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Full Length Research Paper

Comparative analysis of the essential oils from normal and hairy roots of *Panax japonicas* C.A. Meyer

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The essential oils were extracted with steam distillation from normal and hairy roots of *Panax japonicus* C.A. Meyer. The constituents of essential oils were analyzed by gas chromatography mass spectrometry (GC-MS). The results showed that 40 and 46 kinds of compounds were identified from the essential oils of normal and hairy roots; they amount to 93.3 and 95.89% of the total detected constituents, respectively. Both of them own 18 kinds of same compounds, relative peak areas all exceed 50%, except that, the roots alone own 22 kinds of compounds, and the hairy roots alone own 28 kinds of compounds. In the roots, those higher content compounds were hexanoic acid (11.6%), falcarinol (10.04%) and 3-methylbutyric acid (9.56%); however, in the hairy roots, they were caproic acid (13.92%), spathulenol (9.96%), 1H-cycloprop azulene (9.15%). These compounds have lots of bioactivity, for anticancer, antitumor and antibiosis among others. The result showed tremendous value on producing the medical components with the skill of hairy roots.

Key words: *Panax japonicus,* essential oils, gas chromatography mass spectrometry.

INTRODUCTION

Panax japonicus C.A. Meyer is attached to the family of Araliaceae, and born in dank regions and distributed in China, Japan and Korea. In China, it is distributed in Yunnan, Guizhou, Sichuan and Hubei province (Committee of Guizhou herbal, 1989; Committee of Chinese herbal, 1978). Recent research showed that the southwest of China was its biodiversity center (Yang, 1981). *P. japonicus* has integrated function of notoginseng and ginseng recorded by chinese pharmacopeia in 2008, and is used for fracture, haematemesis, cough, bleeding wound, arthralgia and so on (Committee of Chinese pharmacopeia, 2008), therefore it is worth developing for the wealth of folk-medicines. Lots of achievements have been made in the biological character (Lin et al., 2008, 2007, 2006), chemical compositions (Cai et al., 1982, 1984; Zou et al., 2002), bioactivity (Han et al.,2005; Zou et al., 2002), pharmacognosy (Zhang et al., 2009) and genetic engineering (You et al., 2007; Choi et al., 2008; Smolenskaya et al., 2007), but less has been explored about the essential oils of *P. japonicus*.

The technology of hairy roots culture is a new skill which was developed in the process of cell culture in the late1980s. To be more specific, the ways is that the T-DNA of Ri plasmid from *Agrobacterium rhizogenes* is integrated into the DNA of plants, and makes plants produce hairy roots (Chilton et al.,1982). To make the hairy roots synthesize new and valuable secondary metabolites, is a new trend in scientific research and production, which can help solve the problems of lacking raw materials in cosmetics, insecticide and industry. In the paper, in order to study the essential oils of the normal and hairy roots about *P. japonicas,* firstly, the transformation system of its hairy roots was established by genetic engineering; secondly, the constituents of the essential oils about them were detected by gas chromatography mass spectrometry (GC-MS) and analyzed by

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Abbreviations: GC-MS, Gas chromatography mass spectrometry; **PCR,** polymerase chain reaction; **MVA,** mevalonate; **MS,** Murashige and Skoog; **CTAB,** cetyl trimethyl ammonium bromide.

contrast.

MATERIALS AND METHODS

Plant materials

P. japonicus C.A. Meyer was collected from Zhijin County, Guizhou, China (Prof. Cheng identified the plant).

Induction of hairy roots

Explants were tender stem of *P. japonicus*, and pre-cultured for 2 weeks on Murashige and Skoog (MS) medium. The explants were removed from the medium, and placed for 30 min in conical flasks with the C58C1 bacterial suspension, the explants were blotted and transferred to the modified MS medium. After 3 days, the explants were transferred to MS medium containing 400 $mg \cdot L^{-1}$ cefotaxime so as to kill the residual C58C1 *A. rhizogene*s, controls were not co-cultivated with C58C. Cefotaxime concentration was then halved from 400 to 50 $mg \cdot L^{-1}$ each week, and finally cultured free C58C1 were transferred to B_5 medium with 0.2% Phytagel (Sigma) (Dhakulkar et al., 2005). Hairy roots came into being mainly from the cut surfaces of the explants. The hairy roots were separated from the explants when they attained 4 ~ 5 cm in length, and placed on B5 medium for further growth. All the cultures were maintained in absolute darkness at 25 ~ 28°C.

