Comparison of cytotoxic extracts from fruiting bodies, infected insects and cultured mycelia of Cordyceps formosana

ARTICLE *in* FOOD CHEMISTRY · FEBRUARY 2014

Impact Factor: 3.39 · DOI: 10.1016/j.foodchem.2013.09.001 · Source: PubMed

6 AUTHORS, INCLUDING:

Chunru Li
Anhui Agricultural University (AHAU)
28 PUBLICATIONS **172** CITATIONS
SEE PROFILE

Guanhu Bao
Anhui Agricultural University (AHAU)
40 PUBLICATIONS **343** CITATIONS
SEE PROFILE

Zengzhi Li
Anhui Agricultural University (AHAU)
114 PUBLICATIONS **1,051** CITATIONS
SEE PROFILE

Food Chemistry 145 (2014) 1066–1071

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Comparison of cytotoxic extracts from fruiting bodies, infected insects and cultured mycelia of *Cordyceps formosana*

Rui-Li Lu, Guan-Hu Bao, Feng-Lin Hu *, Bo Huang, Chun-Ru Li, Zeng-Zhi Li

Anhui Agricultural University, Hefei, 230036 Anhui, China

ARTICLE INFO

Article history:
Received 10 January 2013
Received in revised form 23 May 2013
Accepted 2 September 2013
Available online 13 September 2013

Keywords:
Cordyceps formosana
Mycelia
CHO cells
Cytotoxicity
HPLC-PAD–HRMS
Structure

ABSTRACT

A resazurin method was employed to test and compare cytotoxicity of extracts from fruiting bodies, insects and cultured mycelia of *Cordyceps formosana* against Chinese hamster ovary (CHO) cells. Results showed that the cultured mycelia had much stronger cytotoxicity than that of the fruiting bodies and infected insects. This suggestes that using cultured mycelia to substitute a natural *Cordyceps* may result in poisoning. A combined method of HPLC-PAD–HRMS and cytotoxic analysis revealed that the most toxic compound (Compound **1**) was found mainly in the cultured mycelia and also a small amount in the infected insect body of the *Cordyceps*, but not in the fruiting body. The second toxic compound (Compound **2**) was found in all structures of *Cordyceps* and in cultured mycelia. Different contents of the toxic compounds resulted in the different cytotoxicity of the extracts. Compound **1** and Compound **2** were prepared with preparative HPLC as yellow and orange powders, respectively. Cytotoxic tests showed that the median lethal dose (LD_{50}) against CHO cells of Compound **1** was 18.3 ± 0.2 and 103.7 ± 5.9 μg/mL for Compound **2**. Compound **1** and Compound **2** were identified as rugulosin and skyrin by HRMS, UV and NMR data. The two compounds were never previously isolated from the genera *Cordyceps* and *Hirsutella* and their cytotoxicity against CHO cells was also reported for the first time.

1. Introduction

Cordyceps species are distributed worldwide, however, only *Cordyceps sinensis* whose Chinese named is Dong Chong Xia Cao has been traditionally used in China as a tonic food or medicine to promote health and longevity (Anon, 1985; Anon, 2010; Hu & Li, 2007; Pu & Li, 1996). Modern pharmacologic research has revealed that *C. sinensis* exerts powerful antioxidant properties that contribute to its wide range of anti-aging and adaptogenic health benefits (El et al., 2012; Jim, 2012; Leung et al., 2009; Li et al., 2006a; Li et al., 2006b; Yang et al., 2011). *C. sinensis* was also shown to fight fatigue, enhance performance and promote lung health by enhancing production of mitochondrial adenosine triphosphate (ATP), the universal energy molecule (Gao et al., 2010). The supply of natural *C. sinensis* is not meeting demand and it cannot yet be commercially cultured. The mycelium of the genuine anamorph of the *Cordyceps* grows very slowly. Therefore, substitutes are being sought (Shrestha et al., 2012; Guo et al., 2006; Hu et al., 2009; Leung et al., 2006; Ng & Wang, 2005; Yan et al., 2010). In order to identify a safe substitute, a cytotoxic screening program was launched on 12 natural *Cordyceps* spp and their anamorphic mycelia. Among them the water extract of *Cordyceps formosana* showed no significant toxicity. At the same time its anamorph, *Hirsutella huangshanensis* from the same genus as that of *C. sinensis*, can grow much faster (Zuo et al., 2008). It seemed that they could be candidates as substitutes for *C. sinensis*. However, further toxic tests revealed that the extracts from mycelia of *H. huangshanensis* had much higher toxicity. This indicates that a strain which is isolated from a low toxicity natural *Cordyceps* is not necessarily safe. This suggests that caution is in order in the production of *C. sinensis* substitutes, because more than 11 species of fungi, belonging to 10 genera, were isolated from *C. sinensis* including *Hirsutella sinensis*, the genuine anamorph of *C. sinensis* and some possible contaminating fungi (Liang, 2001; Russell & Paterson, 2008).

