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Biological activities of extracts and beauvericin from *Cordyceps cateniannulata* CPA14V

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Abstract Antioxidant activities *in vitro* were recorded for antimicrobial and cytotoxic activities (liver cancer cells (HepG2), prostate cancer cells (PC-3) and normal cells (Vero)) of the extract of ethanol (CCM), *n*-hexane (CCH), dichloromethane (CCD), ethyl acetate (CCE), and water (CCW), and a cyclooligomer depsipeptide, beauvericin (CC1) from *Cordyceps cateniannulata* CPA14V. The results showed that CCD contained the main compound, beauvericin (CC1) and exhibited higher activities than the other extracts. Especially, CCD and CC1 displayed good cytotoxicity against HepG2 and PC-3 cell lines with IC_{50} values from 19.17 to 45.29 μg/mL; the CCM and CCD exhibited good free radical scavenging capacity and antioxidant activity with SC_{50} values of 102.95 and 86.87 μg/mL, respectively; CC1 demonstrated potent antimicrobial activities with MIC value of 100 μg/mL (for *Aspergillus niger, Escherichia coli, Fusarium oxysporum, Pseudomonas aeruginosa* and *Staphylococcus aureus*) and MIC value of 200 μg/mL (for *Bacillus subtillis*, *Candida albicans* and *Saccharomyces cerevisiae*), CCM inhibited *E. coli* and *C. albicans* with MIC values of 200 μg/mL and *A. niger* with MIC values of 100 μg/mL. In addition, all samples did not show cytotoxicity to normal cells (Vero) at the tested concentrations.

Keywords: *Cordyceps cateniannulata*, Beauvericin, Antioxidant, Antimicrobial, Cytotoxic

Introduction

Cordyceps is a genus of parasitic fungi grown on larva of some insects. The genus includes about 400 species, distributed mainly in tropical and subtropical forests, mostly in China, Japan, Nepal, Bhutan, Korea, Thailand, and Vietnam (Sung *et al*., 2007). Some species of the *Cordyceps* genus have been widely used in medicine. Many chemical compounds of *Cordyceps* genus have been

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studied including alkaloids, flavonoids, steroids, phenolic, nucleosides, cyclic peptides. Among them, the cyclic peptides call much attention of scientists. Notably, cordycepin, adenosine, guanosine, cordymin, γ-aminobutyric acid (GABA), exopolysaccharides, cordysinin A-E have been applied in pharmaceuticals. Studies reported that *Cordyceps* spp. possess activities like anti-inflammatory, antifungal, hypoglycemic, antithrombotic, spermatogenic, antidiabetic, antiproliferative, antiarteriosclerosis, hypolipidemic, antifibrotic, anticancer, antitumor, antibacterial, immunomodulatory, antimalarial, etc. (Das *et al*., 2021; Wang *et al*., 2018).

Cyclooligomer depsipeptides (CODs) are natural bioactive compounds found in bacteria, fungi, plants, algae, sponges, and other marine organisms. Among the groups of CODs, those originated from fungi, especially parasitic fungi, are important group, accounting for the largest proportion of the known CODs. Cyclooligomer depsipeptides from the entomopathogenic fungi exhibit broad spectra of biological activities, including phytotoxic, cytotoxic, antiviral, insecticidal, antimalarial capabilities, anti-tumor, inhibit the activities of certain enzymes and limit the formation of amyloid in Alzheimer's disease. In particular, recent studies showed that CODs from these fungi inhibited the growth of different human cancer cell lines and have significant potential in cancer prevention and treatment (Süssmuth *et al*., 2011; Wang *et al*., 2018).

