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Comparison of Cuminaldehyde Contents from Cell Suspension Cultures and Seeds of [*Bunium persicum* (Boiss.) B. Fedtsch.]

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Abstract

The cell suspension culture and seed samples of *Bunium persicum* were extracted by supercritical fluid, hydrodistillation and solvent methods and analyzed by Gas Chromatography. In this study to compare the different methods of extractions, cuminaldehyde was targeted as one of the Black zira essential oil constitute. For callus induction the germinated seeds were cultured as explants on Murashige and Skoog medium supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid and 0.5 mg/l kinetin (treatment A) as well as 2 mg/l α-naphthalene acetic acid and 0.5 mg/l 6-benzyl aminopurine (treatment B) and followed by cells suspension cultures establishment for the first time. The results of cell culture showed that cells from treatment B have a growth rate higher than A. All extracts were dissolved in 1 ml hexane and analyzed by Gas Chromatography. According to the Gas Chromatography analysis, cuminaldehyde was not detected in the supercritical fluid samples, while it was present in hydrodistillation and solvent extract. Cuminaldehyde percentage in cell and seed solvent extracts was 4.65% and 18.61% respectively. Gas Chromatography results also showed that no cuminaldehyde is present in media extracts, means no cuminaldehyde has been secreted into the medium.

Keywords: cuminaldehyde, gas chromotography, hydrodistillation, solvent, supercritical fluid extraction

Introduction

Plant cell cultures are an attractive alternative source to whole plant for production of high-value secondary metabolites (Ravishankar *et al*., 1999). There are numerous plant secondary metabolites with interesting biological activities which have found applications in pharmaceutical industries, cosmetics, biopesticides and agrochemicals, flavours or food additives, odours and fragrances, and natural pigments (Goossens *et al*., 2003). Several successful studies on commercial production of secondary metabolites by plant cell suspension cultures have been reported such as shikonin from *Lithospermum erythrorhizon*, berberine from *Coptis japonica* (Fujita, 1988) and taxol from *Taxus* species (Blechert and Guenard, 1990).

Black zira [*Bunium persicum* (Boiss.) B. Fedtsch.] (Zireh Irani, Zireh kuhi, Black cumin), is one of the important medicinal and aromatic plants in Iran that belongs to Apiaceae family. Black zira as a perennial herb grows in Northern areas of Khorasan, Kerman, East of Zagros to Bandar Abbas and South of Alborz in Iran. It is also distributed in central Asia, Pakistan, Afghanistan, Keshmir, and Pamir (Azizi, 2005). The seeds are consumed widely as a condiment. In an indigenous remedy, seeds are regarded as stimulants, carminatives and found to be useful in diarrhea and dyspepsia treatment (Foroumadi *et al*., 2002). In addition, the plant is used for culinary purposes, flavoring foods and beverages (Pourmortazavi *et al*., 2005). It is also reported that Black zira seeds contain essential oils (up to 7%) rich in monoterpene aldehydes. The main constitutes of essential oils are cuminaldehyde, *p*-mentha-1,3-dien-7-al and *p*-mentha-1,4-dien-7-al (Thappa *et al*., 1991). Cuminaldehyde, *4-isopropylbenzaldehyde*, is a natural organic compound with the molecular formula $C_{10}H_{12}O$. It is a benzaldehyde with an isopropyl group substituted in the 4-position (Fig.1). It has a pleasant smell and contributes to the aroma of Apiaceae family oils. It is also used commercially in perfumes and other cosmetic products applied in skin areas likely to be exposed to sunshine, excluding rinse-off products (Foroumadi *et al*., 2002). In addition, it has inhibitory effect on tyrosinase (Parvez1 *et al*., 2007), toxic activity against *Acantboscelides obtectus* (Regnault-Roger and Hamraoui, 1995), suppressant melanin formation in cultured murine melanoma cells (Nitoda *et al*., 2008) and the most inhibition of β-carotene oxidation and lipid peroxidation (Sharififar *et al*., 2010).

