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The Dormancy Mechanism and Bioactivity of Hydroquinone Extracted from Podophyllum hexandrum Royle Seed

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Abstract

Ethyl ether extraction from seed of *Podophyllum hexandrum Royle* (*P.hexandrum*) was separated by GC-MS and nineteen compounds were identified including fatty acid, alkenes, etc. most of which had been proved to have inhibited bioactivities that could be the primary reason of unhealthy germination for *P.hexandrum* seeds. Hydroquinone was one of the nineteen compounds and the first time identified from *P.hexandrum* seed in this experiment which had never been reported about its bioactivity before. The further study of its bioactivities was conducted on wheat and vitro culture of embryo of *P.hexandrum* seed showed that hydroquinone played a positive role and shortened the cycle in growth and development compared to IAA, NAA, BAP, GA_3, Which indicates that hydroquinone has similarity to auxin on plant growth and development.

Keywords: *P.hexandrum* seed; Dormancy mechanism; Hydroquinone; Bioactivity identification; Auxin.

Abbreviations: GC-MS - Gas chromatograph mass spectrometer; MS – Murashige and Skooge medium; IAA - Indole-3-acetic acid; GA3 - Gibberellic acid; NAA - Naphthaleneacetic acid; BAP - Benzyladenine; RL-Radical Length; SL=Seedling Length; RFW - Radical fresh weight; SFW - Seedling fresh weight; RDW - Radical dry weight; SDW - Seedling dry weight.

1. Introduction

P.hexandrum is a perennial herb, which grows in the Himalayan region and western of China [1,2]. Podophyllotoxin is a natural product mainly existed in the rhizome and roots of *P.hexandrum* and as well as its congeners and derivatives has pronounced biological activity mainly as anticancer, antineoplastic and anti-HIV drugs [3-5]. As the market demand of podophyllotoxin is increasing and the natural resource has been facing endangered [6,7]. In addition, the chemical synthesis of podophyllotoxin is very complicated and rather difficult [8,9]. So podophyllotoxin will ultimately depend upon the supply of raw materials. Both of cultivation and biotechnological are two mainly means for production of podophyllotoxin [10,11]. However, plants grown from rhizome cutting of *P.hexandrum* were estimated to take at least to produce rhizome in fair sizes and plants raised from seedling would take even longer [12]. In addition, the seed dormancy period is very long and the germination percentage is unhealthy [12,13]. In our early research the seed dormancy could be broken in 500mg/L GA_3 solution 36h following cold stratification 90d with germination percentage 81.11% [13]. Although the dormancy has been broken, how to hasten the growth and development of *P.hexandrum* is a key to meet the demand of podophyllotoxin.

The main purpose of this study was to separate the ethyl ether extraction from seed of *P.hexandrum* by GC-MS in order to find the materials and methods that could overcome the difficult of slow growing and hasten the growth and development of *P.hexandrum*.

2. Materials and Methods

2.1 Seed collection

P.hexandrum seeds were collected from the forest (2100m) located in Huichuan, Weiyuan area, Gansu province, China, 1000-seed weight 20.65g.

2.2 Extraction by ethyl ether

Seeds weighted 3.0g were grinded to powder, first the power was packed and soaked by ethyl ether at 4℃, 12 h and then extracted by apparatus, Soxhlet's at 40℃, 24h. At last the extraction was condensed to 3mL for preparation.

2.3 Separation of ethyl ether extraction

Instrument: Ammerican Finnigan Company Trace DSQ GC-MS. Condition of GC: 1N NOWAX quartz capillary column with 30m×0.32cm×0.25mm; column temperature 50~190℃; procedure temperature 5℃/min; carrier gas He; vaporizer temperature 280℃. Condition of MS: Ion source was EI and temperature was 200℃; ionizing voltage 70eV; electric current of collection 300μA; electric current of emission 1mA; resolution 600; mass range 10~500.

