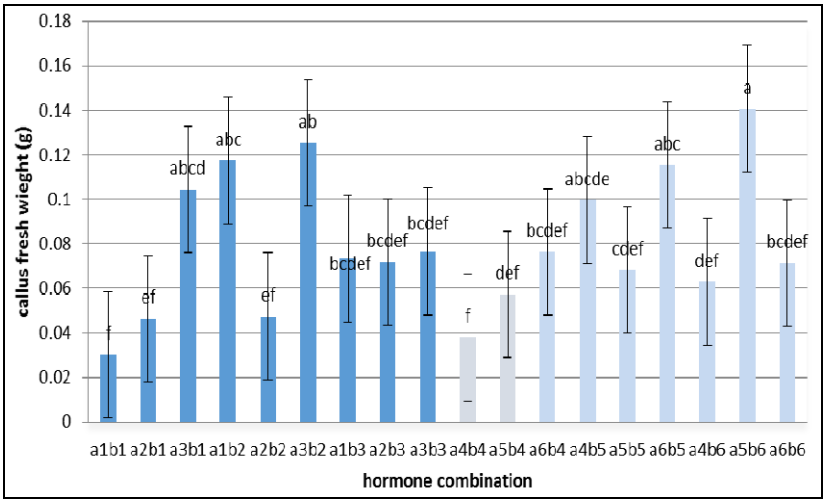

Fig 2: Effect of various hormonal combinations on callus fresh weight after 30 days of incubation.

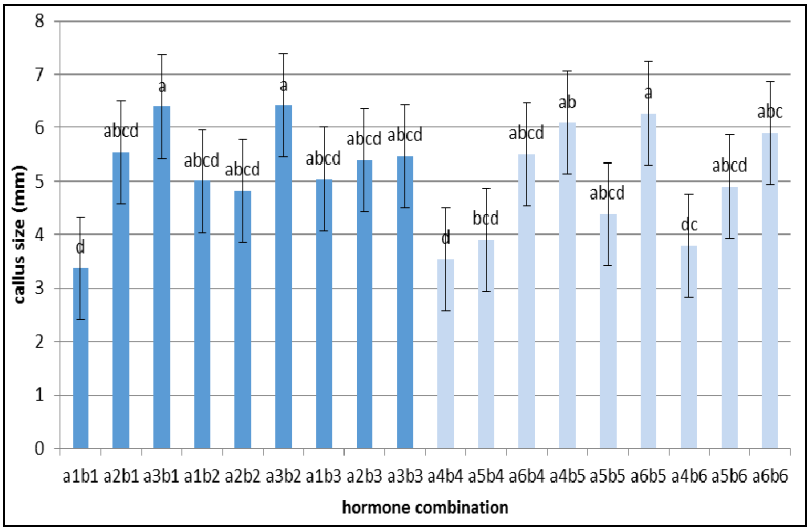

Fig 3: Effect of various hormonal treatments on callus size after 30 days of incubation.

3.2. Influence of hormonal treatments and antioxidants on cell growth in liquid medium

In this part of experiment, to avoid callus browning, it was decided to supplement the callus culture medium with antioxidants. Therefore the MS medium with hormonal treatment of 2, 4-D (2 mgL^{-1}) + Kin (0.5 mgL^{-1}) or NAA (2 mgL^{-1}) + BA (0.5 mgL^{-1}) in combination with. PVP, PVPP or PVP+PVPP were used. All induced calli were cut in to smaller fragments and transferred into 20 mL liquid MS or B_5 media with hormonal and antioxidant treatments. Cell culture in MS medium containing combination of NAA+BA yielded more dried weight of cells (0.1048 g/20 mL) compared to the cell cultures grown in MS medium containing combination of 2,4-D+ Kin (0.0787 g/20mL). The effect of antioxidants on dry weight was significant so that PVP had induced the highest dried weight (0.1016 g/20 mL) than other two treatments while in terms of fresh weight, the combination of PVP+PVPP had caused the highest weight.

The interaction effects of antioxidants and hormonal treatments on fresh and dried cell weight are shown in fig 4. Combination of NAA+BA and PVP gave rise to the highest cell dried weight whereas the lowest one was belonged to the combination of 2, 4-D+ Kin and PVPP. Among various concentrations of antioxidants in relation to two hormonal treatments on cell wet weight, the highest wet weight was obtained at medium containing NAA+ BA along with PVP while combination of NAA+ BA and PVP+ PVPP was produced the least wet weight.

