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Antioxidant and Cytotoxic Effects of *Ophiocordyceps sinensis* and Identification of Bioactive Proteins Using Shotgun Proteomic Analysis

Running title: Antioxidant, Cytotoxicity and Bioactive Proteins of *Ophiocordyceps sinensis*

Boon-Hong Kong[1], Chee-Sum Alvin Yap[1], Muhammad Fazril Mohamad Razif[1], Szu-Ting Ng[2], Chon-Seng Tan[2] and Shin-Yee Fung[1,3,4*]

[1]Medicinal Mushroom Research Group, Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]LiGNO Biotech Sdn. Bhd., Jalan Perindustrian Balakong Jaya 2/2, Taman Perindustrian Balakong Jaya 2, 43300 Balakong Jaya, Selangor, Malaysia
[3]Centre for Natural Products Research and Drug Discovery (CENAR), University of Malaya, 50603 Kuala Lumpur, Malaysia
[4]University of Malaya Centre for Proteomics Research (UMCPR), University of Malaya, 50603 Kuala Lumpur, Malaysia

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SUMMARY

Research background. *Ophiocordyceps sinensis,* a highly valued medicinal fungus is close to extinction due to overexploitation. Successful cultivation of *O. sinensis* fruiting body (OCS02®) shows the cultivar consists promising nutritional value and numerous bioactive compounds. Antioxidant and antiproliferative properties and biologically active proteins of the OCS02® are investigated for possible development into nutraceuticals.

Experimental approach. The chemical compositions of the OCS02® cold water extract was determined, and the antioxidant activities were examined using ferric reducing, DPPH$^{\bullet}$ and $O_2^{\bullet-}$ scavenging assays. MTT cytotoxic assay was performed to assess the antiproliferative activity of the extract. Bioactive proteins in the active fraction of the extract were identified using liquid chromatography (LC) and tandem-mass spectrometry (MS/MS).

*Correspondence author:
Phone: +603 7967 5745
Fax: +603 7967 4957
E-mail: syfung@um.edu.my, syfung@ummc.edu.my

Results and conclusions. The OCS02® extract exhibited strong $O_2^{\bullet-}$ scavenger ((18.40±1.12) mol TE/g extract) and potent cytotoxicity towards A549 cells (IC_{50}=(58.17±6.79) μg/mL). High molecular mass polysaccharides, proteins and protein-polysaccharide complexes could have contributed to the antioxidant and cytotoxic selectivity of the OCS02®. LC-MS/MS analysis identified several potential cytotoxic proteases and an oxalate decarboxylase oxdC protein which may exhibit protection effects on kidneys.

Novelty and scientific contributions. The findings reported the promising superoxide anion radical scavenging capacity, cytotoxic effect and presence of bio-pharmaceutical active proteins in of OCS02® extract, thus demonstrates the potential of OCS02® to be developed into functional food.

Key words: *Ophiocordyceps sinensis,* antioxidant, cytotoxicity, bioactive proteins, protein-polysaccharide complexes

INTRODUCTION

Ophiocordyceps sinensis or *Cordyceps sinensis* (in Chinese known as Dong Chong Xia Cao or “worm in winter and grass in summer”) is an insect parasitizing fungus in the Ascomycetes family (*1*). *O. sinensis* is a traditional Tibetan, Chinese and Indian medicinal fungus found in Tibetan Plateau, China and Indian Himalaya (*2*). This fungus is commonly used as a functional food to reduce inflammation in the body, to improve respiratory system, libido and erectile function, and to treat liver, cardiovascular and chronic kidney diseases (*3,4*). It is also used as a type of herbal tonic to restore energy and promote general health (*3,5*).

Many scientific studies have shown that *O. sinensis* contains numerous bioactive compounds such as cordycepin, polysaccharides, sterol-type compounds, unsaturated fatty acid and peptides. These compounds exerted various bio-pharmacological activities including anti-inflammatory, immunomodulatory, antiproliferative, anti-aging and antioxidants, as well as protective effects on the respiratory, hepatic, renal and cardiovascular systems (*6*). The use of *O. sinensis* as a medicinal health supplement is a global trend. However, natural production of this fungus is limited and overexploitation to meet high market demand has led to near extinction of the species (*7*). Efforts in cultivation of *O. sinensis* using artificial media have been the most promising approach for mass production of *O. sinensis* for development into nutraceuticals. The artificially cultured fruiting bodies, mycelia and fermented mycelial products were shown to possess comparable bio-pharmaceutical properties with the wild type including antioxidants, anti-inflammatory, antitumor, immunomodulatory, anti-hyperglycaemic and enhancement of neuromuscular activity (*8-11*).