2.4 Bioactivity identification of Hydroquinone on wheat

The wheat seeds were soaked in tap water for 1h, then rinsed in 70% ethanol for 10s and five times in sterile water and subsequently immersed in 0.1% $HgCl_2$ for 5min. After rinsing them again in sterile water five times they were inoculated on the basic medium (MS) supplemented with 3.0% sucrose and 0.5% agar with different concentrations of Hydroquinone, IAA, NAA, BAP and GA_3, the pH of the medium was adjusted to 5.8 and the temperature 25±1℃ with 24 h natural light. After 4 days note and measure the wheat RL, SL, RFW, SFW, RDW and SDW. Each treatment inoculated three wheat seeds and repeats three times at the same condition. The data and results were analyzed by SPSS11.5.

2.5 Bioactivity of Hydroquinone on vitro culture of seed embryo of *P.hexandrum*

The seeds of *P.hexandrum* were firstly cleaned and soaked in tap water 6h and then rinsed in 70% ethanol for 10s and then five times in sterile water, subsequently immersed in 0.1% $HgCl_2$ for 20min. Then soaked in sterile 400mg/L GA_3 for 36h to break the seed dormancy and make the embryo developed so as to separate them easily [13], then the seeds were rinsed as before. At last the seeds embryo were separated and inoculated on MS medium supplemented with 2.0% sucrose, 0.4% agar with different growth regulators in the following concentrations[mg/L]: M1: 0.5 IAA; M2: 0.5 GA_3; M3: 0.5 Hydroquinone. The pH of the mediums was adjusted to 5.8 with the temperature 22±1℃ and 24 h lights (1000LX).

3. Results and Discussion

3.1 The separation and identification results of ethyl ether extraction

Nineteen compounds were separated and identified from the extraction of ethyl ether by GC-MS (Figure 1) after the treatments of extraction from *P.hexandrum* seed and there were seven kinds fatty acid, three kinds paraffin hydrocarbon, three kinds alcohol aldehyde, etc existed in the seed, majority of which had been proved to have inhibition bioactives to all of plants seed germination, that including (Z,Z)-9,12-Octadecadienoic acid, Hexadecanoic acid, Oleic acid and 1-Phenanthrenemethanol [14], which relative content in *P.hexandrum* seed reached 26.708%, 17.162%, 8.312% and 5.815%, respectively (Table 1), the only four of which occupied the content of 57.997%. When raising the relative content of germinating inductions such as GA_3 and cold stratification that could change the relative ratio of inhibition and induction could promote the seed germination of *P.hexandrum* [13]. It fully demonstrated that the relative highly content of inhibition compounds of seed germination could be the primary reason of unhealthy *P.hexandrum* seed germination.

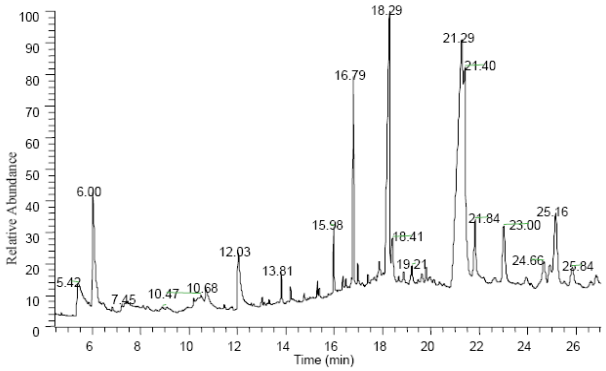

Figure 1. Ion proceeding of ethyl ether extract by GC-MS.

Table 1. Separations of ethyl ether extract by GC-MS.

Peak	Retention time (min)	Compounds	Molecular formula	Relative content (%)
1	5.42	DL-methyltartronic acid	$C_4H_6O_5$	3.705
2	6.00	Dotriacontane	C32H66	7.848
3	7.45	Hexadecane	C16H34	0.875
4	10.47	Octadecane	C18H38	1.917
5	10.68	Hydroquinone	C6H6O2	1.685
6	12.03	2,2'-dithiobis-ethanol	C4H10O2S2	4.014
7	13.81	Lauric acid	C24H46O3	0.785
8	15.98	Tetradecanioc acid	C14H28O2	1.685
9	16.79	1,2-Benzenedicarboxylic,bis(2-methylpropyl)ester	$C_{16}H_{22}O_4$	5.995
10	18.29	Hexadecanoic acid	$C_{16}H_{32}O_2$	17.162
11	18.41	Pentadecanoic acid	$C_{15}H_{30}O_2$	1.801
12	19.21	3,5,5-trimethyl-4-(3-oxobutyl)-2-Cyclohexen-1-one	$C_{13}H_{20}O_2$	0.901
13	21.29	(Z,Z)-9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	26.708
14	21.40	Oleic acid	$C_{18}H_{34}O_2$	8.312
15	21.84	Octadecanoic acid	$C_{18}H_{36}O_2$	3.152
16	23.00	Cis-Z-alpha-bisabolene epoxide	$C_{15}H_{24}O$	3.705
17	24.66	1-Heptatriacotanol	$C_{37}H_{76}O$	2.007
18	25.16	1-Phenanthrenemethanol	$C_{20}H_{30}O$	5.815
19	25.84	7,11-Hexadecadienal	$C_{16}H_{28}O$	1.930