The safety of *C. sinensis* has passed a long history of safe usage, but the isolates are not necessarily safe. Apart from isolates from *C. sinensis,* several strains isolated from other *Cordyceps* were also used as substitutes of *C. sinensis.* For example, *Paecilomyces militaris* and *Paecilomyces tenuipes* were certificated as tonic foods in some eastern countries of Asia (Nam et al., 2011; Lin and Li, 2011). Despite that most of the substitutes were claimed to contain similar biochemical compositions and pharmacological properties as that of *C. sinensis* (Das et al., 2010; Huang et al., 2009; Li et al., 2006a; Li et al., 2006b; Zhong et al., 2009; Zhou et al., 2009), some

* Corresponding author. Tel.: +86 551 5786887.
E-mail address: hufenglin@hotmail.com (F.-L. Hu).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.09.001

of these still lack systematic chemical and toxicity data or only have data on some common nutrients such as fatty acids, protein, essential amino acids and nucleosides etc. (Li et al., 2006a; Li et al., 2006b; Russell & Paterson, 2008). Compared with nutrients, nowadays people pay more attention to food safety (Yamashoji et al., 2013). Therefore, we studied the cytotoxicity of extracts from fruiting bodies, infected insects and cultured mycelia of *C. formosana* against CHO cells. Our results show that even the genuine anamorph of a *Cordyceps* does not necessarily have the same components and toxicity as that of the natural *Cordyceps*, and suggests that special attention should be taken on safety in the substitution of natural *Cordyceps* especially *C. sinensis* with cultured mycelia. At the same time we provide some chemical and toxicological information on *C. formosana*.

2. Materials and methods

2.1. Chemicals and equipment

Solvents and chemicals were of analytical or high performance liquid chromatography (HPLC) grade. Calf serum and trypsin were products of the Gibco Company. DMEM/F_{12} and resazurin were from the Sigma Company. A microplate reader (Spectra Max M2) was from the Molecular Device Company. The Agilent 6210 time-of-flight LC/MS system with a Agilent 1100 HPLC, a photodiode array detector (PAD), a high performance time-of-flight (HRTOF) MS with an electrospray ionisation (ESI) source and an Agilent workstation were from the Agilent Company. The Analytical column (Atlantis, 5 μm, 150 × 3.9 mm i.d.), semi-preparative column (Atlantis, 5 μm, 150 × 7.8 mm i.d.), and preparative column (5 μm, 150 × 19 mm i.d.) were products of the Waters Company. The Preparative HPLC with a UV detector and a high-pressure pump was from the Younglin Company. The centrifugal concentrator was a product of the Christ Company. The R502 rotary evaporator was from the Shanghai Senko Company. The freeze dryer was fromthe Laboconco Company. An Advance 400 nuclear magnetic resonance (NMR) spectrometer was from the Bruker Company.

2.2. Methods

2.2.1. C. formosana sampling and mycelia preparation

C. formosana was collected in the Gu-Liu-Jiang nature protection area of Anhui Province, China, and identified by two of us (Prof. Huang and Li). Its hosts are tenebrionid-larvae and its anamorph is *H. huangshanensis*. Spores of the *C. formosana* were collected and cultured in a liquid medium containing 40 g/L glucose, 10 g/L yeast extract, and 10 g/L peptone. After 15 days fermentation under shaking at 180 rpm and 25 °C, the anamorphic mycelia were filtrated from the fermentation broth, washed with distilled water two times and then lyophilized.

2.2.2. Extraction

Three portions of 50 g of freeze dried fruiting body, insect body or cultured mycelia of *C. formosana* were weighted. They were extracted with 500 mL distilled water, methanol or ethyl acetate for 24 h in a rotary shaker (150 rpm) at room temperature. The suspensions were filtered, and the filtrates were condensed under reduced pressure (1.0 kPa) and dried in a freeze dryer.

2.2.3. CHO cells toxicity assay

The dried extracts were dissolved in 12.5% aqueous DMSO solution at the concentrations of 200, 400, 800, 1600 and 3200 μg/mL, respectively, and filtered through a 0.22 μm pore size disposable filters. Samples with median lethal dose (LC_{50}) < 20 μg/mL will be retested at the concentrations 50, 100, 150, 200, 250, 300 and 350 μg/mL, respectively.

The Chinese hamster ovary (CHO) cells were cultured with DMEM/F_{12} medium contained 10% calf serum at 37 °C, 5% CO_2 incubator. Upon entering the period of logarithmic mitosis, the CHO cells were trypsinized and transferred from culture flasks into 96-well plates, with 4.0×10^5 cells in 100 μL cultural medium in each well, while leaving three blank wells (with just cultural medium without cells), at a 37 °C, 5% CO_2 incubator for 24 h. As cells were stable and adherent for 24 h, the old medium was replaced by 90 μL fresh culture medium and 10 μL of the sample solution. At the same time, cell and cell less control (without sample) groups were set up. Then 20 μL 0.05% resazurin was added into each well and the cell plates were incubated for two more hours. The fluorescence intensity was detected by the microplate reader, with excitation wavelength of 530 nm andemission wavelength of 590 nm. Cytotoxicity was calculated:

$$\text{Cytotoxicity}(\%) = [1 - (\text{FLUsample} - \text{FLU100}\%)/(\text{FLU0}\% - \text{FLU100}\%)] \times 100\%.$$

Every test had five repeats.

2.2.4. HPLC–MS analysis

Three microliters of the extract solution (1 mg/mL) were automatically injected onto the analytical Atlantis column. The column was eluted with a water/methanol (both contain 0.1% formic acid) gradient from 70:30 to 0:100 for 20 min, and 100% methanol for further 15 min. Flow rate was 0.8 mL/min. The eluates were monitored with a PAD at full length scan from 200 to 600 nm, and a HRTOF-MS detector under negative and positive mode. Drying Gas of the ESI ion source was 12 L/min, 325 °C. Nebulizer pressure was 35 psig. Capillary voltage was 3500 V for negative mode and 4000 V for positive mode. Fragmentor voltage was 175 V for negative mode and 215 V for positive mode. Scan range was 50–1200 amu.