Polymerase chain reaction analysis of hairy roots

DNA was extracted with the cetyl trimethyl ammonium bromide (CTAB) method (Murray et al., 1980) from the hairy roots as well as from control non-transformed roots. Polymerase chain reaction (PCR) primers (Invitrogen Biotechnology Co., Ltd) were used for amplification of a 550 bp fragment of the rolB gene. The sequence of each primer was as follows (forward primer 5′-ATGGATCCCAAATTGCTATTCCCCCACGA-3′ and reverse primer 5′-TTAGGCTTCTT TCATTCGGTTTACTGCAGC-3′). The PCR (Eppendorf) reactions were carried out in a total 50 µL volume and consisted of 200 ng of DNA, 10 $pm.\mu L^{-1}$ primer, 200 µM dNTP, 1 U of Taq DNA polymerase, 1×PCR buffer and 2 mM $MgCl_2$. PCR conditions were 94°C for 5 min (initial denaturation), 42 cycles of 94°C for 1 min, 52.5°C for 1.5 min and 72°C for 2 min and a final extension at 72°C for 10 min.

Extraction of essential oils

The normal and hairy roots of *P. japonicus* were dried in air and milled into crude powder. The essential oils were extracted from the powder with steam distillation method described in the Chinese Pharmacopoeia (Committee of Chinese pharmacopeia, 2008), and were stored in a deep freezer.

Analysis of essential oils

Gas chromatography-mass spectrometry (GC/ MS)-QP2010 was applied to measure the contents of the essential oils. The specific description of gas chromatography (GC) condition was as followed: Carrier gas, helium at flow rate of 0.5 $mL \cdot min^{-1}$; sample size, 2 µL injected by the splitles injection technique; fused capillary silica column OV-1701 (30 m × 0.33 mm × 0.25 µm); temperatures: injector, 260°C, detector, 280°C, column, 50°C. The temperature was programmed from 50 to 260°C at a rate of 10°C / min. The MS was taken at 70 eV. The MS scan parameters included a mass range of m/z 10 ~ 500, a scan interval of 0.5 s, a scan speed of 1000 $amu \cdot s^{-1}$, and a detector voltage of 1.20 kV. Mass spectral data was examined by NIST147 library search and then compared with standard published data.

RESULTS AND DISCUSSION

Induction of hairy roots

The stems were infected by C58C1 bacterial suspension after 6~8 days, the inferior extremity of stems began to oncoides, after 8 ~15 days, and then hairy roots come into being. The emergences of hairy roots were concerned with the morphous of stems. The rule is that the superior extremity occurs first, and the inferior extremity followed. These hairy roots have lots of features, for example, white, thickly-grown, disgeotropism and so on (Figure 1).

Confirmation of transgenic status of hairy roots

Integration of the T-DNA into *P. japonicus* genome was confirmed on the molecular level by PCR with primers constructed on the sequences of *rolB* genes. *A. rhizogenes* (colony PCR) served as the positive control and DNA from the non-transformed roots served as the negative control. All transformants showed presence of the 550 bp rolB amplified product, while the non-transformed roots have no 550 bp. The result showed that the T-DNA have integrated into *P. japonicus* genome (Figure 2).

Yield essential oils

The yields of the essential oils from the normal and hairy roots of *P. japonicus* are 0.451 and 0.380%, respectively.

Constituents of essential oils

The essential oils are pale white, and their constituents were identified by GC-MS given in Table 1. There were 40 compounds in the essential oils of normal root samples, amounting to 93.3% of the total detected constituents. Among them, hexanoic acid (11.6%), falcarinol (10.04%), 3-methylbutyric acid (9.56%), 2-ethoxy-propane (6.56%) and [4.1.0]-3-heptene-2-isopropenyl-5-isopropyl-7,7-dimethyl-Bicyclo (5.89%) amount to 43.65% of the oils. In the essential oils of hairy root samples, there were 46 kinds of compounds, amounting to 95.89% of the total detected constituents. Among them, caproic acid (13.92%), spathulenol (9.96%) and 1H-cycloprop azulene (9.15%) were representative compounds, adding up to 33.03% of the oils. In the oils of

Figure 1. Induction and cultivation of the hairy roots about *P. japonicus* (a: The hairy roots of *P. japonicus* inducted by C58C1 *A. rhizogenes*; b: the monoclone hairy roots; c: The liquid culture of the monoclone hairy roots).