In order to extract from cells suspension cultures different solvents including hexane (Brown and Charlwood, 1986), ethyl acetate (Yamada *et al*., 2002) and methanol (Ceoldo *et al*., 2009) were used. The extraction of essential oil components using solvent at high pressure, supercritical fluids, has received much attention in the past several

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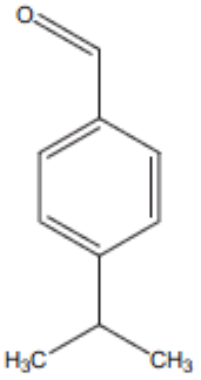

Cuminaldehyde

Fig. 1. Chemical structures of cuminaldehyde

years, especially in food, pharmaceutical and cosmetic industries because of presenting an alternative to conventional processes such as organic solvent extraction and hydrodistillation (Assis and Lancas, 1999; Barnabas *et al*., 1994; Doraiswamy *et al*., 1999; Eikani *et al*., 1999; Fekete *et al*., 1996).

The aim of this research was callus induction and consequently for the first time, establishment of cell suspension culture of Black zira in order to compare the different extraction methods of essential oil from the cell and seed samples. For that purpose, the percentage of cuminaldehyde in extracts was targeted.

Materials and methods

Plant materials and Callus induction

The seeds of Black zira were collected from the experimental farm of Ferdowsi University of Mashhad (36°16' N and 59°36' E), Iran. Seeds were washed in running tap water and then disinfected by immersion in 70% (v/v) ethanol for 1 min followed by 30 min in 1% (v/v) sodium hypochlorite for surface sterilization. To remove the adherent residue, seeds were finally rinsed with sterile distilled water for three times. Sterilized seeds were cultured on simple medium containing 0.5% sucrose and 0.7% agar for solidification. pH was adjusted on 5.8 using 1N NaOH or 1N HCL before autoclaving at 121°C for 20 min. All the cultures were incubated in the dark conditions at 4°C for 3 months.

The germinated seeds were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 2 mg/l 2,4-D and 0.5 mg/l Kin (treatment A) and 2 mg/l NAA and 0.5 mg/l BA (treatment B) and 0.7 % agar. Cultures were incubated at 23±2°C in dark conditions for callus induction. Calli were subcultured in a similar medium at 4-week intervals.

Chemicals

Kinetin (Sigma-Aldrich, Milano, Italy), 6-benzyl aminopurine (Sigma-Aldrich, Milano, Italy), α-naphthalene acetic acid (Merck, Darmstadt, Germany) and 2,4-dichlorophenoxy acetic acid (Merck, Darmstadt, Germany) were chemicals for callus induction and establishment of cell suspension cultures. Cuminaldehyde standard (Merck, Darmstadt, Germany) and hexane solvent (Merck, Darmstadt, Germany) were used for standardization and extraction respectively.

Suspension cultures

For establishment of cell suspension cultures, 25-day-old yellow callus (300 mg fresh weight) was inoculated into 100 ml Erlenmeyer flasks containing 15 ml MS liquid medium similar to that used for callus induction cultures. All cultures flask were then placed on a shaker (120 rpm) at 23±2°C in dark conditions. The cells suspension cultures were subcultured every 7-10 days in 15 ml of fresh medium for 6 months. After establishment of the cell cultures, to get the real suspension cultures, the cells were sieved with a 210 μm sterile stainless steel filter (Fig. 2).

Determination of the cell growth rate

Determination of the rate of cell growth in terms of fresh weight and dry weight was carried out by harvesting weekly three replications in each treatment. The cells