Beauvericin, a bioactive compound first found in *Beauveria bassiana* and then in some other species of *Cordyceps*. Beauvericin is a cyclohexadepsipeptide with alternating of three N-methylphenylalanyl and three D-hydroxyisovaleryl residues (Wang *et al*., 2014). Beauvericin has been reported with biological activities such as insecticidal, reduction of amyloid plaque formationanti-cholesterol, antiplatelet aggregation, chemosensitizer activities, apoptosis, antimicrobial, antiviral, cytotoxic activities, antiangiogenic activities and inhibition of metastasis prostate (Hamill *et al*., 1969; Wang and Xu, 2012). Beauvericin is considered a chemical marker of the Cordycipitaceae family and also promising medicinal potential applications (Sood *et al*., 2017).

During the study of the genus *Cordyceps* spp. both *in vivo* or *in vitro*, a number of notable secondary metabolites have been found, such as nucleosides, sterols flavonoids, cyclic peptides, phenolics, bioxanthracenes, polyketides and alkaloids (Olatunji *et al*., 2018). However, according to Kuo *et al*., it is rare that CODs are found in the genus *Cordyceps* (Kuo *et al*., 2002). Nevertheless, Wang *et al*. (2014) reported that *Cordyceps cicadae* was able to synthesize beauvericin E, beauvericin J, beauvericin and beauvericin A, which indicated that *C. cicadae* could be a source of CODs of the new hexadepsipeptide group (Wang *et al*., 2014). In another study, Rachmawati *et al*. (2017) also determined the beauvericin-producing ability of *C. militaris* species (Rachmawati *et*

al., 2017). Furthermore, the chemical composition and biological activities of *C. cateniannulata* has not been studied. This study was indicated to chemical structure of beauvericin, isolated compound from *C. cateniannulata* by modern spectral analysis techniques, extraction of bioactive fractions and their activities as antioxidant, antimicrobial and cytotoxic activities.

Materials and methods

Sample processing and extraction

The fungus *C. cateniannulata* CPA14V was isolated from insect samples collected in Copia Nature Reserve (Son La Province) in 24/12/2016. The strain was identified by morphological and DNA sequences analysis of ITS, LSU and Rpb1 fragments (Van *et al*., 2021).

The fungal freeze-dried biomass (25g) was extracted with ethanol by the sonication for 3 times at room temperature. The combined extracts were then vacuum evaporated to give the total residue (**CCM**; 16.5g), which was then suspended in water and successively partitioned with *n*-hexane, dichloromethane and ethyl acetate to obtain *n*-hexane (**CCH**; 3.1g), dichloromethane (**CCD**; 4.2g), ethyl acetate (**CCE**, 2.5g) and water (**CCW**; 5.1g) extracts.

Isolation, purification and determining chemical structures of beauvericin

The **CCD** (4.0g) was fractionated on a silica gel column eluting with dichloromethane:methanol (99:1→1:1, v/v) to obtain five fractions (from **CCD1→CCD5**). The fraction **CCD2** (1.2g) was chromatographed on a reversed phase column (RP-18), eluting with methanol:water (3:7) to obtain three fractions (**CCD2.1→CCD2.3**). The fraction **CCD2.1** (0.4g) was subjected to separation on a Sephadex LH-20 eluting with methanol and recrystallized in acetone to obtain compound **CC1** (powder, white color, R_f= 0.38 (dichloromethane:methanol 20:1), Mp 95-96 ^oC, 22.6 mg). The process of isolation of beauvericin (CC1) from dichloromethane extract of *C. cateniannulata* is CPA14V shown in the Figure 1.

Structure of the beauvericin was elucidated on the basis of 1D, 2D NMR spectroscopic data, HRESIMS, GC-MS and comparison with reported data as well as some physical properties such as melting point (Mp), polarity ($[\alpha]_D$).

Biological activity test methods

The cytotoxic activities were tested by MTT method applied for liver cancer cells (HepG2), prostate cancer cells (PC3) and normal cells (Vero) (Meerloo *et al*., 2011; Mosmann, 1983; Yang *et al*., 2017).