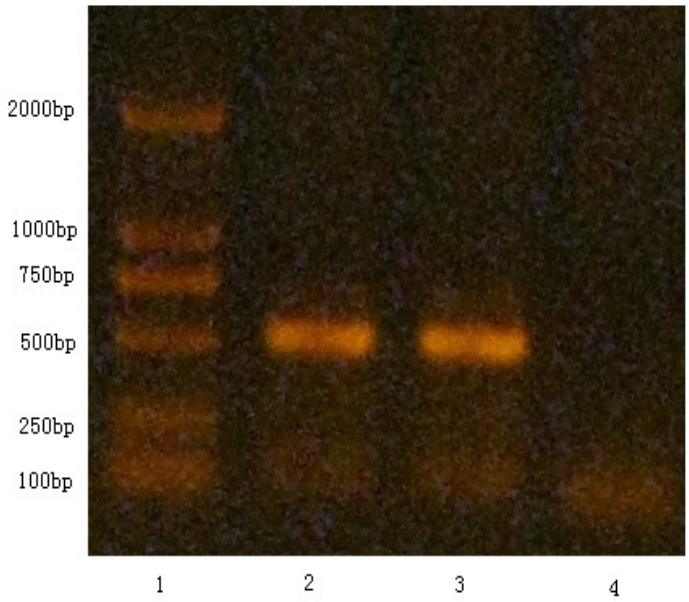

Figure 2. PCR amplification of the *rol*B gene (Lane 1: DNA Marker; Lane 2 and 3: DNA from normal roots, Lane 4 : DNA from hairy roots).

the normal and hairy roots, there were 18 kinds of same compounds, their relative peak areas were 59.86 and 59.84%, all over half of the oils (Figure 3). However for the different compounds, the high content were 3-methylbutyric acid (9.56%) and [4.1.0]-3-heptene,2-isopropenyl-5-isopropyl-7,7-dimethyl-Bicyclo (5.89%) of roots; 1, 2, 3, 4, 4a, 5, 6, 8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-Naphthalene (3.18%) and 3,7,11-trimethyl-, (Z ,E)- 1,3,6, 10- dodecatetraene (3.18%) of hairy roots.

The hairy roots of *P. japonicus* were induced by C58C1 *A. rhizogenes* for the first time. The PCR result showed that the T-DNA of C58C1 *A. rhizogenes* was integrated into *P. japonicus* genome. The essential oils of the normal and hairy roots about *P. japonicus* were extracted with steam distillation methods, the yields of them were 0.451 and 0.380%, respectively. By GC-MS, there were 40 kinds of compounds in normal roots, and 46 kinds of compounds in hairy roots, they amounted to 93.3 and 95.89% of the total detected constituents, respectively. Both of them own 18 same compounds, relative peak areas all exceeded 50%, except that, the normal roots alone own 22 kinds of compounds, and the hairy roots own 28 kinds of compounds, their relative peak areas were all at lower level. The above results showed that the genotype plays an important role in the course of biosynthesizing essential oils of *P. japonicas*; at the same time, the environments could affect the compositions and

Table 1. Constituents of the essential oils from the roots and hairy roots of *P. japonicus*.