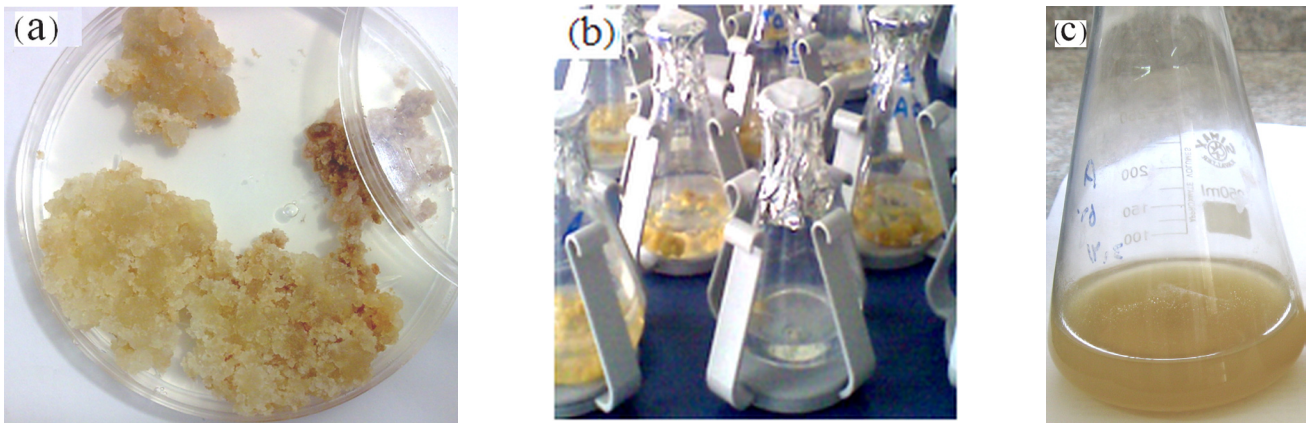

Fig. 2. Callus culture of *Bunium persicum* in MS solid medium, 3 month after inoculation (a) and Cell suspension cultures. Aggregative cells, 8 weeks after culture (b) and separated cells after sieve (c)

were separated from medium through vacuum filtration using a pre-weighed filter paper (Whatman no. 4). The filtrated cells were washed three times with distilled water to remove medium components and then any excess water removed before fresh weight measurement. To measure the dry weight, cells were placed in an oven at 40 °C until constant weight and the dry cell weight was then calculated.

Extraction of intracellular cuminaldehyde by solvent

Hundred g of fresh cells of A and B treatments were dried in an oven at 40°C. Dried cells of A and B and 2 g seeds as control were powdered in a mortar and pestle and consequently extracted in the presence of hexane. The filtrate extract was concentrated by rotary evaporated at 40°C and finally kept at 4 °C until use for Gas Chromatography analysis. The crude extract weight from A, B cells and seeds were 21.7 mg, 24.2 mg and 31 mg respectively.

Supercritical fluid extraction (SCF)

In this study, 7 g of dried B cells and 2 g seeds were powdered and extracted with Supercritical CO_2 under 200 atm pressure and 45°C temperature for 60 min static followed by 30 min dynamic (single run for each plant). A Dura flow manual variable restrictor (Ki) was used in the SCF system to collect the extracted analytics. The supercritical CO_2 flow rate through the Dura flow restrictor was approximately 3 ml/min (compressed). The extracted analytics were collected in a 30 ml ethanol in a 50 ml volumetric flask. Final extracts weight from B cells and seed were 27 mg and 50 mg respectively.

Hydrodistillation

The samples (50 g seed, 4 g and 6 g dried A and B cells respectively) were extracted by hydrodistillation system for 1.5 h, using a Clevenger-type apparatus, according to the European Pharmacopoeia (1975). The volatile distillate was collected over anhydrous sodium sulphate and refrigerated until use for Gas Chromatography analysis. The yield of the seed oil was 2.9% v/w based on plant dry weight.

Determination of secretory cuminaldehyde

In order to determine the concentration of secreted cuminaldehyde into the media, 10 ml filtrated medium culture of A and B treatments were freeze-dried. The dry weight for extracts from A and B medium was 18.5 mg and 22.3 mg respectively.

Gas Chromatography (GC) Analysis

For comparison of different extraction methods, cuminaldehyde was targeted as one of the Black zira essential oil and constitutes.

Gas Chromatography was performed on a Varian PU-4500 chromatograph, with a FID and a CP Sil 5CB column (30 m × 0.25 mm i.d., 0.25 mm film thickness). The oven temperature was programmed from 80°C to 200°C at 8°C/min and kept 20 min at 200°C. The carrier gas was Ar with a flow rate of 1.1 ml/min. Injector and detector temperature were set at 200°C and 205°C respectively. The injection volume for the seed samples and cuminaldehyde standard was 0.5 µl and for the media and cell samples, 5 µl.