3.2 Bioactivities of different hydroquinone concentrations

Because hydroquinone was the first time identified, the bioactivities of different hydroquinone concentrations were conducted on wheat by measuring the RL, SL after culturing 4 days. The results (Table 2) showed that the growth of wheat seedling could be promoted with the concentration increasing by 1.0 mg/L and then inhibited with the concentration increasing and the treatment of 1×10^{-1} mg/L hydroquinone had no difference with 1.0 mg/L. The RL, SL under the 1.0 mg/L treatment could reach 4.43cm, 4.53cm respectively by the 6th days and increased by 2.42, 4.16 times compared to control, which indicated that hydroquinone possessed bioactivities to promote plant growth and development and had similarity to plant growth regulators.

Table 2. Identification of different hydroquinone concentrations on wheat (±Values represents std deviation).

Concentration (mg/L)	4th days		6th days	
	RL(cm)	SL(cm)	RL(cm)	SL(cm)
Control	1.02±0.14ab	0.62±0.07ab	1.83±0.12bc	1.09±0.06bc
1×10^{-3}	1.34±0.26a	0.76±0.13a	2.17±0.39b	1.41±0.41bc
1×10^{-2}	1.48±0.23a	0.83±0.10a	2.48±0.17b	2.11±0.19b
1×10^{-1}	1.83±0.42a	0.90±0.18a	4.22±0.64a	4.39±1.40a
1.0	1.55±0.41a	0.73±0.25ab	4.43±0.68a	4.53±0.45a
1×10	1.08±0.55ab	0.52±0.31ab	2.47±0.74b	1.05±0.25bc
1×10^{2}	0.39±0.20bc	0.25±0.12bc	0.69±0.16cd	0.28±0.09c
1×10^{3}	0c	0c	0d	0c

Note: Different letters follow the same line numbers show the different significances at 0.05 levels, the same as below.

3.3 Comparison bioactivities between hydroquinone and other hormones

Further study on the bioactivity of hydroquinone was conducted by comparing to NAA, IAA, GA_3 and BAP with 1×10^{-1}mg/L, 1.0mg/L, respectively. The results (Figure 2 and Table 3) showed that the growth and development of wheat seedling was significantly promoted from 4th days to 8th days compared to other hormones and the hydroquinone of the RDW and SDW at the concentration of 1×10^{-1} mg/L could increase by 18.18%, 44.44% and 52.17%, 75.00% compared to both NAA and BAP at the concentration of 1.0 mg/L. All of the data and statistical analysis (Table 3) indicated that the hydroquinone could stimulus the wheat seedling growth and development and hasten the nutrient substances absorption and accumulation, which further demonstrated hydroquinone had the similarity biology characteristics to plant regulators.

3.4 Effect of hydroquinone on vitro culture of embryo of P.hexandrum seed

Considering the hydroquinone was separated from the seed of P.hexandrum and since the relative content of it was low compared to other separated compounds. So the 0.5 mg/L high concentration of hydroquinone, IAA, GA_3 was respectively selected at the basic of before study on the vitro culture of embryo of P.hexandrum seed [7,15]. The results (Figure 3) showed that after culturing 30 days, the embryo growth (Figure 3, M3) of P.hexandrum seed was significantly hastened compared to IAA (Figure 3, M1) and GA_3 (Figure 3, M2), and the seedling (M3) was strong and healthy with the root developed. In addition; after culturing 40 days, the bulb of seedling (Figure 3, M3-1) began to rosette euphylls that needs 1 year to grow and develop at natural Condition that showed the growth and development of P.hexandrum was hastened and the cycle of the growth and development shortened by adding hydroquinone at a degree concentration.