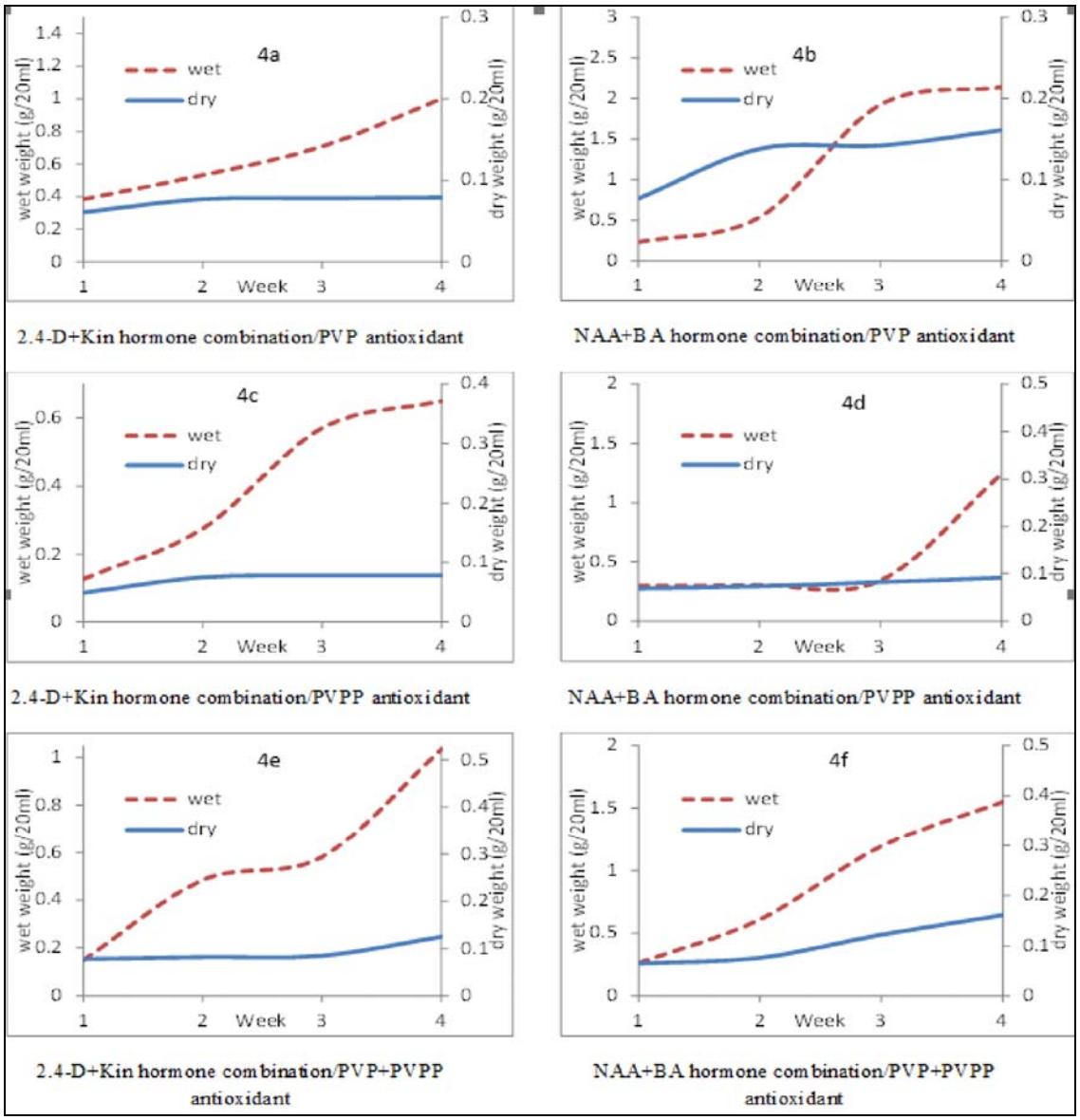

Fig 4: The effect of two hormonal treatments (2, 4-D+ Kin and NAA+BA) supplemented with PVP, PVPP and PVP+PVPP on cell biomass (fresh and dry weight) after 4 weeks of incubation.