Results and discussion

Effect of plant growth regulators on cell growth

Bunium persicum callus cultures were derived from the germinated seeds on MS medium supplemented with two treatments, A (2 mg/l 2,4-D and 0.5 mg/l Kinetin) and B (2 mg/l NAA and 0.5 mg/l BA), within 10-12 days of incubation in dark conditions. Yellow, friable and fresh callus were observed on both combinations. There are many reports for the successful application of different plant growth regulator combinations for callus induction and cell suspension cultures in several species by other researcher (Abenavoli *et al*., 2003; Ceoldo *et al*., 2009; Narayan *et al*., 2005). In this investigation the best growth of *Bunium persicum* callus was obtained on B treatment. However, other study indicated that callus of Black zira have a well growth on MS medium containing 2 mg/l NAA and 2 mg/l Kin (Vlizadeh *et al*., 2006). Wakhlu (1990) recom-

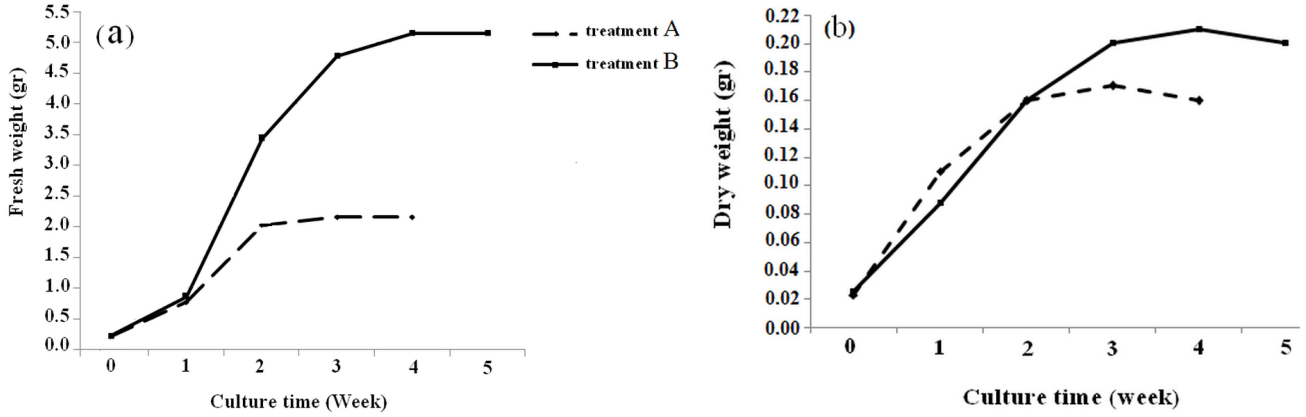

Fig. 3. Effect of different combinations of phytohormone on fresh (a) and dry (b) weight of cell from suspension cultures of *Bunium persicum* in a 5-week period in MS liquid medium. A (2 mg/l 2,4-D and 0.5 mg/l Kinetin) and B (2 mg/l NAA and 0.5 mg/l BA)

mended the same medium but with hormonal combination (2 mg/l 2,4-D and 4 mg/l Kin).

Well-developed callus was used as an initial material for establishment of the cells suspension cultures which were grown on MS liquid medium similar to that used for callus induction cultures. The growth pattern of the cells in terms of fresh and dry weight in a 5-week period in both treatments of A and B were investigated (Fig. 3). The time course study on fresh and dry weight of the cells revealed that the biomass in treatment A increases up to 3 weeks while in case of treatment B, it happened up to 4 weeks. The maximum fresh and dry weight in treatment A (2.16 g and 0.17 g respectively) was obtained after three weeks whereas in treatment B (5.16 g and 0.21 g respectively) occurred after four weeks. Thus treatment B promoted rapid growth of cells similar to callus cultures in solid medium.

Effect of solvent extraction on concentration of cuminaldehyde

According to GC chromatogram, the retention time of cuminaldehyde (standard) was obtained at 6.8 min (Fig. 4). The percentage of cuminaldehyde in hexane extracts from seed, A and B cell samples was found to be 18.61%, 4.63% and 4.73% respectively (Tab. 1). The cuminaldehyde content in the cells of both treatments (A and B) was almost equal and but in seeds it was 4-fold more. Cuminaldehyde is known as one of the major constitute of seed essential oil of Black zira which possesses multiple biological activities (e.g. antifungal, antioxidant and anti-melanoma) (Nitoda *et al*., 2008; Shahsavari *et al*., 2008; Takayuki *et al*., 2007). Brown and Charlwood (1986) reported that in *Pelargonium fragrans* (Willd.), the undifferentiated callus accumulated negligible amounts of monoterpene components, whose numbers and concentrations of these components are more than what in parent plants.

Based on our GC results no cuminaldehyde was present in the hexane extract of treatments A and B media. It is therefore can be concluded that no cuminaldehyde even at low concentration has been excreted into the medium (Tab. 1).