Recent studies have demonstrated that a laboratory-cultured of *O. sinensis* fruiting body (OCS02®), by LiGNO Biotech Sdn. Bhd. (Selangor, Malaysia) is safe for consumption. No toxic effects were reported from an oral administration of 1000 mg/kg of OCS02® in rats in subacute toxicity assessment and no heavy metal was detected in the sample (*12,13*). It is rich in proteins and minerals, and contains high amounts of bioactive compounds including cordycepin, amino acids and glucans (*13*). Therefore, it is important to investigate the bio-pharmaceutical properties of OCS02® to support the development of this strain into functional food and nutraceutical. Previous study showed that the OCS02® cold aqueous extract possessed immunomodulatory properties that attributed to its polysaccharide and protein contents (*14*). Herein, we aim to further examine the antioxidant and antiproliferative properties of OCS02® water extract, and to identify the potential bioactive proteins in the extract. The bio-pharmaceutical active proteins found in OCS02® could play a role as potential new drug candidates.

MATERIALS AND METHODS

OCS02® and extract preparation

The OCS02® was cultured using solid state fermentation technology with rice-based medium used as substrate (LiGNO Biotech Sdn. Bhd., Selangor, Malaysia). This cultivated species was authenticated by its partial small subunit ribosomal gene (*12*). A mixture of 30 g freeze-dried OCS02® powder and 600 mL distilled water was stirred at 4 °C for 24 h to extract the heat-labile substances. The unextracted materials was pelleted using a refrigerated centrifuge (8000 × *g*, 4 °C, 30 min) (Sorvall Biofuge Primo R, Thermo Scientific, USA) and the water extract was filtered using a grade 1 filter paper (Whatman®, GE Healthcare Bio-sciences AB, Sweden). The freeze-dried cold water extract (CWE) was kept at -20 °C and dissolved in distilled water for further analysis.

Fractionation of OCS02® CWE

The CWE of OCS02® was fractionated using gel filtration (Sephadex^{TM} G-50) (GE Healthcare Life Sciences, USA) column chromatography (length of 40 cm, diameter of 2.5 cm). The fractions were eluted by gravity using 0.05 M ammonium acetate buffer (Merck, Darmstadt, Germany). Fractions of 3 different molecular masses (low, medium and high) were collected according to protein and carbohydrate peak profiles. Bradford's assay was performed to determine the protein content of the fractions (*15*). Carbohydrate content was estimated using phenol sulfuric acid assay (*16*).

Isolation of proteins from high molecular mass (HMW) fraction

Proteins were precipitated from the HMW fraction using 100 % saturation ammonium sulphate (Sigma-Aldrich, Missouri, USA), where the HMW fraction was dissolved in water with ammonium sulphate was gradually added until 100 % saturation reached, followed by continuous stirring for an hour at 4 °C. The precipitated proteins and non-protein component (supernatant) were retrieved by centrifugation and desalted using the Sartorius centrifugal concentrator, Vivaspin® 15R (Germany) of molecular mass cut-off value of 5 kDa.

Total phenolic content

The OCS02® CWE and Sephadex-G50 fractions phenolic content was determined using Folin-Ciocalteu assay (*17*). Briefly, Folin–Ciocalteu's phenol reagent (Merck), 1:10, (500 µL) was mixed with sample (10 µL) and incubated at ambient temperature (~22 °C) for 5 min. Three-hundred and fifty microliters of sodium carbonate (115 µg/mL) was pipetted into the mixture and further incubated for 2 h. Gallic acid (Sigma-Aldrich) at concentrations ranged from 20-200 µg/mL was used as standard. The absorbance values (765 nm) were recorded using a plate spectrophotometer (Bio-Rad-Model 680, California, USA).

Antioxidant assays

Antioxidant activity of OCS02® CWE and its fractions was assessed using ferric reducing antioxidant power (FRAP) (*18*) and superoxide anion radical ($O_2^{\bullet-}$) scavenging (*19*) assays. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical (DPPH$^{\bullet}$) scavenging capacity was assessed using the method of Cos *et al.* (*20*), with sight adjustments. Twenty-five microliters of sample (0-16 mg/mL) were mixed with 150 µL of DPPH (Sigma-Aldrich) solution (40 µg/mL methanol). The sample was then incubated for 30 min in dark ambient condition (20-22 °C), followed by measurement of the absorbance values at 515 nm. Different concentrations (0-2 mg/mL) of Trolox (Sigma-Aldrich) were used to generate a standard curve.