The results of this study confirmed that MS medium is a suitable one for callus induction and cell growth of *K. odoratissim.* For this purpose the superiority of MS medium to the other media has been proposed by some other researchers (Narayan *et al*. 2005) [19]. It is also found that a combination of auxin and cytokinin can improve the process of callus induction. Although some researchers believe application of auxin alone is sufficient for callus induction of herbaceous explants (George and Sherrington (1984)), our results revealed that, a combination of auxin and cytokinin can also induce considerable callus on explants of *K. odoratissim*. Later, another researcher) reported that the presence of auxin (2,4-D alone was not sufficient for callus formation on *C. officinalis* samples (Grzelak & Janiszowska 2002) [10]. In some cases, for stimulation of callus growth a cytokinin was combined with auxin (Keng *et al*. 2008). For example, in *Zingiber officinale*,

MS medium with a combination of NAA (0.1 mgL^{-1}) and Kinetin (1-2 mgL^{-1}) was used for callus initiation (Jamil *et al*. 2007) [12]. In this study treatments a_1b_1 and a_4b_4 (without cytokinin), the lowest callusing frequency, callus weight and size were observed. This demonstrate though cytokinin is not necessarily required for callus induction, it can improve callus induction and subsequent growth when it is in combination with auxin. Application of 2,4-D as an effective hormone on callus induction and growth has been published in other members of Apiaceae family (Nath & Buragohain 2005) [20]. Sarkheil, *et al*. (2009) [23] obtained the highest callus induction percentage in *Foeniculum vulgare* Mill. Afify *et al*. (2011) [1] used ½ MS medium containing 2 and 4 mgL^{-1} 2, 4-D with 0.25 and 0.5 mgL^{-1} BAP for callus induction of *Foeniculum vulgare*. They observed high rate of callus induction was on the medium with 2, 4-D at 0.5 mgL^{-1} and kinetin at 1.0 mgL^{-1} which is along to our results. The results of other study showed that the highest callusing (82.49%) was observed on florets explants of *Calendula officinalis* L in the MS medium supplemented with 2.0 mgL^{-1} which is also confirm our results (Legha *et al*. 2011) [15].

Cell culture systems are useful for obtaining uniform and fast growing cells. These cells can be used for studying biochemical aspects of secondary metabolites. Based on findings of Watts *et al*. (1984) [26], 2, 4-D and NAA are the best auxin hormones to enhance cell growth in the suspension cultures of Celery. Browning is a process which occurs as a result of production of phenolic compounds and their oxidation. Accumulation of phenolic compounds in the cell and medium over the time caused reduction in cell growth and ultimately cell death (Haq & Zafar 2005) [11]. In fact, this process is a plant protection strategy which is a problem when incidence in *in vitro* condition. Chattopadhyoy *et al*. (2001) [7] prevented browning and pH drops of the medium by addition of PVP. PVP has been reported by them to act as an inhibitor of polyphenol oxidase which oxidizes polyphenolic compounds (Chattopadhyay *et al*. 2001) [7].

Presence of auxin (2.4-D) in suspension cultures of *Thalictrum minus* led to a rapid increase in cell number (Nakagawa *et al*. 1986) and also stimulated carotenoid biosynthesis in *D.carota* L.(Mok *et al.*,1976).

3.3. Identification of components in cells

Thirty five compounds were identified from the cell extract of *k. odoratissima* grown on media containing 2, 4-D+Kin that constitutes 99.96% of its extract (Table 1).the main compounds were Acetic acid, octyl ester (5.112%), Octyl-.beta.-D-glucopyranoside (4.282%), Heptadecane (4.172%), alpha.-d-Riboside, 1-O-dodecyl- (7.702%), Ergost-5-en-3-ol, (3.beta.)- (4.162%) and Stigmasterol (4.162%).

Twenty eight compounds were identified (99.98%) in the cell extract of *k. odoratissima* grown on media containing NAA+BA (Table 2). Hexane, 2,2,3-trimethyl- (10.747%), Benzene, 1-methoxy-4-(1-propenyl)- (5.767%), Cyclohexasiloxane, dodecamethyl- (6.757%), Heptasiloxane, hexadecamethyl- (6.607%) and Benzo(a)heptalen-9(5H)-one, 6,7-dihydro-10-hydroxy-1,2,3-trimethoxy-7-(methylamino)-, (S)- (5.657%) were the main compounds.

