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Original article

Analysis of volatiles and 18S rRNA gene of *Haplophyllum canaliculatum* in *in vitro* cultures

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Abstract

Background and objectives: *Haplophyllum canaliculatum* is an endemic and endangered Iranian plant from Rutaceae family. The object of this work was to study the volatile production in established shoot and callus cultures of *Haplophyllum canaliculatum* as well as isolation, identification and sequencing of 18S rRNA gene from callus culture. **Methods:** Shoot and callus cultures of *H. canaliculatum* were established from seedlings and shoot cultures, respectively. Both cultures were transferred to MS medium supplemented with α-naphthalene acetic acid (α-NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kn). Volatiles from fresh callus and shoot cultures were extracted and analyzed by GC/MS. For 18S rRNA gene study, DNA content was extracted using PCR procedure. The study of sequence similarities was performed using NCBI database and GeneDoc software. **Results:** GC/MS analysis of *H. canaliculatum* showed that shoot cultures mainly contained piperitone (10.92%), and β-caryophyllene (12.67%) in addition to three alkaloids, while calli cultures of *H. canaliculatum* mainly contained methylated salicylate (31.55%), alkane structures like tetradecane (24.31%) and hexadecane (12.95%). Gene analysis showed 98% homology with certain species of Rutaceae, Meliaceae, Simaroubaceae, Burseraceae and Cneoraceae. **Conclusions:** Our results showed that the hydrocarbon in addition to methyl salicylate biosynthetic pathway in calli cultures and terpene as well as alkaloid biosynthetic pathway were active in *H. canaliculatum* shoot cultures. Moreover, the obtained sequences could be used as a "DNA barcoding" tool through the concept of one sequence one species for the practical identification of this species.

Keywords: GC/MS, *Haplophyllum canaliculatum*, *in vitro* culture, 18S rRNA gene, volatiles

Introduction

The family Rutaceae contains approximately 150 genera with about 900 species [1]. The genus *Haplophyllum* belongs to this family with about 70 species [2]. Having 14 endemic and 30 species in total, Iran has the most number of *Haplophyllum* species in the world [3]. It has been reported that this genus contains essential oils, alkaloids, fixed oils, coumarins, sterols, flavonoids and lignans [4-7].

In Iranian traditional pharmacy, *Haplophyllum*

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spp. ("*Sodab"* in Persian) are used as tonic, coagulant and wound healing agents. There are many reports about pharmacological activities of different *Haplophyllum* spp. Such as anti leishmania (*H. myrtifolium* and *H. bucharicum*) [8,9], cytotoxic (*H. stapfianum*, *H. tuberculatum*, *H. acutifolium* and *H. viridulum*) [10], antioxidant (*H. tuberculatum*) [11], anti-Alzheimer, skin diseases and type II diabetes [12], heart rate reducer (*H. tuberculatum*) [13], and topical anti-inflammatory properties (*H. hispanicum*) [14]. There is no report about *in vitro* cultures of *H. canaliculatum* and its components as an Iranian native and endangered plant.

Molecular markers are used as tools for estimating the phylogenetic relationships of different kinds of organisms. Genes encoding ribosomal RNAs and ribosomal proteins have been highly conserved throughout evolution and have diverged more slowly than other chromosomal genes. Comparison of the nucleotide sequence of 16S ribosomal RNA from a range of biologic sources has revealed evolutionary relationships among widely divergent organisms [15]. Because of the broad spectrum of applications of 16S rRNA, using PCR to amplify the complete region of this gene for further analyses will be very useful [16-18].

In the present study, production of volatile components in established shoot and callus cultures of *H. canaliculatum* PTCS 001 was studied. The sequencing data might be used as a "DNA bar-coding" tool for practical identification and also characterization of various species. Therefore, isolation, identification and sequencing of 18S rRNA gene of *Haplophyllum* callus cultures were investigated and genetic homology of this species between Rutaceae families was studied. Homology between the hypothetical proteins of amplified sequence with other available proteins from NCBI database was studied too.

Experimental

Plant material

Fruits of *Haplophyllum canaliculatum* Boiss. (Rutaceae) were collected from Shiraz, Fars, Iran. The plant material was identified by Mohammad Soltani. Voucher specimens have been deposited at Shiraz Faculty of Pharmacy Herbarium (No. 258).