Comparison of different extraction methods based on cuminaldehyde concentration in seed samples

By comparing the cuminaldehyde content in hydrodistillation and solvent extract, it was revealed that cuminaldehyde percentage in seed extracts were significantly different and was 58.87% and 18.61% respectively. It was also found that no cuminaldehyde is present in SCF extract of seeds (Tab. 1).

Obviously cuminaldehyde percentage as a target compound, in hydrodistillation was in much higher amount than solvent extraction samples and was also the major

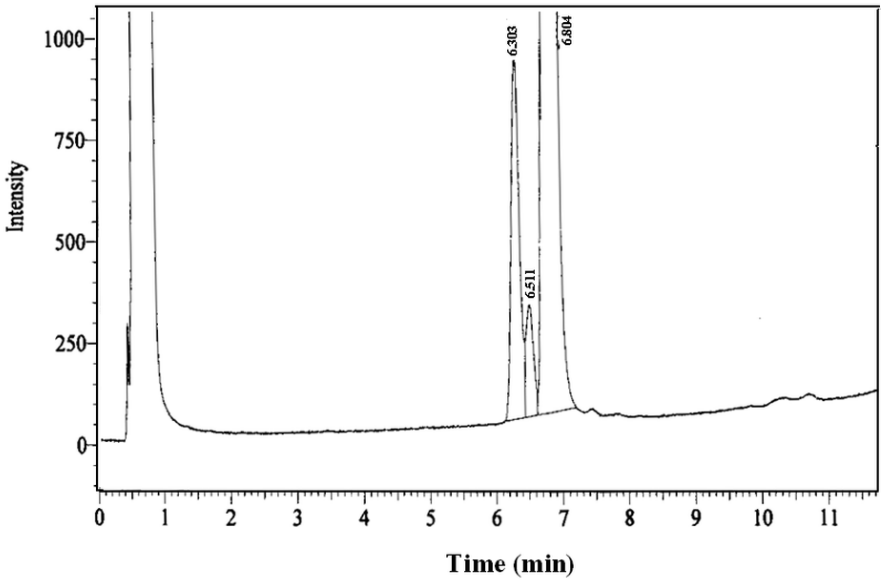

Fig. 4. GC chromatogram of the cuminaldehyde standard (Arrow shows the cuminaldehyde pick)

Tab. 1. Comparison of cuminaldehyde percentage in seed, cell suspension cultures and medium of *Bunium persicum* in three extraction methods

Extraction Methods	Samples				
	Seed	Cell Suspension Culture		Medium	
		Treatment A	Treatment B	Treatment A	Treatment B
Hexane	18.61%	4.63%	4.73%	Not detected	
Hydrodistillation	58.87%	Not detected		Not detected	
Supercritical Fluid	Not detected	Not detected		Not detected	

constituted in hydrodistillation samples. It means that the composition and even the concentration of constitutes of the extracts can be influenced by different extraction methods (Anklam *et al*., 1998; Azizi and Davareenejad, 2009; Mortazavi *et al*., 2010; Pourmortazavi *et al*., 2003, 2004; Salehi *et al*., 2008; Stashenko *et al*., 1996).

Pourmortazavi *et al*. (2005) have also compared the percentage of cuminaldehyde obtained from hydrodistillation and SCF. They reported that cuminaldehyde was detected in both extraction methods, though in SCF it was at lower level. In our results no cuminaldehyde was found in SCF extract. On the other hand the hydrodestillation- derived cuminaldehyde in our experiment was markedly higher than that reported by them in the same extraction method. These differences can be attributed to the harvest time and conditions, climatic and seasonal factors. Technical differences can also be a reason for obtaining different results, for example static and dynamic times in SCF extraction in those two investigations were different. In reports of Agarwal *et al*. (1991) and Abduganiew *et al*. (1997), that black cumin seed were extracted with hydrodistillation, cuminaldehyde was also the major component.

Conclusion

In conclusion, the results of this study have shown that for production of more biomass, the treatment B is more suitable than A. It was also found that cells of treatment A are more susceptible to browning than cells of treatment B. Results also demonstrated, though hydrodistillation is a suitable method for extraction of cuminaldehyde from seeds, it is not favored for cell samples. According to the obtained results of this study for isolation of cuminaldehyde from cell samples, the hexane extract is recommended. The results have shown that two different hormonal treatments have no significant effect on cuminaldehyde content in cell samples of *Bunium persicum*.

Acknowledgements

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