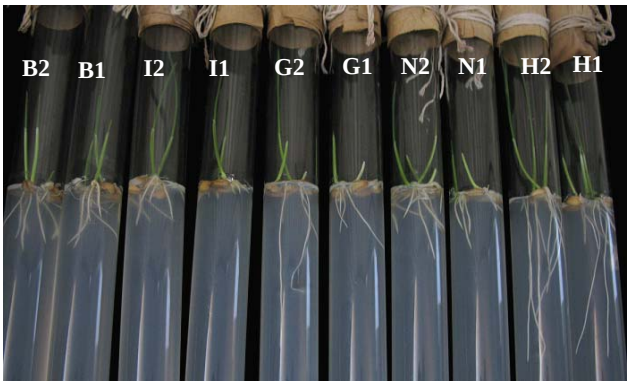

Figure 2. Compare bioactivities between hydroquinone and other hormones on wheat (6th days). BAP, B2=1.0 mg/L; B1=1×10^{-1} mg/L. IAA, I2=1.0 mg/L; I1=1×10^{-1} mg/L. GA_3, G2=1.0 mg/L; G1=1×10^{-1} mg/L. NAA, N2=1.0 mg/L; N1=1×10^{-1} mg/L. Hydroquinone, H2=1.0 mg/ L; H1=1×10^{-1} mg/ L.

Table 3. Comparison bioactivities between hydroquinone and other hormones on wheat (±Values represent std deviation).

Tr	4th days		5th days		6th days		7th days		8th days					
	RL (cm)	SL(cm)	RL (cm)	SL(cm)	RL (cm)	SL(cm)	RL(cm)	SL(cm)	RL(cm)	SL(cm)	RFW(g)	SFW(g)	RDW(g)	SDW(g)
B2	0.57±0.15cd	0.49±0.01bc	1.03±0.25cde	1.36±0.35ab	1.45±0.13bc	2.09±0.46a	1.91±0.51bc	2.52±0.76a	2.23±0.48cd	3.55±0.48ab	0.073±0.030a	0.108±0.062a	0.018±0.006a	0.020±0.012a
B1	0.40±0.21d	0.52±0.33bc	1.20±0.22bcde	0.90±0.22b	1.60±0.12bc	2.58±1.45a	1.75±0.09bc	2.94±1.49a	2.54±1.04cd	4.36±2.17ab	0.077±0.028a	0.122±0.030a	0.021±0.007a	0.021±0.006a
I2	0.36±0.04d	0.54±0.07bc	0.48±0.03e	1.15±0.58ab	0.70±0.17c	3.15±2.08a	0.85±0.05c	3.45±2.57a	0.92±0.32d	4.74±3.84ab	0.027±0.026a	0.102±0.076a	0.008±0.006a	0.020±0.014a
I1	0.53±0.15cd	0.56±0.16bc	0.83±0.30de	1.10±0.59ab	1.02±0.30bc	2.79±1.49a	1.38±0.35bc	3.57±1.29a	1.52±0.37cd	3.93±1.37ab	0.028±0.026a	0.089±0.090	0.011±0.010a	0.015±0.014a
G2	1.17±0.14abc	0.65±0.19bc	1.78±0.43bc	1.37±0.33ab	2.90±1.09ab	2.66±1.22a	3.11±1.19ab	2.90±1.39a	3.44±0.66abc	3.53±1.20ab	0.045±0.009a	0.133±0.045a	0.015±0.004a	0.020±0.007a
G1	0.98±0.18bcd	0.92±0.08ab	1.31±0.13bcde	1.37±0.08ab	1.75±0.68bc	2.38±0.93a	2.48±0.92bc	2.78±1.00a	3.18±1.19bcd	2.93±0.54ab	0.035±0.009a	0.103±0.010a	0.012±0.001a	0.017±0.003a
N2	1.11±0.08bc	1.25±0.22a	1.49±0.16bcd	1.53±0.25ab	1.82±0.73bc	2.95±1.41a	1.99±0.75bc	3.06±1.41a	2.19±0.78cd	3.55±1.55ab	0.079±0.042a	0.130±0.070a	0.022±0.012a	0.024±0.013a
N1	0.37±0.12d	0.34±0.14c	0.97±0.15cde	0.78±0.20b	1.33±0.18bc	1.73±0.50a	1.52±0.16bc	1.92±0.40a	1.81±0.29cd	2.59±0.30b	0.052±0.020a	0.107±0.007a	0.013±0.004a	0.020±0.003a
H2	1.48±0.50ab	0.73±0.25abc	2.12±0.35ab	1.27±0.08ab	4.43±0.68a	4.53±0.45a	4.63±0.61a	4.67±0.51a	5.41±0.87ab	6.11±1.58ab	0.052±0.008a	0.194±0.020a	0.020±0.001a	0.030±0.005a
H1	1.83±0.42a	0.90±0.18ab	2.74±0.71a	2.23±0.80a	3.88±1.21a	4.39±1.39a	4.71±1.18a	4.69±1.38a	5.81±1.39a	7.67±0.80a	0.076±0.009a	0.240±0.048a	0.026±0.005a	0.035±0.007a