2.2.5. HPLC–MS-cytotoxicity combined analysis

The ethyl acetate extracts from the fruiting body, insect body and anamorphic mycelia of *C. formosana* were dissolved in mixed solvent of methanol and ethyl acetate (8:2) respectively.

Thirty microliters of the extract solution (10 mg/mL) were automatically injected onto the semi-preparative Atlantis column. The column was eluted at 4 mL/min using the same solvent system and gradient as that of the analytical HPLC. The eluates were monitored with a PAD at full length scan from 200 to 600 nm, and 10% of the eluates were monitored with the MS detector through a distribution valve. The other 90% eluates were automatically collected every minute. The injection was repeated 10 times. The tubes were combined according to peaks of the HPLC–MS and condensed with the centrifugal concentrator and then freeze dried. The dry components were dissolved in 12.5% DMSO for CHO cell toxicity assays.

2.2.6. Cytotoxic compounds preparation and structure identification

Two hundred microliters of the extract at a concentration of 10 mg/mL were injected into preparative HPLC. The column was eluted at 20 mL/min using the same solvent system and gradient as that of the analytical HPLC. Fractions were automatically collected according to the HPLC peaks and evaporated under reduced pressure (1 kPa) and lyophilized.

After purity and cytotoxicity was confirmed, the purified compounds were dissolved in DMSO-d6 for 1D and 2D NMR (^1H, ^{13}C, DEPT 135, COSY and HMBC) tests. The structures were elucidated with the aid of the MS, UV and NMR data.

Table 1
Cytotoxicities of extracts on CHO cells.

Name of the sample	LD_{50} (μg/mL) of different extracts		
	Methanol extract ethyl	Acetate extract	Aqueous extract
Fruiting body	–	436.75 ± 27.36	–
Insect body	215.61 ± 9.80	158.32 ± 6.90	–
Mycelia	76.10 ± 3.14	52.44 ± 1.67	394.82 ± 21.24

3. Results and discussion

3.1. Cytotoxicity comparison of the extracts

Cytotoxicity of the crude extracts was tested according to Section 2.2.3. The LD_{50} of the extracts were calculated and listed in Table 1.

In the Table 1 we can see that the water extracts of *C. formosana* (fruiting body and infected insect) had no significant toxicity to CHO cells, but the methanol and ethyl acetate extracts did. This means the Cordyceps may contain organic solvent soluble toxins. Therefore, it is not a safe candidate substitute for *C. sinensis*. At the same time, despite that the anamorph of *C. formosana* (*H. huangshanensis*) comes from the same genera as that of *C. sinensis* (*Hirsutella sinensis*), and can much easier be cultured much easier (Zuo et al., 2008), the strong toxicity of the cultured mycelia indicates that it should not be used as a substitute of *C. sinensis*.

Table 1 showes that the cytotoxicity of the extracts were very different; the cultured mycelia had much stronger cytotoxicity than the *Cordyceps*. This toxic difference should make people aware that there are differences between the teleomorphic and anamorphic stages of a fungus, because many people thought a *Cordyceps* (telemorph) and its cultured mycelia (anamorph) were the same, and many researchers even mistakenly called or used cultured mycelia (anamorphic) as cultured *Cordyceps* (telemorphic) (Chen et al., 2010; Choi et al., 2010; Wang et al., 2009; Yang et al., 2011; Zhang et al., 2011). Confusing cultured mycelia with *Cordyceps* may result in poisoning. If the mycelia were not cultured with a genuine anamorphic strain the situation may be even worse because a *Cordyceps* can be contaminated by a variety of fungi (Liang, 2001; Russell & Paterson, 2008).

The significant toxic difference between the fruiting body, infected insect and anamorphic mycelia of the *Cordyceps* is somewhat puzzling, for the fruiting body and infected insect were from the same *Cordyceps*, meanwhile, the infected insect of the *Cordyceps*, which was filled with mycelia, was similar to cultured mycelia. In order to understand the differences, the extracts were submitted to chemical components and toxicity analysis.