Table 1: Components in the cell extract of *k. odoratissima* grown on media containing 2, 4-D+Kin (relative abundance, %)

No	compounds	RT	%
1	2,2'-Bioxirane	3.879	2.082
2	Hexanal	4.328	3.112
3	Furfural	4.751	1.622
4	1-Butanol, 2-amino-3-methyl-, (.+/-.)-	5.467	3.342
5	6-Oxa-bicyclo [3.1.0] hexan-3-one	5.994	1.912
6	Hexanoic acid	6.776	2.592
7	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	9.587	2.262
8	Hepta-2,4-dienoic acid, methyl ester	9.734	2.682
9	Acetic acid, octyl ester	10.359	5.112
10	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	10.815	1.862
11	Dodecane, 2,6,11-trimethyl-	11.567	3.592
12	2,4-Decadienal	12.115	1.722
13	Formic acid, decyl ester	14.164	2.442
14	Octyl-.beta.-D-glucopyranoside	14.343	4.282
15	Eicosane	14.453	2.202
16	Phenol, 2,4-bis(1,1-dimethylethyl)-	14.672	1.622
17	Heptadecane	15.695	4.172
18	.beta.-Allyloxypropionic acid	15.785	2.222
19	.alpha.-d-Riboside, 1-O-dodecyl-	16.076	7.702
20	Pentadecanoic acid	19.318	3.892
21	Cyclopentadecanone, 2-hydroxy-	19.385	2.432
22	l-(+)-Ascorbic acid 2,6-dihexadecanoate	19.609	2.512
23	9,12-Octadecadienoic acid (Z,Z)-	21.266	1.492
24	9-Octadecenoic acid, (E)-	21.309	1.872
25	Octadecanoic acid	21.49	1.782
26	Ethyl Oleate	21.561	1.352
27	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	21.603	3.434
28	Disulfide, di-tert-dodecyl	21.679	2.692
29	Eicosane	21.818	1.942
30	Benzoic acid, undecyl ester	21.906	2.622
31	Eicosanoic acid	23.4	3.402
32	Ergost-5-en-3-ol, (3.beta.)-	27.315	4.162
33	Stigmasterol	28.161	4.162
34	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	28.384	1.792
35	.beta.-Sitosterol	29.728	3.882
	Total		99.96

Table 2: Components in the cell extract of *k. odoratissima* grown on media containing NAA+BA (relative abundance, %)

No	Compound	RT	%
1	Hexane, 2,2,3-trimethyl-	3.037	10.747
2	11-Dimethyl-1,2,3,4-tetrahydro-1,4-methanophenazine-1-carboxylic acid, methyl ester	3.625	2.517
3	1-Butynyl 2,3-epoxypropyl sulfide	3.72	2.777
4	Benzene, 1-methoxy-4-(1-propenyl)-	11.716	5.767
5	Cyclohexasiloxane, dodecamethyl-	12.178	6.657
6	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane	14.45	2.187
7	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	18.227	1.977
8	Docosa-8,14-diyn-cis-1,22-diol, bis (trimethylsilyl) ether	18.884	2.437
9	21.xi-methyl-17-isocholest-16-en-3.beta.-ol	19.498	1.927
10	Dibutyl phthalate	19.678	2.147
11	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	19.788	1.897

12	Heptasiloxane, hexadecamethyl-	24.093	6.607
13	1,2-Benzenedicarboxylic acid, diisooctyl ester	25.716	1.447
14	Octyl-.beta.-D-glucopyranoside	26.351	3.267
15	tert-Butyl 2-aminophenylcarbamate ditms	26.501	3.027
16	Eicosane	26.614	3.577
17	Heneicosane, 3-methyl-	26.794	3.257
18	trans-4,4'-Dimethoxy-beta-methylchalcone	26.855	3.207
19	Octyltrichlorosilane	26.941	2.587
20	Heptasiloxane, hexadecamethyl-	27.071	3.567
21	(4-Methoxy-phenyl)-carbamic acid o-tolyl ester	27.225	2.467
22	3,4-Dimethyl-2-oxo-5-(1H)pyrrolylideneacetic acid, .alpha.-cyano-, t-butyl ester	27.285	4.167
23	4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyramine	27.365	2.427
24	Terephthalic acid, dodecyl 2-ethylhexyl ester	27.5	4.067
25	Benzo(a)heptalen-9(5H)-one, 6,7-dihydro-10-hydroxy-1,2,3-trimethoxy-7-(methylamino)-, (S)-	27.67	5.657
26	Purine-2,6-dione, 8-(3-ethoxypropylamino)-1,3-dimethyl-3,9-dihydro-	28.065	3.337
27	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	28.378	2.487
28	Heptasiloxane, hexadecamethyl-	28.512	3.787
	Total		99.98