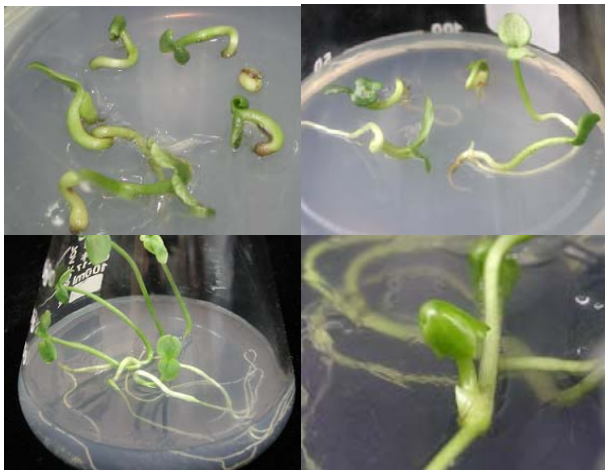

Figure 3. Effect of hydroquinone on vitro culture of seed embryo of P.hexandrum. M1: Seedling cultured with 1.0 mg/L IAA after 30 days; M2: Seedling cultured with 1.0 mg/L GA_3 after 30 days; M3: Seedling cultured with 1.0 mg/L Hydroquinone after 30 days; M3: Seedling developed from bulb cultured on M3 after 40 days.

4 Conclusions

- Majority of nineteen compounds including seven kinds fatty acid, three kinds paraffin hydrocarbon, three kinds alcohol aldehyde, etc separated and identified from P.hexandrum seed had been proved to have inhibition bioactive to majority of plants seed germination and the relative high content of (Z,Z)-9,12-Octadecadienoic acid, Hexadecanoic acid, Oleic acid and 1-Phenanthrenemethanol could be the primary reason of unhealthy seed germination and dormancy mechanism of P.hexandrum.
- Hydroquinone was the first time identified from P.hexandrum seed and the concentrations 1×10^{-1} ~ 1.0 mg/L of hydroquinone possessed bioactivities and could stimulus the wheat seedling growth and development and hasten the nutrient substances absorption and accumulation that showed hydroquinone had the similarity biology characteristics to plant regulators.
- Adding 0.5 mg/L concentration of hydroquinone in the MS medium on the vitro culture of embryo of P.hexandrum seed could hasten and shorten the cycle of the growth and development of P.hexandrum, which will provide an important foundation for the use of biotechnology to obtain podophyllotoxin in the near future.
- The ratio of hydroquinone conspicuously lower compared to (Z,Z)-9,12-Octadecadienoic acid, hexadecanoic acid, oleic acid, dotriacontane, etc was the main reason that couldn't promote the seed germination of P.hexandrum. The growth and development of seed embryo of P.hexandrum could be promoted when the concentration of hydroquinone increased by proper degree (1×10^{-1} ~ 1.0 mg/L). In addition, the bioactivity mechanism of Hydroquinone still needs further studying.

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