Cell culture and MTT cytotoxicity assay

American Type Culture Collection (ATCC®, Virginia, USA) of human breast (MCF7, MDA-MB-231), lung (A549) and prostate (PC3) adenocarcinoma cell lines, and human normal lung (NL20) cell line were purchased for this study. RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) was used to culture MCF7, PC3 and A549 cell lines. MDA-MB-231 and NL20 cell lines were maintained in DMEM medium (Nacalai Tesque, Kyoto, Japan) and Ham's F12 medium (Lonza, Basel, Switzerland), respectively. All the media contained 10 % of serum from foetal bovine and cells were allowed to proliferate in a 37 °C incubator with 95 % humidity and 5 % of CO_2.

To examine the cytotoxicity effect of OCS02® CWE and its fractions, overnight seeded cells (at optimal density) in 96-well microplate were treated with various concentrations (15.6-500 mg/mL) of samples (200 µL) for 72 h. After 72 h post-treatment, MTT reagent was added into each well at a final concentration of 0.45 µg/mL and incubated for 4 h at 37 °C. The mixture of spent medium and MTT reagent was discarded, and DMSO (200 µL) was used for dissolution of purple formazan crystals prior to measurement of the absorbance values (570 nm). Concentration of the extract and fractions that was required to inhibit 50 % of cell proliferation (IC_{50}) was calculated from the curves plotted using the cell viability percentage over the tested sample concentrations.

Identification of proteins using LC-MS/MS

Proteins isolated from HMW were resolved on an SDS-PAGE gel under reducing condition. The separated protein bands were excised into 10 gel sections, where the gel sections were destained, reduced with dithiothreitol, alkylated with iodoacetamide and tryptic digested with trypsin protease (Thermo Scientific™, Pierce™, USA) (*21)*. Analysis was performed using an Agilent 1260 HPLC-Chip/MS Interface, coupled with Agilent 6550 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, California, USA), following the protocol as described previously (*21,22*). NCBI database of *Ophiocordycipitaceae* (non-redundant) was used for mass spectra searches that performed using the Agilent Spectrum Mill MS Proteomics Workbench software packages (http://spectrummill.mit.edu/). The spectrum mill settings applied including molecular ion (MH+) scan (100-3200 Da), complete carbamidomethylating of cysteines, peptides and protein scores greater than 6 and 20, respectively, scored peak intensity above 60 %, and the significant number of distinct peptide is greater than or equal to two.

Statistical analysis

All data was expressed as mean±standard deviation (SD). Differences between the mean values in the experiment groups analysed using one-way analysis of variance (ANOVA) and Tukey HSD post hoc test (IBM SPSS Statistics 22) (*23*) were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Antioxidant activity

Antioxidant activities including ferric reducing power, DPPH$^{\bullet}$ and $O_2^{\bullet-}$ scavenging assay were performed on the OCS02® cold water extract (CWE) and its fractions of different molecular masses (Table 1). The CWE demonstrated low FRAP and DPPH$^{\bullet}$ scavenging capacities compared to rutin and quercetin (positive controls). However, the capability of the OCS02® CWE to scavenge DPPH$^{\bullet}$

is ten times stronger (0.015 mmol TE/g extract) than water extracts from reported *O. sinensis* and other mushrooms (0.0013-0.0049 mmol TE/ g extract) (*24*). The extract also demonstrated stronger superoxide radical scavenger capability (18.4 mmol TE/g extract) than other reported *Lignosus* spp. mushrooms (9.61-9.90 mmol TE/g extract) (*25,26*). Three different molecular masses (HMW, MMW, LMW) fractions of the OCS02® CWE collected from Sephadex G-50 fractionation also demonstrated weak ferric ions reducing and DPPH$^{\bullet}$ scavenging activities, with HMW is the weakest DPPH$^{\bullet}$ scavenger. Howbeit, HMW was the most potent $O_2^{\bullet-}$ scavenger among the fractions with the activity was stronger than the crude CWE and comparable to the positive controls. This superoxide scavenging property is of great significance as it implies that OCS02® can be beneficial as an antioxidant supplement to aid in prevention of superoxide anion radicals-induced oxidative stress and related diseases. The antioxidant activity of the OCS02® was not correlated to its phenolic content. For instance, MMW exhibited equal or lower $O_2^{\bullet-}$ scavenging activity compared with CWE and HMW, respectively, although it contains 2 times higher phenolic content (Table 1). A few studies have reported that the antioxidant activity of the *O. sinensis* are mostly contributed by polysaccharides (*27,28*). Thus, the strong $O_2^{\bullet-}$ scavenging activity of OCS02® could be attributed to carbohydrates or polysaccharides that are abundantly present in the HMW (Table 1). The synergistic effects between phenolics, proteins and protein-polysaccharide complexes could have also contributed to the antioxidative activities of the OCS02®.