We believe that the differences between essential oils and cell components are acceptable due to different conditions in case of environments and nutrients. Goldhaber *et al.* (2012) [9] also concluded that there are dramatic variations in components and contents among essential oils from *ligusticum porter* plant and its cell suspension culture. Our results from the cells of *K. odoratissima* showed that phthalides compounds like Z-ligustilide which are produced in large quantities in the wild plant, were not produced in both types of cells. Stahl-Biskup and Wichtmann (1991) [25] shown that they weren't produced in callus culture of *Levisticum officinale*. They mentioned for biosynthesis of phthalides, a minimum of differentiation is necessary. However, 3-n-butylphthalide and sedanolide were accumulated in cells of *A. graveolens* grown in liquid MS medium supplemented with 2,4-D+ Kin (Watts *et al*. 1984) [26].

β-sitosterol and stigmasterol are phytosterols that were produced in cells grown in supplemented media with 2,4-D+ Kin this means that it can be potentially used as phytosterols source. Phytosterols are phytochemicals naturally occurring in plants and are applied in medicine and cosmetics and taken as food additives to lower cholesterol (Christiansen *et al.*, 2003) [8]. Kaufman *et al.* (1999) [13] reported β-sitosterol as an antitumor and hypoglycaemic compound. Stigmasterol is a structural component of the lipid core of cell membrane and precursor of many secondary metabolites including plant steroid hormones or acts as carriers in acyl, sugar and protein transport (Balbaa *et al.*, 2008) [5]. Ali *et al.* (2002) [3] showed that stigmasterol has a promotive effect on growth, yield and structure of rice plants.

4. Conclusion

Cell culture systems are useful for obtaining uniform and fast growing cells. These cells can be used for studying biochemical aspects of secondary metabolites. Our results reveal that no phthalides were present in cells, suggesting that probably differentiation is essential for their biosynthesis. Some of identified components like Ergost-5-en-3-ol, (3.beta.)-, Stigmasterol, beta.-Sitosterol, Hexane, 2, 2, 3-trimethyl-, trans-anethole, Cyclohexasiloxane, dodecamethyl-, and Heptasiloxane have pharmaceutical properties. Present study has provided new information about *in vitro* production of secondary metabolites from *K. odoratissima* for the first time, Therefore, manipulation of the plant cell culture potentially able to enhance desirable bioactive compounds that could be considered.

5. Acknowledgments authors would like to give thanks to Prof. Fuji Yoshiharu from Tokyo University of Agriculture and Technology for providing facilities necessary to achieve the results of research.

6. References

1. Afify AE-M, El-Beltagi HS, Hammama AAE-A, Sidky MM, Mostafa OFA. Distribution of trans-anethole and estragole in fennel (*Foeniculum vulgare* Mill) of callus induced from different seedling parts and fruits. Not Sci Bio. 2011; 3(1):79-86.
2. Ahmadi F, Kadivar M, Shahedi M. Antioxidant activity of *Kelussia odoratissima* Mozaff In model and food systems. Food Chem. Elsevier 2007; 105:57-64.
3. Ali Z A, Abd El-Wahed MS, Amin AA. Effect of stigmasterol on growth, productivity and anatomical structure of rice plants. Egypt. J Appl Sci. 2002; 17(12):508-530.
4. Askari-Khorasgani O, Mortazaeinezhad F, Otroshy M, Golparvar AR. Breaking seed dormancy of endangered medicinal plant *Kelussia odoratissima* using zygotic embryo culture technique. *Tech.* J Eng Appl Sci. 2013, 15.
5. Balbaa LK, Nahed G, Abd El A, Youssef AA. Physiological Effects of Stigmasterol and Nicotinamide on Growth, Flowering, Oil Yield and Some Chemical Compositions of *Tagetes erecta* L. Plant. J Appl Sci Res. 2008; 3(12):1936-1942.
6. Beck JJ, Chou S-C. The structural diversity of phthalides from the Apiaceae. J Nat Prod ACS Publications. 2007; 70:891-900.
7. Chattopadhyay S, Srivastava AK, Bhojwani SS, Bisaria VS. Development of suspension culture of *Podophyllum hexandrum* for production of podophyllotoxin. *Biotechnol. Lett*. Springer 2001; 23:2063-2066.
8. Christiansen L, Karjalainen M, Seppänen-Laakso T, Hiltunen R, Yliruusi J. Effects of β-sitosterol on precipitation of cholesterol from non-aqueous and aqueous solutions. Int. J Pharm. 2003; 254(2):155-166.
9. Goldhaber-Pasillas D, Bye R, Chávez-Ávila VM, Mata R. In vitro morphogenetic responses and comparative analysis of phthalides in the highly valued medicinal plant *Ligusticum porteri* Coulter & Rose. *Plant Growth Regulation*. 2012; 67(2):107-119.
10. Grzelak A, Janiszowska W. Initiation and growth characteristics of suspension cultures of *Calendula officinalis* cells. *Plant Cell. Tissue Organ Cult.* Springer