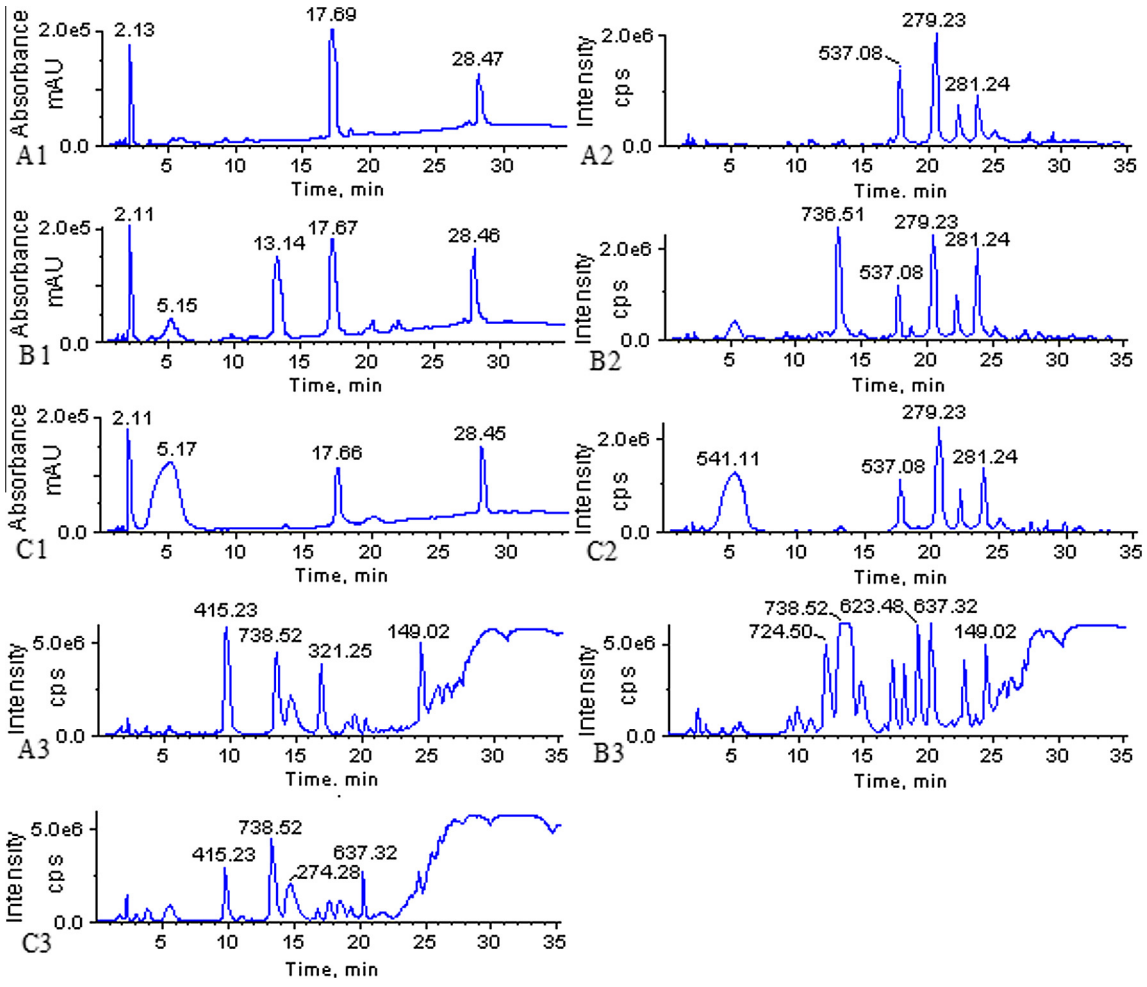

Fig. 1. Chromatograms of HPLC-MS of the extracts A: Fruiting body A1: Uv Chromatogram A2: Ion flow in negative mode A3: Ion flow in positive mode B: Insect body B1: Uv Chromatogram B2: Ion flow in negative mode B3: Ion flow in positive mode C: Anamorphic mycelia C1: Uv Chromatogram C2: Ion flow in negative mode C3: Ion flow in positive mode

Table 2
Cytotoxicity of the collected peaks prepared by semi-preparative HPLC–MS.

Range of collection time (min)	Cytotoxicity (%) at the concentration of 40 μg/mL				
	4.0–6.0	9.0–10.0	12.0–14.0	16.0–18.0	27.0–29.0
Fruiting body		–		15.75 ± 1.39	–
Insect body			–	16.32 ± 1.51	–
Anamorphic mycelia	87.46 ± 3.27			15.81 ± 1.46	–

Note: "–" means no significant cytotoxicity compared to the control group.

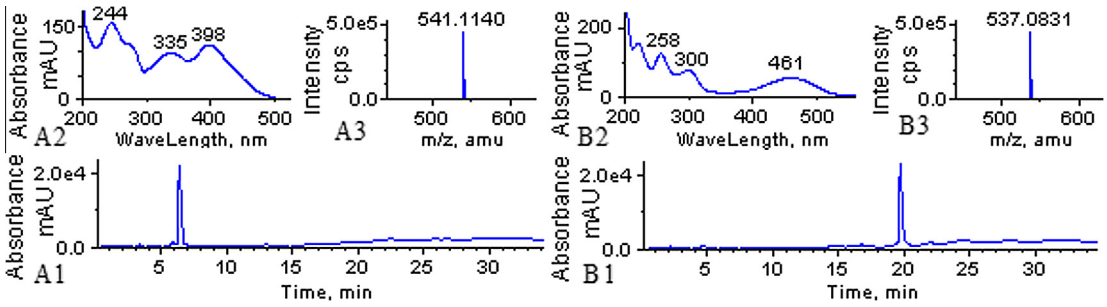

Fig. 2. HPLC-MS analysis of the Compound 1 and Compound 2 A1: HPLC Chromatogram of Compound 1 A2: Uv spectrum of Compound 1 A3: Mass spectrum of Compound 1 B1: HPLC Chromatogram of Compound 2 B2: Uv spectrum of Compound 2 B3: Mass spectrum of Compound 2.

3.2. Cytotoxic components comparison between fruiting body, insect body and cultured mycelia of C. formosana

The HPLC–MS results (Fig. 1A–C) showed that the extracts shared many common peaks for example at Rt 17.6, Rt 28.4, *m*/*z* 279, *m*/*z* 255, *m*/*z* 281, *m*/*z* 738 and 391 etc. This phenomenon is understandable considering that the anamorphic mycelia and the telemorphic *Cordyceps* contained the same genetic resource (Zuo et al., 2008).