Seed germination and culture initiation

For sterilizing the seeds of *H. canaliculatum* PTCS 001 were scarified and washed with ethanol 96% for 1 min and then dipped in sodium hypochlorite 1% for 9 min. They were rinsed three times with sterile distilled water afterwards. Sterile seeds were transferred to sterile coconut medium (15% coconut milk solidified with 0.8% agar). For rapid germination, the seeds were treated with gibberellic acid (GA) 500 mg/L [19]. After seed germination, resulted seedlings were transferred to Murashige and Skoog (MS) media [20] supplemented with 3% sucrose and solidified with agar (0.8%). pH was adjusted to 5.6 with NaOH 1N before autoclaving. Seedlings were incubated in continuous light (4420 lx) at 25±2 ºC. Shoot cultures were produced from the seedlings.

Callus cultures

Calli cultures of *H. canaliculatum* PTCS 001 were initiated from shoot cultures transferred to MS medium supplemented with α-naphthalene-n-acetic acid (α-NAA) 1 mg/L, 2,4-dichlorophenoxyacetic acid (2, 4-D) 0.5 mg/L and kinetin (Kn) 0.5 mg/L and then cultures were incubated in continuous light (4420 lx) at 25±2 ºC [21].

Extraction of volatiles

The volatile compounds of fresh callus and shoot cultures (10 g) were extracted by dichloromethane (20 mL) with shaking for 30 min, then the solvent was evaporated with liquid nitrogen to reduce the volume to 150 µL [21].

Analysis of volatiles

The chemical composition of the dichloromethane extract was determined by Gas chromatography/Mass spectrometry (GC/MS), using a Hewlett-Packard 6890 system. GC was

equipped with a HPA-5MS capillary column (phenyl methyl siloxane, 25 m×0.25 mm i.d.); the oven temperature was programmed from 60 ºC and (without stop) to 260 ºC at a rate of 3 ºC/min, maintained at 260 ºC for 3 min. The carrier gas was helium with a flow rate of 1.2 mL/min. The mass spectrometer was operating in the EI mode at 70 eV. The interface temperature was 280 ºC; mass rang was 35-600 m/z [22].

Identification of compounds

Identification of known compounds was based on computer matching with Wiley 275 and literatures [23]. Relative percentage amounts of the spectra compounds were calculated from total ion chromatograms by the computerized integrator.

18S ribosomal RNA gene sequencing

The sequence of 18S rRNA gene of the selected strain was studied. For this purpose, DNA content was extracted from callus cultures of the *H. canaliculatum* PTCS 001 and then PCR procedure was applied using two sets of primers [24].

DNA extraction

To extract DNA from the callus cultures of the *H. canaliculatum* PTCS 001, 200 mg of lyophilized callus was crashed. Extraction buffer (400 µL) was added and mixed completely. After centrifugation at 12000 rpm at 7 min, 300 µL of the supernatant was transferred to the new tube and isopropanol (300 µL) was added and mixed completely. Then the resulting solution was centrifuged at 12000 rpm for 7 min. The supernatant was removed and the pellet was washed with 1 mL ethanol (70%). After removing ethanol, the resulting pellet was dried completely and 30-50 µL distilled water was added and mixed gently. The resulting supernatant used as template for PCR [24].

PCR amplification

The two oligonucleotide primers used for amplification of *H. canaliculatum* PTCS 001 18S rRNA gene were: 5´-GTCAGAGGTGAAATTCTTGGATTTA-3´ as forward and 5´-AGGGCAGGGACGTAATCAACG-3´ as reverse [25].