2002; 71:29-40.
11. Haq I, Zafar Y. Effect of nitrates on embryo induction efficiency in cotton (*Gossypium hirsutum* L.). African J Biotechnol Academic Journals (Kenya). 2005; 3:319-323.
12. Jamil M, Kim JK, Akram Z, Ajmal SU, Rha ES. Regeneration of Ginger Plant from Callus culture Through Organogenesis and Effect of CO 2. *Biotechnology,* 2007, 6.
13. Kaufman PB, Cseke LJ, Waber S, Duke JA, Brielmann HL. Natural Products from Plants. CRC Press, Florida, 1999, 207-239.
14. Keng CL, See KS, Hoon LP, Lim BP. Effect of Plant Growth Regulators and Subculture Frequency on Callus Culture and the Establishment of *Melastoma malabathricum* Cell Suspension Cultures for the Production of Pigments. *Biotechnology* 2008; 7:678–685.
15. Legha MR, Prasad KV, Singh SK, Kaur C, Arora A, Kumar S. Induction of carotenoid pigments in callus cultures of *Calendula officinalis* L. in response to nitrogen and sucrose levels. Vitr. Cell. Dev. Biol. 2011; 48:99-106.
16. Mao L, Chaode Z, Qingmin S, Qicheng F. Chemical studies of Angelica sinensis. Acta Pharm Sin. 1979; 14:529-534.
17. Miao C, Wu S, Luo B, Wang J, Chen Y. A new sesquiterpenoid from *Ligusticum chuanxiong* Hort. *Fitoterapia*. Elsevier 2010; 81:1088-1090.
18. Nakagawa K, Fukui H, Tabata M. Hormonal regulation of berberine production in cell suspension cultures of *Thalictrum minus*. Plant Cell Rep Springer. 1986; 5:69-71.
19. Narayan MS, Thimmaraju R, Bhagyalakshmi N. Interplay of growth regulators during solid-state and liquid-state batch cultivation of anthocyanin producing cell line of *Daucus carota*. *Process Biochem*. Elsevier 2005; 40:351-358.
20. Nath S, Buragohain AK. Establishment of callus and cell suspension cultures of *Centella asiatica*. Biol. Plant. Springer 2005; 49:411-413.
21. Ramachandra Rao S, Ravishankar GA. Plant cell cultures: chemical factories of secondary metabolites. Biotechnol. Adv. Elsevier 2002; 20:101-153.
22. Saeedi KA, Omidbaigi R. Chemical characteristics of the seed of Iranian endemic plant Kelussia odoratissima. Chem. Nat. Compd. 2010; 46:813-815.
23. Sarkheil P, Omidi M, Peyghambari S, Davazdahemami S. The effects of plant growth regulators and explants on callogensis, regeneration and suspension culture in *Foeniculum vulgare* Mill. Iranian Journal of Medicinal and Aromatic Plants. 2009; 25(3):364-375.
24. Shojaei ZA, Ebrahimi A, Salimi M. Chemical composition of three ecotypes of wild celery (*Kelussia odoratissima*). Journal of Herbs, Spices & Medicinal Plants. 2011; 17(1):62-68.
25. Stahl-Biskup E, Wichtmann E. Composition of the essential oils from roots of some Apiaceae in relation to the development of their oil duct systems. Flavour and Fragrance Journal. 1991; 6(4):249-255.
26. Watts MJ, Galpin IJ, Collin HA. The effect of growth regulators, light and temperature on flavour production in celery tissue cultures. *New Phytol*. Wiley Online Library 1984; 98:583-591.
27. Ziaratnia SM, Ohyama K, Hussein AA-F, Muranaka T, Lall N, Kunert KJ, Meyer JJM. Isolation and identification of a novel chlorophenol from a cell suspension culture of Helichrysum aureonitens. *Chem. Pharm*. Bull 2009; 57:1282-1283.