The antimicrobial activities of the extracts were estimated by using methods of Vander Bergher (1991), and Vlietinck (1999) on 8 tested strains *Aspergillus niger*, *Bacillus subtillis*, *Candida albicans*, *Escherichia coli*, *Fusarium oxysporum*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, and *Staphylococcus aureus* (Vanden Berghe, 1991; Vlietinck, 1999).

The antioxidant activities of the extracts was done by using DPPH (1,1-diphenyl-2-picrylhydrazyl) (Brand-Williams *et al.*, 1995).

The tests were conducted at the Bioactive Compounds Laboratory, Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology.

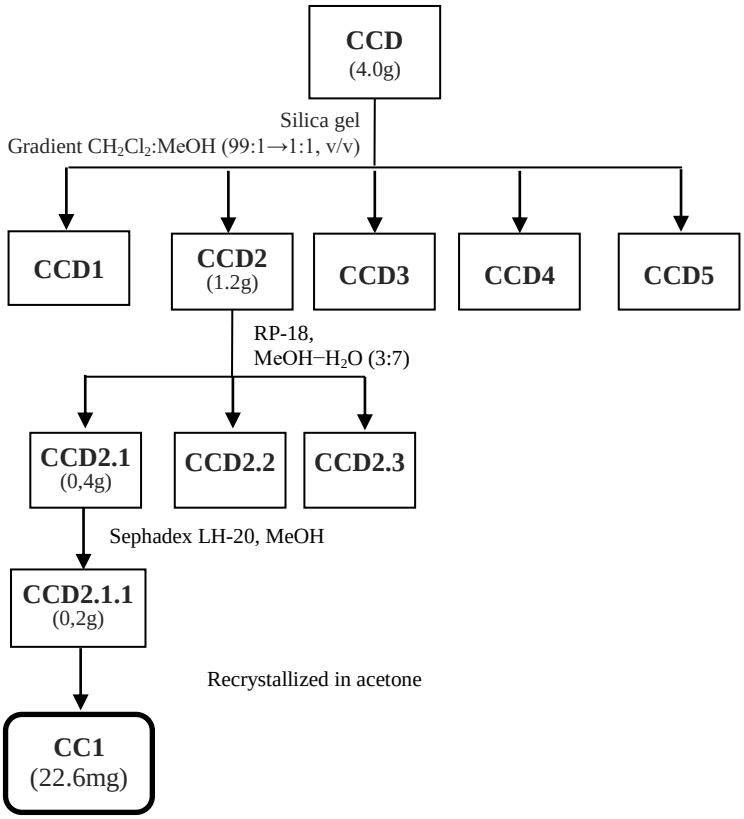

Figure 1. Isolation of beauvericin from *C. cateniannulata* CPA14V

Results

The structure of beauvericin

The compound **CC1** was obtained as a white colored powder. LC/MS Q-TOF spectrum indicated *m/z* 784.41676 [M + H]+, corresponding to the molecular formula as $C_{45}H_{57}N_3O_9$. The ^{1}H-NMR spectrum of **CC1** appeared three methyl singlet signals at δ_H 0.88 (3H, d, *J*= 6.5 Hz, Hγ_1-Hiv), 0.29 (3H, d, *J*= 7.0 Hz, Hγ_2-Hiv) and 3,16 (3H, s, N-C$\underline{H}_3$); one methylene at δ_H 3.40 (1H, dd, *J*= 4.5 and 14.5 Hz, H$_{\beta 1}$-Phe) and 3.05 (1H, m, H$_{\beta 2}$-Phe); three methine at δ_H 1.84 (1H, m, H$_\beta$-Hiv), 4.88 (1H, d, *J*= 9.0 Hz, H$_\alpha$-Hiv), and 5.83 (1H, dd, *J*= 4.5 and 12.5 Hz, H$_\alpha$-Phe); and the aromatic ring protons at δ_H 7,19-7,27 (5H, m, H$\gamma_{2,3,4,5,6}$-Phe).