Nevertheless, there were also many different peaks. The fruiting body had a unique high peak at *m*/*z* 415, while the insect body had a very high peak at Rt 13.1 or *m*/*z* 736 and 738, and the cultured mycelia had a largepeak at Rt 5.1 or *m*/*z* 541. The fruiting body contained unique metabolites is understandable considering that the fruiting body possessed unique form and colour of the sexual stage (Fig. 1) and that epigenetics related to metabolites (Katada et al., 2012). The differences between the insect body and the cultured mycelia seemed perplexing for the cultured mycelia and mycelia in the insect body were all at the same stage of life cycle. However, the mycelia growing environments were different. The mycelia in the insect body had suffered immune attack of the insect. Different growing environments can result in metabolite differences (Abdel et al., 2012; Zhao et al., 2012). Obviously, different metabolites can result in different cytotoxicities of the extracts.

In order to find out the toxic compounds in the extracts, all the peaks of the chromatogram were collected according to the Section 2.2.5, except the solvent peaks such as the ethyl acetate peaks (Rt 2.1) and peaks of known safe compounds such as linoleic acid (*m*/*z* = 279.2330), palmitic acid (*m*/*z* = 255.2331) and oleic acid (*m*/*z* = 281.2490). After evaporation of the solvent in the collected peaks, 5 main components were obtained. Toxicity of the components is listed in Table 2.

There were two toxic components in the extracts (Table 2). The most toxic compound was found mainly in the extract of the cultured mycelia (Fig. 2C). Small amounts of the most toxic compound were also found in the insect body (Fig. 2B), but not in the fruiting body (Fig. 2A). Obviously, the different content of the toxic compounds resulted in the different cytotoxicity of the extracts.

3.3. Cytotoxic compound preparation and identification

3.3.1. Cytotoxic compounds preparation

The two toxic compounds were prepared according to the preparative HPLC method. The most toxic compound (Compound **1**) was a yellow powder prepared from the extract of the cultured mycelia. The second toxic compound (Compound **2**) was an orange powder prepared from the extract of the fruiting body of the Cordyceps. After preparation, the two compounds were submitted for purity and toxicity analysis. The HPLC–MS analysis showed that the two compounds were pure because there was only one peak on each chromatogram, and only one ion group on each mass spectrum (Fig. 2). Results of cytotoxic tests showed that the median lethal dose of the Compound **1** was 18.3 ± 0.2 μg/mL and Compound **2** 103.7 ± 5.9 μg/mL. The purity and toxicity of the compounds proved a successful preparation.

3.3.2. Identification of the toxic Compound *1*

The results of the HPLC–PDA–MS analysis of Compound **1** showed that the high resolution *m*/*z* of anion and cation were 541.1140 and 543.1286, respectively, and the possible molecular formula was $C_{30}H_{22}O_{10}$. Maximum absorbance of the UV spectrum at 244, 272, 335 and 398 nm suggested that there was a long conjugated system in the compound. Database query revealed that there were several known compounds corresponding to the molecular formula $C_{30}H_{22}O_{10}$, but none came from the genus *Cordyceps* or *Hirsutella*. To confirm the structure, Compound **1** was submitted to NMR analysis.

NMR data of the Compound **1** were listed on Table 3. The NMR data were consistent with those reported for rugulosin (Nicolaou et al., 2007; Snider & Gao, 2005). Rugulosin has been isolated from several fungi of the genus *Penicillium*, *Endothia*, *Hypocrella* (anamorph of *Aschersonia*) and lichen (Bouhet et al., 1976; Brunati et al., 2009; Ernst-Russell et al., 2000; Yamazaki et al., 2010), but it has never been isolated from the genera *Hirsutella* and *Cordyceps*. Rugulosin has been reported possessing many bioactivities such as anti-bacterial, anti-insect cells, genotoxicity and hepatocarcinogenicity but cytotoxicity against CHO cells was not previously

Table 3
Atoms assignment of the Compounds **1** and **2**.

Position	^{13}C δ	^{1}H δ *J* (Hz)	DEPT 90°, 135° and HMQC	HMBC ^{1}H to ^{13}C
Compound ***1***				
1,1′	47.74	3.37(2H,brs)	CH	$C_3C_{9a}C_9$, $C_{3'}C_{9a'}C_{9'}$
2,2′	68.45	4.39(2H,brd)	CH	
2,2′-OH		5.42(2H,brs)		
3,3′	58.34	2.77(2H,d,*J* = 5.2 Hz)	CH	$C_1C_2C_{4a}C_9C_{10}$, $C_{1'}C_{2'}C_{4a'}C_{9'}C_{10'}$
4,4′	180.56		C	
4,4′-OH		14.70(2H,s)		
4a,4a′	106.06		C	
5,5′	160.14		C	
5,5′-OH		11.45(2H,s)		
6,6′	123.94	7.19(2H,d)	CH	$C_5C_8C_{10a}$, $C_{5'}C_{8'}C_{10a'}$
7,7′	147.44		C	
8,8′	120.40	7.46(2H,d)	CH	$C_{10a}C_6$, $C_{10a'}C_{6'}$
8a,8a′	131.95		C	
9,9′	193.94		C	
9a,9a′	55.60		C	
10,10′	185.89		C	
10a,10a′	114.15		C	
7,7′-CH_3	21.41	2.43(6H,s)	CH_3	$C_6C_7C_8$, $C_{6'}C_{7'}C_{8'}$
Compound ***2***				
1,1′	160.98		C	
1,1′-OH		12.01		$C_1C_2C_{13}$, $C_{1'}C_{2'}C_{13'}$
2,2′	123.48	7.14(1.1)	CH	$C_1C_4C_{13}C_{15}$, $C_{1'}C_{4'}C_{13'}C_{15'}$
3,3′	148.07		C	
4,4′	120.34	7.27(1.1)	CH	$C_2C_4C_{13}C_{15}$, $C_{2'}C_{4'}C_{13'}C_{15'}$
5,5′	108.83		C	
6,6′	164.24		C	
7,7′	107.12	6.70	CH	$C_2C_5C_6C_8$, $C_{2'}C_{5'}C_{6'}C_{8'}$
8,8′	164.37		C	
9,9′	189.38		C	
10,10′	181.99		C	
11,11′	133.17		C	
12,12′	117.71		C	
13,13′	113.10		C	
14,14′	131.22		C	
15,15′	21.42	2.33	CH_3	$C_2C_3C_4$, $C_{2'}C_{3'}C_{4'}$
6-OH		12.78		$C_5C_6C_7$, $C_{5'}C_{6'}C_{7'}$
8-OH		11.04		