PCR was performed in a final concentration of 50 µL containing 5µL 10×PCR amplification buffer, 0.8 µL Taq DNA polymerase, 1.5µL of each dNTP, 2µL of each primer and 15µL template DNA. Amplification conditions were: initial denaturation at 94 ºC for 5 min; 10 cycles at 94 ºC for 30 S, 50 ºC for 30 S and 72 ºC for 2 min; 20 cycles at 94 ºC for 30 S, 50 ºC for 30 S, and 72 ºC for 2.5 min with a final cycle of 72 ºC for 5 min. Taq polymerase was added to the reaction after the first denaturation step. The lower denaturation temperature (92 ºC) during the 20 cycles step was used to avoid loss of enzyme activity. Samples were electrophoresed in a 1% (w/v) agarose gel using TBE buffer containing 1 µg/mL ethidium bromide. A single 700 bp DNA was cut and extracted from the gel using Core Bio Gel Extraction Kit. The sequence was determined by CinnaGen Company with the primers. Sequence similarity searches of 18S rRNA gene from *H. canaliculatum* PTCS 001 and its hypothetical protein were done, using BLAST through the website of the NCBI and the GeneDoc software version 2.7 [26].

BLAST search of the targeted Gene

The DNA sequence of *H. canaliculatum* PTCS 001 was recorded in the NCBI under the accession number EU379937.1

The amplified sequence which had 630 bp nucleotides, showed 98% homology with eight species of plants from Rutaceae, Meliaceae, Simaroubaceae, Burseraceae and Cneoraceae families. GeneDoc software was used to compare the amplified sequence with other 18S rRNA available genes from NCBI database. Seventy-eight of nucleotides of partial sequence of *H. canaliculatum* PTCS 001 were different from other compared sequences (figure 1).

BLAST analysis of the hypothetical protein

Amplified sequence was translated to protein and compared with the sequence of other available