The^{13}C-NMR, DEPT and HSQC spectra of **CC1** displayed 15 carbon atoms signals, including two cacbonyl groups at δ_C 173.0 (CO-Phe) and 170.9 (CO-Hiv); three methine groups at δ_C 31.2 (C$_\beta$-Hiv), 57.8 (C$_\alpha$-Phe) and 77,2 (C$_\alpha$-Hiv); a methylene group at δ_C 35.5 (C$_\beta$-Phe), three methyl groups at δ_C 17.2 (Cγ_1-Hiv), 18.3 (Cγ_2-Hiv) and 32.2 (N-C$\underline{H}_3$) and carbon atoms of aromatic ring at δ_C from 127.9 to 129.8 ppm (C$\gamma_{2,3,4,5,6}$-Phe). Analytical the NMR spectrum data and MS spectrum of **CC1** showed that it consists of three similar groups, each group has 15 carbon atoms.

The ^{1}H-^{1}H COSY and ^{13}C-^{1}H HMBC spectra interactions of **CC1** was shown in the Figure 2. In the ^{1}H-^{1}H COSY spectrum of **CC1**, the cross peaks between H$_\beta$-Hiv and Hγ_1-Hiv, Hγ_2-Hiv; H$_\alpha$-Hiv and protons of aromatic ring H$\gamma_{2,3,4,5,6}$-Phe were observed. The HSQC spectrum of **CC1** allows to assign values of proton to corresponding carbon. The HMBC spectrum confirmed the correlations between proton H$_\beta$-Hiv (δ_H 1.84), Hγ_1-Hiv (δ_H 0.88), Hγ_2-Hiv (δ_H 0.29), H$_\alpha$-Hiv (δ_H 4.88) with C$_\beta$-Hiv (δ_C 31,2), Cγ_1-Hiv (δ_C 17,2), Cγ_2-Hiv (δ_C 18,3) and C$_\alpha$-Hiv (δ_C 77,2); H$_\alpha$-Phe (δ_H 5.83) with $\underline{C}$O-Phe (δ_C 170.9), N-$\underline{C}$H$_3$-Phe (δ_C 32.2) and C$_\beta$-Phe (δ_C 35.5); H$_\beta$-Phe (δ_H 3.05 và 3.40) with $\underline{C}$O-Phe (δ_C 170.9), C$_\alpha$-Phe (δ_C 57.8), Cγ_1-Phe (δ_C 138.1), Cγ_2-Phe (δ_C 129.8) and Cγ_6-Phe (δ_C 129.8), respectively. Its structure and spectral data were shown in the Figure 2 and the Table 1.

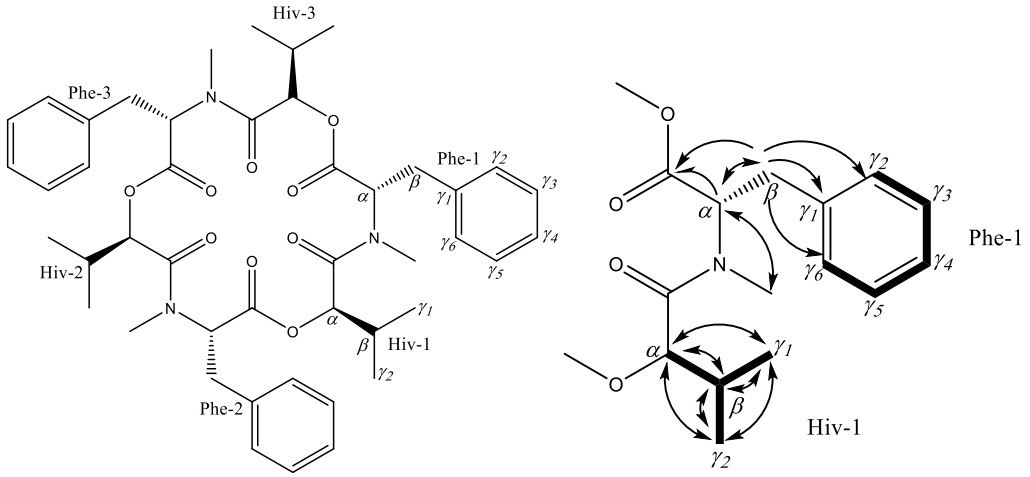

Figure 2. Chemical structure, important ^{1}H-^{1}H COSY and HMBC (H→C) interactions of CC1