reported (Sakai et al., 1992; Stark et al., 1978; Ueno et al., 1980; Watts et al., 2003).

3.3.3. Identification of the Compound *2*

Compound 2 was foundin all parts of the *Cordyceps* and had weak cytotoxicity. HPLC–PDA–MS analysis showed that the high resolution *m/z* of anion and cation of the compound were 537.0831 and 539.0970 respectively, and the possible molecular formula was $C_{30}H_{18}O_{10}$. Maximum absorbance of the UV spectrum at 222, 258, 300 and 461 nm suggested that there was a long conjugated system in the compound. Database query revealed that there were also several known compounds corresponding to the molecular formula $C_{30}H_{18}O_{10,}$ but none came from the genus *Cordyceps* or *Hirsutella*. To confirm the structure, Compound **2** was submitted to NMR analysis.

Results of the NMR analysis were listed on Table 3. The NMR data were consistent with those reported for skyrin. Skyrin has been isolated from several fungi of the genera *Penicillium* and *Hypocrella* (anamorph of *Aschersonia*) and some plants, (Brunati et al., 2009; Lin et al., 2001; Liu et al., 2010) but it has not been previously isolated from *C. formosana* and cultured mycelia of *Hirsutella huangshanesis* (anamorph of *C. formosana*). Skyrin has been reported possessing bioactivities such as anti-bacterial, anti-insect cells, glucagon antagonist and hepatocarcinogenicity, but cytotoxicity against CHO cells has not been previously reported (Brunati et al., 2009; Parker et al., 2000; Stark et al., 1978; Watts et al., 2003).

4. Conclusion

The above research showed that fruiting bodies, infected insects and cultured mycelia of *C. formosana* contained toxins and should not be used as a substitute of *C. sinensis* or *H. sinensis*.

The significant cytotoxicity difference between natural *C. formosana* (fruiting body, infected insect) and cultured mycelia suggests that special attentions should be taken on the toxic difference while using cultured mycelia to substitute the natural *Cordyceps*.

Bioactive guided anysis revealed that the most toxic compound (Compound **1**) was existed mainly in the cultured mycelia and also a small amount in the infected insect of the *Cordyceps*, but not in the fruiting body. The second toxic compound (Compound **2**) was found in all structures of the *Cordyceps* and in cultured mycelia. Cytotoxic tests showed that the median lethal dose (LD_{50}) against CHO cells of Compound **1** was 18.3 ± 0.2 and 103.7 ± 5.9 μg/mL for Compound **2**. Different toxicity and contents of the toxic compounds resulted in the different cytotoxicity of the mycelia and *Cordyceps*.

HRMS, UV and NMR data analysis showed that the Compounds **1** and **2** were rugulosin and skyrin. The two compounds were never previously isolated from the genera *Cordyceps* and *Hirsutella* and their cytotoxicity against CHO cells was also reported for the first time.

Results of the study can be guidance for further utilisation of *C. formosana* and its anamorph, *Hirsutella Huangshanensis*, and suggests that the components and toxicity of fruiting bodies, infected

insects and cultured mycelia of a *Cordyceps* can be very different, and that special attention should be taken on safety in the substitution of natural *Cordyceps* with cultured mycelia.

Acknowledgements

Thanks are due to Dr. Inken Plitzko, Institute of Pharmacy, University of Basel, Switzerland, for the chemical structure confirmation. Financial support was provided by the Ministry of Science and Technology of Anhui Province (Grant Nos. 11040606M69 and KJ2012A107) and the National Natural Science Foundation of China (Grant No. 30871676).

References

Abdel, H. A., Schmidt, H. M., Parra, R., Geisen, R., & Magan, N. (2012). Systems approach to model the relationship between aflatoxin gene cluster expression, environmental factors, growth and toxin production by *Aspergillus flavus*. *Journal of the Royal Society, Interface, 9*(69), 757–767.

Anon (1985). *Dictionary of traditional Chinese medicine*. Shanghai: Science and Technology Press (pp. 767–768) [in Chinese].

Anon (2010). *Pharmacopoeia of the People Republic of China*. Beijing: Science and Technology Press (pp. 75–76) [in Chinese].

Bouhet, J. C., Van Chuong, P. P., Toma, F., Kirszenbaum, M., & Fromageot, P. (1976). Isolation and characterization of luteoskyrin and rugulosin, two hepatotoxic anthraquinonoids from *Penicillium islandicum* Sopp. and *Penicillium rugulosum* Thom. *Journal of Agriculture and Food Chemistry, 24*(5), 964–972.