No.	Compound name	Rt./min	Molecular formula	Relative content (%)	
				Normal roots	Hairy roots
1	2-ethoxy-propane	5. 978	$C_5H_{12}O$	6.56	3.09
2	3-methyl-Butanoic acid	6. 328	$C_5H_{10}O_2$	9.56	-
3	Octanal	7. 025	$C_8H_{16}O$	1.76	0.88
4	propyl-Propanedioic acid	7. 608	$C_6H_{10}O_4$	0.90	0.71
5	3-methyl-Pentanoic acid	8. 051	$C_6H_{12}O_2$	0.56	-
6	(*E*)-2-Octenal	8. 542	$C_8H_{14}O$	0.42	-
7	Benzeneacetaldehyde	9. 825	C_8H_8O	0.49	-
8	Hexanoic acid	10. 448	$C_6H_{12}O_2$	11.60	13.92
9	n-Caproic acid vinyl ester	11. 247	$C_8H_{14}O_2$	1.66	-
10	Heptanoic acid	12. 075	$C_7H_{14}O_2$	2.21	2.77
11	OCtanoic Acid	12. 739	$C_8H_{16}O_2$	4.27	2.77
12	Copaene	12. 748	$C_{15}H_{24}$	1.10	0.56
13	3-ol,3,7-dimethyl-1,7-Octadien	12. 982	$C_{10}H_{18}O$	0.83	-
14	Tricyclo[2.2.10(2,6)]heptane	13. 216	$C_{15}H_{24}$	2.37	-
15	(*E,E*)-2,4-Decadienal	13. 228	$C_{10}H_{16}O$	0.97	0.88
16	*trans*-α-Bergamotene	14. 604	$C_{15}H_{24}$	1.95	-
17	2-methyl-3methylene-2-(4-methyl-3-pentenyl)-(1*S*-exo)-Bicyclo[2.2.1]heptane	14. 667	$C_{15}H_{24}$	1.14	-
18	1H-Cycloprop[e]azulene	14. 920	$C_{15}H_{24}$	4.92	9.15
19	2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-(1*S*-endo)-Bicyclo[2.2.1]heptane	14. 924	$C_{15}H_{24}$	0.59	-
20	7,11-dimethyl-3-methylene-1,6,10-Dodecatriene,	15. 133	$C_{15}H_{24}$	3.72	1.78
21	1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-Naphthalene	15. 275	$C_{15}H_{24}$	1.62	1.78
22	*trans*-α-Bergamotene	15. 876	$C_{15}H_{24}$	1.86	1.05
23	[4.1.0]-3-heptene,2-isopropenyl-5-isopropyl-7,7-dimethyl-Bicyclo	16. 323	$C_{15}H_{24}$	5.89	-
24	γ-Elemene	17. 244	$C_{15}H_{24}$	0.76	3.16
25	α-Calacorene	17. 267	$C_{15}H_{20}$	0.25	-
26	1-(4-hydroxy-3-methoxyphenyl)-Ethanone	18. 307	$C_9H_{10}O_3$	0.66	-
27	(-)-Spathulenol	18. 342	$C_{15}H_{24}O$	2.71	9.96
28	3-ol, 3,7,11-trimethyl- 1,6,10-Dodecatrien-	18. 676	$C_{15}H_{26}O$	0.49	2.11
29	1,5-diisopropyl-2,3-dimethyl-Cyclohexane	18. 789	$C_{14}H_{28}$	0.83	-
30	2-Pentadecyn-1-ol	19. 014	$C_{15}H_{28}O$	0.76	-
31	3,4,5,6-tetramethyl-Octane	19. 029	$C_{12}H_{26}$	0.56	-
32	3,5,6,8a-tetrahydro-2,5,5,8a-tetramethyl-, *trans*-2H-1-Benzopyran	19. 036	$C_{13}H_{20}O$	0.46	0.88
33	[2,1-b]furan-2(1H)-one,decahydro-8-hydroxy-3a,6,6,9a-tetramethyl-Naphtho	19. 474	$C_{16}H_{26}O_3$	0.97	-
34	2,3,5,8-tetramethyl-Decane	19. 951	$C_{14}H_{30}$	0.52	-
35	n-Hexadecanoic acid	21. 822	$C_{16}H_{32}O_2$	3.10	3.50
36	2,3,5,8-tetramethyl-Decane	22. 408	$C_{14}H_{30}$	0.70	-
37	Falcarinol	22. 507	$C_{17}H_{24}O$	10.04	1.70

contents of essential oils.

Table 1 shows that the normal and hairy roots all owned 6 acids compounds, relative contents were 29.1 and 20.17%. It is known that the acids were from the AA-MA pathway, so it was confirmed that the AA-MA pathway has the advantage in synthesizing the essential oil of *P. japonicus*. At the same time, in the essential oils of normal and hairy roots, the alkenes compounds were detected. The former own 10 alkenes compounds and the later own 15, individual contents were 28.10 and 32.64%, the majority of which were the sesquiterpene. Therefore, the mevalonate (MVA) pathway was also one of the major biosynthesis of essential oils in *P. japonicus*. Among the compounds of the essential oils, the caproic acid, 1H-cycloprop azulene and (-)-spathulenol were owned by the roots and hairy roots in common, they were also representative compounds by both.

However, it was entertaining that the relative content of

Table 1. Contd.