Cytotoxic activity of OCS02® CWE and its fractions

An investigation of the *in vitro* cytotoxicity of the OCS02® CWE showed the extract exhibited significant cytotoxicity (IC_{50} of (58.2±6.8) μg/mL) against lung cancer A549 cells (Fig. 1). The extract also exerted weak cytotoxic activity towards MCF7 cells with the IC_{50} value ((371.0±62.0) μg/mL) of approximately 6-fold higher than A549 cells. Our results showed that the CWE was more active in inhibiting the proliferation of oestrogen-dependent MCF7 breast cancer cells compared to the invasive, oestrogen-independent MDA-MB-231 breast cancer cells. There were no observed effects on the MDA-MB-231 and prostate cancer PC3 cells (IC_{50}>1000 μg/mL). Although the CWE exerted good antiproliferative activity on A549 cells, it was cytotoxic to normal lung NL20 cells as well (IC_{50}=(42.37±2.21) μg/mL). The NL20 is an immortalized non-tumorigenic lung cell line derived from human healthy lung epithelial cells through transfection with SV40 large T plasmid (*29*). NL20 cells showed no mutations in K-ras codons, no c-myc gene amplification and activation of dominant oncogenes (*30*) and are commonly used as non-tumorigenic (normal lung cells) lung cell model along with A549 lung adenocarcinoma cell model (*31-35*). Further fractionation of the CWE displayed HMW contributed to the cytotoxic selectivity towards lung cancer A549 cells with selectivity index of 1.8

(Table 2). Yet, separated proteins and non-protein (mostly polysaccharides) components of the HMW were cytotoxic to normal lung cell lines (NL20), which implies the non-selective nature of the cytotoxicity of proteins and polysaccharides toward this cancer cell line. Previous reports have indicated that the polysaccharides from *O. sinensis* act on cancer cells by modulating the immune system rather than exerting direct cytotoxicity toward the cancer cells (*36,37*). A recent work (*14*) done using OCS02® revealed that the HMW consists heteroglycans that stimulated the release of several cytokines/chemokines which are associated with its immunomodulator capability. Hence, this suggests that carbohydrates, the most abundant components in HMW, could act as immunomodulator associated with the antitumor effects on A549 cells.

Protein composition of HMW by LC-MS/MS

To date, limited studies are available for bioactive protein isolation and identification from *O. sinensis*. Studies have shown that fungi contain potential antioxidative and cytotoxic proteins such as manganese-superoxide dismutase, catalase, glutathione transferase, lectin, proteases and fungal immunomodulatory proteins (*38-40*). Our present study using shotgun LC-MS/MS analysis has identified a total of 17 distinct proteins in the protein fraction of HMW (Table 3, Fig. 2a and Fig. 2b). Majority (>50 %) of the proteins *e.g.*, alpha-mannosidase, beta-glucosidase A, beta-1,3-glucanosyltransferase, glycoside hydrolase family protein, transaldolase and WSC domain-containing protein, are involved in carbohydrate metabolism during the development process of *O. sinensis* fruiting body. Study by Park *et al.* (*40*) demonstrated that a trypsin-like protease (CMP) purified from *Cordyceps militaris* has significant inhibitory activity toward human breast MCF7 and bladder 5637 cancer cells. We have identified several proteolysis enzymes including peptidase A1, peptidase family M49 proteins and subtilisin-like proteinase SPM1 in the HMW. These proteases could have contributed to the cytotoxicity of the OCS02®.

O. sinensis water extract has been reported to have protective effects on kidneys including decreased proteinuria, enhanced renal functions and inhibited glomerular sclerosis (*4*). An oxalate decarboxylase oxdC, enzyme that mediates the degradation of oxalate, was identified in the HMW fraction OCS02® CWE. Oxalate, a metabolic end product in humans, if presents in excessive levels can cause calcium oxalate stones or kidney stones. A study has reported that oral administration of oxalate decarboxylase recombinant probiotic bacteria in hyperoxaluria rat models decreased the urinary oxalate level, thereby reducing hyperoxaluria (*41*). Several oxalate decarboxylase enzyme products such as ALLN-177 (clinicaltrials.gov/ct2/show/results/NCT02289755), Nephure™ (clinicaltrials.gov/ct2/show/NCT03661216) and Oxazyme (clinicaltrials.gov/ct2/show/results/NCT01127087) have been undergone clinical trials and

demonstrated promising results with significant reduction of oxalate levels in the OxDC-treatment groups (*42,43*). Presence of oxalate decarboxylase oxdC in the HMW fraction implicates the potential use of OCS02® to improve renal functions.