Brunati, M., Rojas, J. L., Sponga, F., Ciciliato, I., Losi, D., Göttlich, E., et al. (2009). Diversity and pharmaceutical screening of fungi from benthic mats of Antarctic lakes. *Marine Genomics, 2*(1), 43–50.

Chen, W., Zhang, W., Shen, W., & Wang, K. (2010). Effects of the acid polysaccharide fraction isolated from a cultivated *Cordyceps sinensis* on macrophages in vitro. *Cellular Immunology, 262*(1), 69–74.

Choi, J. W., Ra, K. S., Kim, S. Y., Yoon, T. J., Yu, K. W., Shin, K. S., et al. (2010). Enhancement of anti-complementary and radical scavenging activities in the submerged culture of *Cordyceps sinensis* by addition of citrus peel. *Bioresource Technology, 101*(15), 6028–6034.

Das, S. K., Masuda, M., Sakurai, A., & Sakakibara, M. (2010). Medicinal uses of the mushroom *Cordyceps militaris*: current state and prospects. *Fitoterapia, 81*, 961–968.

El Zahraa, Z., El Ashry, F., Mahmoud, M. F., El Maraghy, N. N., & Ahmed, A. F. (2012). Effect of *Cordyceps sinensis* and taurine either alone or in combination on streptozotocin induced diabetes. *Food and Chemical Toxicology, 50*(3), 1159–1165.

Ernst-Russell, M. A., Chai, C. L., Wardlaw, J. H., & Elix, J. A. (2000). Euplectin and coneuplectin, new naphthopyrones from the lichen *Flavoparmelia euplecta*. *Journal of Natural Products, 63*(1), 129–131.

Gao, B. A., Yang, J., Huang, J., Cui, X. J., Chen, S. X., Den, H. Y., et al. (2010). *Cordyceps sinensis* extract suppresses hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells. *Saudi Medical Journal, 31*(9), 974–979.

Guo, F. Q., Li, A., Huang, L. F., Liang, Y. Z., Chen, B. M., et al. (2006). Identification and determination of nucleosides in *Cordyceps sinensis* and its substitutes by high performance liquid chromatography with mass spectrometric detection. *Journal of Pharmaceutical and Biomedical Analysis, 40*(3), 623–630.

Hu, F. L., He, Y. Q., Huang, B., Li, C. R., Fan, M. Z., & Li, Z. Z. (2009). Secondary metabolites in a soybean fermentation broth of *Paecilomyces militaris*. *Food Chemistry, 116*, 198–201.

Hu, F. L., & Li, Z. Z. (2007). Secondary metabolites and their bioactivities of Cordyceps and its related fungi. *Mycosystema, 26*(4), 607–632 [in Chinese].

Huang, L., Li, Q., Chen, Y., Wang, X., & Zhou, X. (2009). Determination and analysis of cordycepin and adenosine in the products of *Cordyceps* spp. *African Journal of Microbiology Research, 3*, 957–961.

Jim, English. (2012). Research supports anti-aging benefits of "Elite Class" of tonic herbs. *Nutrition Review, 7*(1), 1–6.

Katada, S., Imhof, A., & Sassone-Corsi, P. (2012). Connecting threads: Epigenetics and metabolism. *Cell, 148*(1), 24–28.

Leung, P. H., Zhang, Q. X., & Wu, J. Y. (2006). Mycelium cultivation, chemical composition and antitumour activity of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. *Journal of Applied Microbiology, 101*(2), 275–283.

Leung, P. H., Zhao, S., Ho, K. P., & Wu, J. Y. (2009). Chemical properties and antioxidant activity of exopolysaccharides from mycelial culture of Cordyceps *sinensis* fungus Cs-HK1. *Food Chemistry, 114*(4), 1251–1256.

Li, C. R., Li, Z. Z., Fan, M. Z., Cheng, W. M., Long, Y. H., Ding, T., et al. (2006a). The composition of Hirsutella sinensis, anamorph of *Cordyceps sinensis*. *Journal of Food Composition and Analysis, 19*(8), 800–805.

Li, S. P., Zhang, G. H., & Zeng, Q. (2006b). Activity of polysaccharide, with antioxidation, isolated from cultured Cordyceps mycelia. *Phytomedicine, 13*(6), 428–433.

Liang, Z. Q. (2001). Current situation and ponderation of Cordyceps research and exploitation in China. *Acta Edulis Fungi, 8*(2), 53–62 [in Chinese].

Lin, B., & Li, S. (2011). Cordyceps as an Herbal Drug. In I. F. F. Benzie & S. Wachtel-Galor (Eds.), *Herbal Medicine: Biomolecular and Clinical Aspects* (2nd edition. Boca Raton (FL): CRC Press. Chapter 5.

Lin, L. C., Chou, C. J., & Kuo, Y. C. (2001). Cytotoxic principles from *Ventilago leiocarpa*. *Journal of Natural Products, 64*(5), 674–676.

Liu, F., Cai, X. L., Yang, H., Xia, X. K., Guo, Z. Y., Yuan, J., et al. (2010). The bioactive metabolites of the mangrove endophytic fungus *Talaromyces* sp. ZH-154 isolated from Kandelia candel (L.) Druce. *Planta Medica, 76*(2), 185–189.

Nam, S. H., Li, C. R., Li, Z. Z., Fan, M. Z., Kang, S. W., Lee, K. G., et al. (2011). Long-term preservation, regeneration, and cultivation of *Paecilomyces tenuipes* (Peck) Samson (Ascomycetes), an entomopathogenic fungus inoculated into the silkworm larva of Bombyx mori. *International Journal of Medicinal Mushrooms, 13*(1), 83–91.