Table 1. Spectrum data ^{1}H- and ^{13}C-NMR of CC1 and reference compound

Position	**Compound CC1**		**Beauvericin** (Bogner *et al*., 2017)	
	δ_C	**δ_H**(mult., J Hz)	**#δ_C**	**#δ_H** (mult., *J* Hz)
Phe (3 units):				
CO	170.9	-	169.5	-
A	57.8	5.83 (1H, dd, *J*= 12.5, 4.5)	56.5	5.82 (1H, dd, *J*= 12.7, 4.5)
B	35.5	3.40 (1H, dd, *J*= 14.5, 4.5) 3.05 (1H, m)	34.7	3.42 (1H, dd, *J*= 14.8, 4.5) 3.06 (1H, dd, *J*= 14.8, 12.7)
γ_1	138.1	-	136.9	-
γ_2	129.8	7.25-7.28 (1H, m)	128.3	7.26-7.30 (1H, m)
γ_3	129.7	7.25-7.28 (1H, m)	128.3	7.26-7.30 (1H, m)
γ_4	127.9	7.19 (1H, m)	126.2	7.20 (1H, tt, *J*= 6.0, 3.1)
γ_5	129.7	7.25-7.28 (1H, m)	128.3	7.26-7.30 (1H, m)
γ_6	129.8	7.25-7.28 (1H, m)	128.3	7.26-7.30 (1H, m)
N-CH_3	32.2	3.16 (1H, s)	31.2	3.17 (1H, s)
Hiv (3 units):				
CO	173.0	-	172.3	-
A	77.2	4.88 (1H, d, *J* = 9.0)	75.5	4.86 (1H, s)
B	31.2	1.84 (1H, m)	29.8	1.83 (1H, dq, *J*= 8.8, 6.7)
γ_1	19.0	0.88 (3H, d, *J* = 6.5)	17.8	0.87 (3H, d, *J* = 6.6)
γ_2	17.2	0.29 (3H, d, *J* = 7.0)	16.1	0.27 (3H, d, *J* = 6.9)

#δ_H and #δ_C of beauvericin (^{1}H: 300 MHz, ^{13}C: 75 MHz, methanol-d4) (Bogner *et al*., 2017)

Biological activities of C. cateniannulata CPA14V

Evaluation of in vitro cytotoxic activities

The results showed that all samples were not toxic to normal cell line (**Vero**) at concentrations tested (Table 2). The water extract (**CCW**) did not show inhibition of growth of the tested cell lines. The rest of the the extracts exhibited cytotoxic activities against the HepG2 and PC3 human cancer cell lines. Dichloromethane extract (**CCD**) and beauvericin (**CC1**) showed the highest inhibition activities on HepG2 and PC3 with IC_{50} values ranging from 19.17 to 45.29 µg/mL, that was lower concentration of positive control Paclitaxel used in the study.