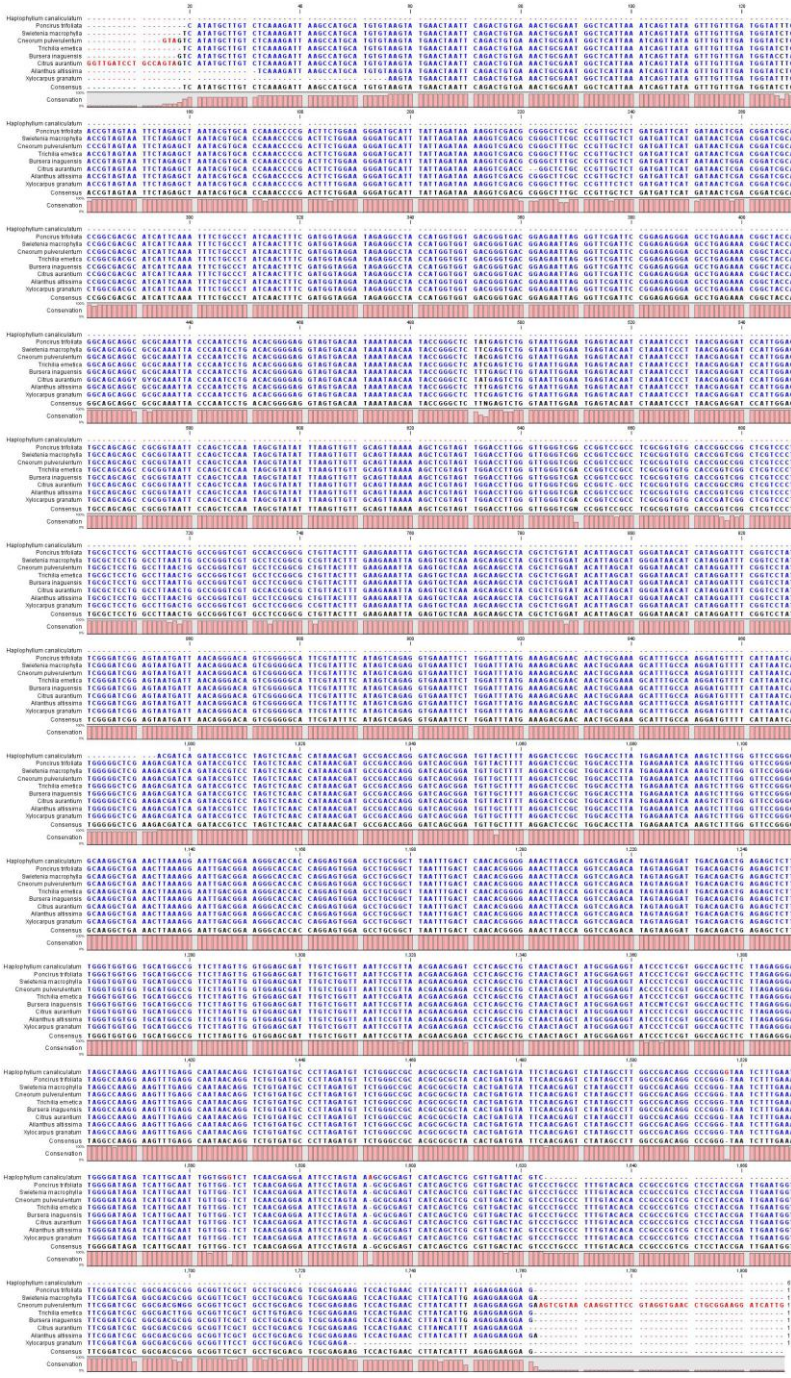

Figure 1. The tabular format of a multiple alignment from nine 18S rRNA genes including the studied *H. canaliculatum* PTCS 001 strain and eight related plant strains from the NCBI database using the CLC sequence viewer software, version 7.7. Sequence names appear at the beginning of each row and the residue position is indicated by the numbers at the top of the alignment columns. The level of sequence conservation is shown on a color scale with red residues being the least conserved and blue residues being the most conserved. Besides a pink colored plot in percent scale, shows the conservation extent of each domain.

proteins from NCBI Database. The hypothetical protein of amplified sequence showed homology with the eight available proteins from NCBI database. This hypothetical protein showed 96% homology with rRNA intron-encoded homing endonuclease from *Oryza sativa*. The hypothetical amino acid sequence of *H. canaliculatum* gave 62 amino acids 60 of which showed the same with amino acid sequence of *O. sativa* in frame (+2) (figure 2).

Results and Discussion

Volatile composition of shoot and callus *in vitro* cultures of *H. canaliculatum* an endemic plant from Iran, were shown in table 1. This is the first time that *H. canaliculatum in vitro* cultures were established and production of secondary metabolites in the cultures was investigated. To the best of our findings *H. patavinum* is the only species of this genus which its tissue cultures were carried out [27]. GC/MS analysis of *H. canaliculatum* showed that shoot cultures of this plant contained piperitone (10.92%), and β-caryophyllene (12.67%). In calli cultures of *H. canaliculatum*, methylated salicylate (31.55%) and major alkanes like hexadecane (12.95%) and tetradecane (24.31%) were the most abundant compounds in *H. canaliculatum* callus cultures. Three Indole and quinoline components were detected in the dichloromethane extract of shoot culture as a differentiated tissue (table 1). Quinoline and quinolinone alkaloids like atanin haplacutine A–F, as well as of acutine, haplamine, eudesmine, and 2-nonylquinolin-4(1H)-one have been reported from *Haplophyllum* spp. Earlier [10,28]; although, no such alkaloids were detected in the *H. canaliculatum* calli culture as an undifferentiated tissue.

Low percentage of monoterpenes in callus cultures indicate that organogenesis is the essential factor for producing monoterpenes in cultures [29]. There is a reverse relationship between differentiation and accumulation of secondary metabolites with growth rate of the cultures [30]. In the present study, calli showed more growth rate than shoot cultures and therefore differentiation and lower terpenes accumulation was lower in such cultures. Furthermore, in differentiated cultures there were more storage pools which is another explanation for the presence of monoterpenes along with differentiation [21]. It has been reported that methyl salicylate roles as an airborne signal which activates the expression of defense-related genes [31]. Here *Haplophyllum* callus culture as a heterogeneous tissue synthesized methyl salicylate (31.55 %) as a reaction, too. As a whole, more than 68% hydrocarbon structures as the active biosynthetic pathway in *H.canaliculatum* in addition to methyl salicylate were detected. However, shoot culture produced mono- and sesquiterpenes (>36 %) in addition to indole and quinoline alkaloids.