Ng, T. B., & Wang, H. X. (2005). Pharmacological actions of Cordyceps, a prized folk medicine. *Journal of Pharmacy and Pharmacology, 57*(12), 1509–1519.

Nicolaou, K. C., Lim, Y. H., Piper, J. L., & Papageorgiou, C. D. (2007). Total syntheses of 2,2′-epi-cytoskyrin A, rugulosin, and the alleged structure of rugulin. *Journal of the American Chemical Society, 129*(13), 4001–4013.

Parker, J. C., McPherson, R. K., Andrews, K. M., Levy, C. B., Dubins, J. S., Chin, J. E., et al. (2000). Effects of skyrin, a receptor-selective glucagon antagonist, in rat and human hepatocytes. *Diabetes, 49*(12), 2079–2086.

Pu, Z. L., & Li, Z. Z. (1996). *Insect mycology*. Hefei: Anhui Science and Technology Press (pp. 1–714). Hefei: Anhui Science and Technology Press [in Chinese].

Russell, R., & Paterson, M. (2008). Cordyceps a traditional Chinese medicine and another fungal therapeutic biofactory? *Phytochemistry, 69*, 1469–1495.

Sakai, M., Abe, K., Okumura, H., Kawamura, O., Sugiura, Y., Horie, Y., et al. (1992). Genotoxicity of fungi evaluated by SOS microplate assay. *Natural Toxins, 1*(1), 27–34.

Shrestha, B., Han, S. K., Sung, J. M., & Sung, G. H. (2012). Fruiting body formation of *Cordyceps militaris* from multi-ascospore isolates and their single ascospore progeny strains. *Mycobiology, 40*(2), 100–106.

Snider, B. B., & Gao, X. (2005). Efficient syntheses of rugulosin analogues. *Journal of Organic Chemistry, 70*(17), 6863–6869.

Stark, A. A., Townsend, J. M., Wogan, G. N., Demain, A. L., Manmade, A., & Ghosh, A. C. (1978). Mutagenicity and antibacterial activity of mycotoxins produced by *Penicillium islandicum* Sopp and *Penicillium rugulosum*. *Journal of Environmental Pathology and Toxicology, 2*(2), 313–324.

Ueno, Y., Sato, N., Ito, T., Ueno, I., Enomoto, M., & Tsunoda, H. (1980). Chronic toxicity and hepatocarcinogenicity of (+) rugulosin, an anthraquinoid mycotoxin from penicillium species: preliminary surveys in mice. *Journal of Toxicological Sciences, 5*(4), 295–302.

Wang, Y., Wang, M., Ling, Y., Fan, W., Wang, Y., & Yin, H. (2009). Structural determination and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps sinensis*. *American Journal of Chinese Medicine, 37*(5), 977–989.

Watts, P., Kittakoop, P., Veeranondha, S., Wanasith, S., Thongwichian, R., & Saisaha, P. (2003). Cytotoxicity against insect cells of entomopathogenic fungi of the genera *Hypocrella* (anamorph *Aschersonia*): possible agents for biological control. *Mycological Research, 107*(5), 581–586.

Yamashoji, S., Yoshikawa, N., Kirihara, M., & Tsuneyoshi, T. (2013). Screening test for rapid food safety evaluation by menadione-catalysed chemiluminescent assay. *Food Chemistry, 138*(4), 2146–2151.

Yamazaki, H., Koyama, N., Omura, S., & Tomoda, H. (2010). New rugulosins, anti-MRSA antibiotics, produced by *Penicillium radicum* FKI-3765-2. *Organic Letters, 12*(7), 1572–1575.

Yan, W. J., Li, T. H., Lin, Q. Y., Song, B., & Jiang, Z. D. (2010). Safety assessment of *Cordyceps guangdongensis*. *Food and Chemical Toxicology, 48*(11), 3080–3084.

Yang, M. L., Kuo, P. C., Hwang, T. L., & Wu, T. S. (2011). Anti-inflammatory principles from *Cordyceps sinensis*. *Journal of Natural Products, 74*(9), 1996–2000.

Zhang, J., Yu, Y., Zhang, Z., Ding, Y., Dai, X., & Li, Y. (2011). Effect of polysaccharide from cultured *Cordyceps sinensis* on immune function and anti-oxidation activity of mice exposed to 60Co. *International Immunopharmacology, 11*(12), 2251–2257.

Zhao, J., Avula, B., Chan, M., Clément, C., Kreuzer, M., & Khan, I. A. (2012). Metabolomic differentiation of maca (*Lepidium meyenii*) accessions cultivated under different conditions using NMR and chemometric analysis. *Planta Medica, 78*(1), 90–101.

Zhong, S., Pan, H., Fan, L., Lv, G., Wu, Y., Parmeswaran, B., et al. (2009). Advances in research of polysaccharides in Cordyceps species. *Food Technology and Biotechnology, 47*, 304–312.

Zhou, X., Gong, Z., Su, Y., Lin, J., & Tang, K. (2009). Cordyceps fungi: Natural products, pharmacological functions and developmental products. *Journal of Pharmacy and Pharmacology, 61*, 279–291.

Zuo, D. P., Li, C. R., Huang, B., He, Y. Q., Fan, M. Z., & Li, Z. Z. (2008). Molecular identification for connection of *Cordyceps formosana* and its *Hirsutella* anamorph. *Mycosystema, 27*(2), 224–229 [in Chinese].