Table 2. *In vitro* cytotoxic activities of the extracts from *C. cateniannulata* CPA14V

No.	Samples	HepG2 cells		PC3 cells		Vero cells	
		Cell inhibition rate (%)	IC_{50} (µg/mL)	Cell inhibition rate (%)	IC_{50} (µg/mL)	Cell inhibition rate (%)	IC_{50} (µg/mL)
1	**CCM**	80.55 ±3,1	60.12	88.61 ±0,6	80.97	22.86 ±0.1	>100
2	**CCH**	53.17 ±2.0	95.64	78.33 ±1.8	69.37	31.31 ±1.5	>100
3	**CCD**	96.56 ±0.9	22.90	90.33 ±1.5	45.29	26.25 ±1.3	>100
4	**CCE**	56.49 ±1.9	93.48	46.64 ±2.1	>100	35.43 ±2.0	>100
5	**CCW**	42.25 ±2.4	>100	44.36 ±2.3	>100	40.75 ±2.2	>100
6	**CC1**	97.02 ±1.8	19.17	95.16 ±0.8	23.52	46.53 ±1.8	>100
Paclitaxel 50nM		54.2 ±1.8	47.2 nM	64.09 ±2.4	40.74 nM	39.16 ±2.6	>100 nM

CCM showed moderate inhibitory activities with IC_{50} values of 60.12 µg/mL (for HepG2) and 80.97 µg/mL (for PC3). The **CCH**, **CCD** and **CCE** extracts were not much effective against the HepG2 and PC3 cells with IC values ranging from 22.90 to 95.64 µg/mL. The dichloromethane extract (**CCD**) showed the best activity with IC_{50} value of 22.90 µg/mL (for HepG2) and 45.29 g/mL (for PC3). The finding in this study was the first record of beauvericin with ability of inhibition on the PC3 cells. It is confirmed that the strain is a source of beauvericin production.

Evaluation of antioxidant activity

Follow the ectraction scheme, the CCM was the crude extract (ethanol extract) that was then refined by other solvents, showed relatively strong antioxidant activity (Table 3), with SC 72,68 ±2,8% and SC_{50} 102,95 µg/mL. However, among the 5 extracts, there was only one CCD that showed slightly higher antioxodant activity (SC 79,07 ±2,6 % and SC_{50} 86,87 µg/mL) than CCM.

The other 4 extracts expreessed week or no antioxidant activity (SC were less than 20% and SC_{50} were greater than 200 μg/mL). The interesting finding here was that CC1 took more than 50% of weight of CCD but CCD still showed high antioxidant activity. The remaining compound possiblly did neutreulised oxidant activity of CC1, which did not show antioxidant activity. Beauvericin is a mycotoxin and normally possesses oxidant properties.

Table 3. *In vitro* antioxidant activity of the extracts from *C. cateniannulata* CPA14V

No.	Sample	Ability to neutralize free radicals (SC, %)	SC_{50} (μg/mL)
	Control (+) [ascorbic acid]	86,53 ± 0,3	12,6 μg/mL
	Control (-) [DPPH/EtOH+ DMSO]	0,0 ±0,0	-
1	**CCM**	72,68 ±2,8	102,95
2	**CCH**	1,54 ±0,1	>200
3	**CCD**	79,07 ±2,6	86,87
4	**CCE**	2,88 ±1,5	>200
5	**CCW**	19,26 ±1,8	>200
6	**CC1**	1,23 ±0,6	>200

Antimicrobial activities of the extracts

The results showed that the compound beauvericin (CC1) exhibited the best antimicrobial activity against all bacterial and fungal strains tested with MIC values of 100 μg/ml (for *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus niger* and *Fusarium oxysporum*) and MIC value of 200 μg/ml (for *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Candida albicans*). The crude (CCM) exhibited against *E. Coli* and *C. albicans* with MIC value of 200 μg/ml and *A. Niger* with MIC value of 100 μg/ml. The antimicrobial activities of the extracts of *n*-hexane (CCH), dichloromethane (CCD), ethyl acetate (CCE), water (CCW) from *C. cateniannulata* CPA14V showed that the dichloromethane extract was exhibited the best antimicrobial activity with MIC value of 100 μg/ml (for *E. coli*, *A. niger* and *F. oxysporum*) and MIC value of 200 μg /ml (for *P. aeruginosa*, *S. aureus* and *C. albicans*). The *n*-hexane extract only showed antifungal activity against *A. niger* with MIC value of 200 μg/ml. The ethyl acetate (CCE), water (CCW) extracts did not show any activity.