No.	Compound name	Rt./min	Molecular formula	Relative content (%)	
				Normal roots	Hairy roots
38	8-en-2-one- Oxacycloheptadec	22. 519	$C_{16}H_{28}O_2$	1.18	
39	Octadecanoic acid	22. 962	$C_{18}H_{36}O_2$	0.87	0.97
40	Tetratetracontane	23. 086	$C_{44}H_{90}$	1.49	-
41	Heptanal	23. 158	$C_7H_{14}O$	-	0.71
42	2-methyl-1-Hepten-6-one	23. 681	$C_8H_{14}O$	-	0.38
43	1,7-Octadiene	23. 705	C_8H_{14}	-	0.47
44	3,3,5-trimethyl-1,4-Hexadiene	24. 175	C_9H_{16}	-	0.47
45	5-(pentyloxy)-, (E)-2-Pentene	24. 197	$C_{10}H_{20}O$	-	1.23
46	(*Z*)2-Nonenal	25. 196	$C_9H_{16}O$	-	1.13
47	1-methyl-4-(2-methyloxiranyl)-7-Oxabicyclo[4.1.0]heptane	25. 162	?	-	0.72
48	Tridecane	26. 301	$C_{13}H_{28}$	-	0.64
49	Aristolene	27. 563	$C_{15}H_{24}$	-	1.13
50	1,2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-methylene-H-Benzocycloheptene	27. 781	$C_{15}H_{24}$	-	1.05
51	Aromadendrene	27. 782	$C_{15}H_{24}$	-	1.70
52	Thujopsene	28. 432	$C_{15}H_{24}$	-	2.18
53	1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,Naphthalene	28. 901	$C_{15}H_{24}$	-	3.08
54	3,7,11-trimethyl-, (*Z,E*)-1,3,6,10-Dodecatetraene	29.135	$C_{15}H_{24}$	-	1.18
55	3,7,7-trimethyl-11-methylene-Spiro[5.5]undec-2-ene	29.421	$C_{15}H_{24}$	-	1.95
56	2,4a,5,6,7,8-hexahydro-3,5,5,9-tetramethyl-,(*R*)-1H-Benzocycloheptene	29.737	$C_{15}H_{24}$	-	3.12
57	2,10-dimethyl-Undecane	29.929	$C_{13}H_{28}$	-	1.21
58	3-Dodecylcyclohexanone	30.275	$C_{18}H_{34}O$	-	1.54
59	Epiglobulol	30.438	$C_{15}H_{26}O$	-	0.64
60	2,4-Undecadien-1-ol	30.638	$C_{11}H_{22}O$	-	1.29
61	9-octyl-Heptadecane	30.674	$C_{25}H_{52}$	-	1.5
62	4-Methoxy-2(1H)-quinolone	31.018	$C_{10}H_9NO_2$	-	1.29
63	2,4-bis(1,1-dimethylethyl)-Phenol	31.329	$C_{14}H_{22}O$	-	0.97
64	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	31.650	$C_{20}H_{30}O_4$	-	1.29
65	Tridecane, 1-iodo	31.849	$C_{13}H_{27}I$	-	1.38
66	2-Tetradecyne	31.865	$C_{14}H_{26}$	-	0.72
67	Eicosane	31.941	$C_{20}H_{42}$	-	1.42

- = Not detected.

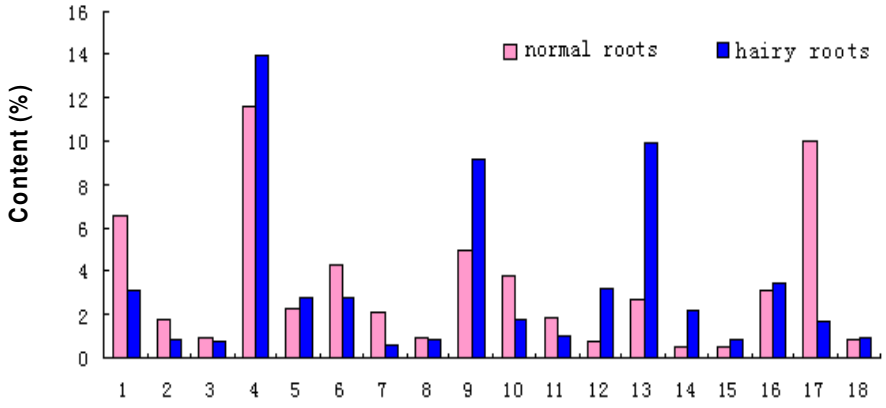

Figure 3. The same compound of essential oils from normal and hairy roots of *P. jappicus* (1, 2-ethoxy-propane; 2, Octanal; 3, propyl-propanedioic acid; 4, hexanoic acid; 5, heptanoic acid; 6, octanoic acid; 7, copaene; 8, (E,E)-2,4-decadienal; 9, H-cycloprop[e] azulene; 10, 7,11-dimethyl-3-methylene-1,6,10-dodecatriene; 11, trans-α-bergamotene; 12, *γ*–elemene; 13, (-)- spathulenol; 14, 3-ol,3,7,11-trimethyl-1,6,10-dodecatrien; 15, 3,5,6,8a-tetrahydro-2,5,5,8a-tetramethyl-,trans-2H-1-benzopyran; 16, n-hexadecanoic acid; 17, **f**alcarinol; 18: octadecanoic acid).

essential oils of the hairy roots exceed the one of the normal roots. Resent study showed that they have lots of bioactivity, for example, caproic acid for relieving fever; (-)-spathulenol for anticancer, antitumor and antibiosis1H-cycloprop and azulene for analgesia (Tsuneki et al., 2005; Ma et al., 2008). So, there was tremendous value on developing medicine in applying the feature of the short growth cycle and the high content ingredient of hairy roots of *P. japonicus*.

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