Moreover, in this study 18S rRNA gene was sequenced and its homology with other submitted 18S rRNA genes were investigated. The partial sequence of 18S rRNA gene sequence of this species has been shown in figure 3. This gene sequence didn't have 100% homology with any submitted sequences. But it showed 98% homology with *Poncirus trifoliate* (Rutaceae); *Citrus aurantium* (Rutaceae); *Xylocarpus granatum* (Meliaceae); *Swietenia macrophylla* (Meliaceae); *Trichilia emetica* (Meliaceae); *Ailanthus altissima* (Simaroubaceae); *Bursera inaguensis* (Burseraceae) and *Cneorum pulverulentum* (Cneoraceae). Sequence data for plastid *rbc*L and *atp*B from members of Burseraceae, Cneoraceae, Meliaceae, Rutaceae, and Simaroubaceae were analyzed cladistically to evaluate the familial and sub familial circumscriptions of Rutaceae, previously [32]. The analysis showed that Rutaceae is paraphyletic with Simaroubaceae and Cneoraceae, forming a clade sister to all other circumscription of the family. This analysis indicated that Simaroubaceae and Meliaceae are out-group closest to Rutaceae. As the results of bioinformatics analysis showed, there was 96% homology between the hypothetical protein and rRNA intron-encoded homing endonuclease from *Oryza sativa*.

Table 1. Volatiles from shoot and callus cultures of *Haplophyllum canaliculatum*

	Compound	KI	Percentage		References[1]
			Shoot	Callus	
1	β-Pinene	981	4.27	-	[34, 35]
2	Decane	999	-	7.94	
3	*o*-Cymene	1030	-	0.20	
4	δ-Carene	1032	4.86	-	[34, 36]
5	Undecane,5-methyl	1156	-	0.20	
6	Undecane,3-methyl	1170	-	0.25	
7	Dodecene	1187	-	2.98	
8	Methyl salicylate	1195	-	**31.55**	
9	Piperitone	1258	**10.92**	-	[34]
10	Bornyl acetate	1285	0.66	-	[37]
11	Tridecane	1295	-	0.25	
12	Tridecane,5-methyl	1350	-	0.79	
13	Tridecane,3-methyl	1366	-	0.72	
14	Tetradecene	1386	-	5.34	
15	Tetradecane	1396	-	**24.31**	[36]
16	β-Caryophyllene	1415	**12.67**	-	[34-37]
17	α-Humulene	1449	0.97	-	[34, 36]
18	Phenol,2,6-bis(1,1-dimethylether)	1502	-	0.18	
19	Pentadecane,5-methyl	1545	-	0.43	
20	Caryophyllene oxide	1577	2.08	-	[34, 36]
21	Hexadecene	1587	-	2.93	
22	Hexadecane	1594	-	**12.95**	
23	10,10-Dimethyl-2,6-dimethylene bicycle(7.2.0)undecan-5-beta-ol	1643	1.52	-	
24	Octadecane,1-(ethenyloxy)	1647	-	0.12	
25	Octadecene	1788	-	1.62	
26	Octadecane	1795	-	4.84	[36]
27	Neophytadiene	1838	1.14	-	
28	Hexadecanoic acid	1980	1.78	-	[36]
29	Eicosane	1994	-	2.40	[35, 36]
30	1-Hydroxy xanthone	2236	3.09	-	
31	9,9a-dihydro-7-methoxy-3.α,6methyl-3H- pyrrolo[1,2-a]indole-5,8-dione	2279	**14.23**	-	
32	3,9,10a-tetrahydro-7-methoxy-3.α.,6-dimethylpyrrolo[1,2-a]quinoline-5,8-dione	2379	**14.98**	-	[10, 28]
33	7-(1,1-Dimethylpropynoxy)-4-methoxy furo[2,3-B]quinoline	2499	**4.41**	-	[10, 28]
34	Docosanoic acid, methyl ester	2513	0.76	-	
35	Tetracosanoic acid, methyl ester	2638	1.06	-	
	Identification (%)		**79.40**	**100**	
	Grouped components:				
	Monoterpenes		**20.71**	**0.20**	
	Sesquiterpenes		**15.72**	**-**	
	Hydrocarbons		**1.14**	**68.07**	
	Others		**41.83**	**31.73**	

[1]Represents the volatile components in at least one *Haplophyllum* species.