Table 4. *In vitro* antimicrobial activities of the extracts from *C. cateniannulata* CPA14V

Samples	Minimum inhibitor concentration (MIC: µg/ml)							
	Gram(-) bacteria		Gram (+) bacteria		Strains		Yeast	
	E.coli	*P. aeruginosa*	*B. subtillis*	*S. aureus*	*A. niger*	*F. oxysporum*	*S. cerevisiae*	*C. albicans*
CCM	200	>200	>200	>200	100	>200	>200	200
CCH	>200	>200	>200	>200	200	>200	>200	>200
CCD	100	200	>200	200	100	100	>200	200
CCE	>200	>200	>200	>200	>200	>200	>200	>200
CCW	>200	>200	>200	>200	>200	>200	>200	>200
CC1	100	100	200	100	100	100	200	200

Discussion

The structure of beauvericin from *C. cateniannulata* CPA14V was investigated. The combination of spectral data (MS, ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, NOESY, HSQC and HMBC) together with the corresponding data of beauvericin in the references (Bogner *et al*., 2017; Zhang *et al*., 2016) confirmed that CC1 was identified as a cyclic hexadepsipeptide, a mycotoxin named (3S,6R,9S,12R,15S,18R)-3,9,15-tribenzyl-4,10,16-trimethyl-6,12,18-tri(propan-2-yl)-1,7,13-trioxa-4,10,16-triazacyclooctadecane-2,5,8,11,14,17-hexone or beauvericin and first studied from *Beauveria bassiana* (Wang *et al*., 2014).

The beauvericin (**CC1**) displayed strong anticancer activity with IC_{50} values of 19.17 µg/mL (for HepG2) and 23.52 µg/mL (for PC3). The results were compared to that of Juan-Garcá *et al*. (2019) and Ivanova *et al*. (2006) where beauvericin showed the inhibition activities on HepG2 with IC_{50} values of 0-25µM (Ivanova *et al*., 2006; Juan-Garcá *et al*., 2019). The finding was the first record of beauvericin to inhibit the PC3 cells. The strain was confirmed to be a source of beauvericin production.

Beauvericin has reported to be a mycotoxin and normally possesses oxidant properties. The crude extract or interaction of compounds in the extracts of organisms possessed different properties to the pure compounds individually (Dzoyem *et al*., 2017; Mallebrera *et al*., 2017).

The research findings showed that beauvericin exhibited the best antimicrobial activity against all tested bacterial and fungal strains with MIC values of 100 µg/ml for *E. coli*, *P. aeruginosa*, *S. aureus*, *A. niger* and *F. oxysporum* and MIC value of 200 µg/ml for *B. subtilis*, *S. cerevisiae* and *C.*

albicans). The antibacterial and antifungal activities of beauvericin of different sources were reported (Olleik *et al*., 2019).

It is concluded that *Cordyceps cateniannulata* CPA14V is a potential candidate for beauvericin production with productivity of 0.8% of CDW when applying the described extraction procedure. The beauvericin (CC1) chemical structure was identified by 1D, 2D NMR spectroscopic data, HR-ESI-MS and comparison with reported data and some physical properties such as melting point (Mp). This beauvericin showed strong anticaner activities on HepG2 and PC-3 cell lines but did not harm Vero cell line (IC50 > 100 µg/mL). This compound also showed strong antibacterial and antifungal activities against both Gram negative and Gram positive bacteria and three tested fungi. None of the extract was toxic to Vero cells. The dichloromethane extract (CCD) containing the main compound cyclooligomer depsipeptide beauvericin (CC1) exhibited better cytotoxic, antioxidant and antimicrobial activities than the other fractions. This suggests further investigation on the chemical composition and biological activities of *C. cateniannulata* CPA14V for appication in different aspects in medicine and agriculture.

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