Although further investigations should be performed when the genome sequencing for other related plant species become available. Our results also agreed with previous data and showed close relationships between mentioned families and Rutaceae. These gene fragments are commonly used to study phylogenetic relationships between species and could be

Haplophyllum canaliculatum: RSDTVLVSTINDADQGSADVTFRTPLAPYEKSKSLGSGGSMVARLKLKGIDGRAPPGVEPAA: 62

Oryza sativa: RSDTVLVSTINDADQGSADVAYRTPPAPYEKSKSLGSGGSMVARLKLKGIDGRAPPGVEPAA: 66

RSDTVLVSTINDADQGSADV RTP APYEKSKSLGSGGSMVARLKLKGIDGRAPPGVEPAA

Figure 2. The multiple alignment sequence analysis of the hypothetical amino acid obtained from 18S rRNA sequence of the studied *H. canaliculatum* PTCS 001 and *Oryza sativa* as the most relative amino acid sequence in NCBI data bank using GeneDoc software version 2.7. Sequence names appear at the beginning of each row. The level of sequence conservation is shown on a color scale with pink residues being the conserved and white residues being the non-conserved domains.

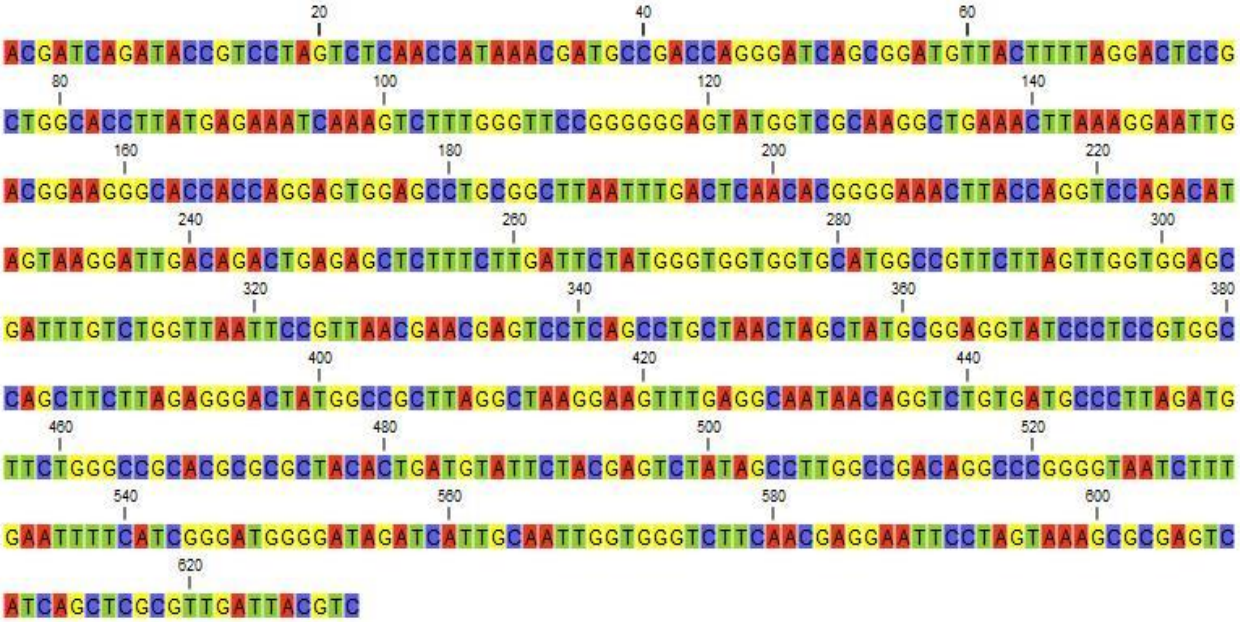

Figure 3. 18S rRNA sequence of *H. canaliculatum* PTCS 001 deposited in NCBI data bank under the accession number EU379937.1**.** CLC sequence viewer software version 7.7 was used for annotating and shading the sequence.

applied to study several species of plants that present uncertain taxonomic affiliations. Moreover, the obtained sequences could be used as a "DNA barcoding" tool through the concept of one sequence, one species [33] for practical identification of plants.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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