CONCLUSIONS

The extract from cultivated fruiting bodies of *O. sinensis*, OCS02®, was shown to have promising antioxidant and cytotoxic activity with high content of polysaccharides, proteins and phenolics. The strong superoxide anion radical scavenging of OCS02® CWE is possibly mainly attributed to its high molecular mass polysaccharides content. The CWE inhibited proliferation of lung cancer A549 cells and oestrogen-dependent breast cancer MCF7 cells. The selective cytotoxicity of the HMW fraction toward the A549 cells are associated with the proteins and protein-polysaccharides complexes. Several bioactive proteins with potential cytotoxic properties and kidney protection effects including proteases and oxalate decarboxylase oxdC were found in the HMW, implying that the HMW has the potential for development into dietary supplements as adjuvant therapy. Despite that, a more detailed study is required to gain better insights of the bio-pharmaceutical properties of HMW. Our future study will focus on investigation of the cytotoxic activity of HMW *in vivo*, isolation of protein of interest and investigation of the bio-pharmaceutical properties and underlying molecular mechanisms of specific proteins for drug discovery.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest. Szu Ting Ng is employed by Ligno Biotech Sdn. Bhd. and Chon Seng Tan is the Technical Advisor to LiGNO Biotech Sdn. Bhd., Balakong Jaya, Selangor, Malaysia.

AUTHORS' CONTRIBUTIONS

Boon-Hong Kong participated in performing experiments, data collection and analysis, wrote the article and critical revision. Chee-Sum Alvin Yap took part in conducting experiments. Shin-Yee Fung and Muhammad Fazril Mohamad Razif conceived the study, wrote the article and revising the

manuscript. Szu-Ting Ng and Chon-Seng Tan provided the sample material. All authors have read and approved the final version of the article.

ORCID ID

B.H. Kong https://orcid.org/0000-0003-3255-9960
C.S.A. Yap none
M.F. Mohamad Razif https://orcid.org/ 0000-0002-3951-8136
S.T. Ng none
C.S. Tan none
S.Y. Fung https://orcid.org/0000-0002-9288-7328

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Table 1. Chemical composition and antioxidant activity of the OCS02® cold water extract and its fractions

Sample	Chemical composition			Antioxidant activity		
	w(protein)/%	*w*(carbohydrate)/%	*w*(phenolics as GAE)/(mg/g)	FRAP/(mmol/(min·g))	Trolox equivalent antioxidant capacity, TEAC/(mmol/g)	
					DPPH	Superoxide anion
CWE	(2.12±0.34)^{a}	(41.47±6.10)^{a}	(6.67±0.64)^{a}	(0.0022±0.0002)^{a}	(0.015±0.0001)^{a}	(18.40±1.12)^{a}
HMM	(3.79±0.86)^{a}	(80.28±9.54)^{b}	(5.19±1.70)^{ab}	(0.0008±0.0002)^{a}	(0.003±0.0007)^{b}	(22.57±1.85)^{b}
MMM	(3.32±1.45)^{a}	(27.95±5.11)^{a}	(14.80±0.82)^{c}	(0.0031±0.0003)^{a}	(0.014±0.0002)^{a}	(15.53±0.19)^{a}
LMM	N.D.	(0.64±0.30)^{c}	(3.33±0.64)^{b}	(0.0008±0.0002)^{a}	(0.017±0.0003)^{a}	(2.57±0.45)^{c}
Rutin	-	-	-	(2.5725±0.1069)^{b}	(1.265±0.0054)^{c}	(29.12±1.30)^{d}
Quercetin	-	-	-	(0.7388±0.0229)^{c}	(1.214±0.0031)^{d}	(25.36±0.40)^{b}

Protein and carbohydrate contents were estimated based on dry weight (*w*/*w*). All the values were expressed as mean±SD (*N*=3). Means in the same column with different letters are significantly different, according to analysis of variance and post hoc Tukey HSD test ($p<0.05$). Rutin and quercetin were used as positive controls in antioxidant assay. Abbreviations: CWE=cold water extract, HMM=high molecular mass, MMM=medium molecular mass, LMM=low molecular mass; N.D.=not detected

Table 2. Cytotoxicity (IC_{50}/(μg/mL)) of OCS02® CWE fractions against human lung adenocarcinoma and normal cell lines

Sample	A549	NL20	Selectivity index
HMM	157.3±10.1	279.0±70.1	1.8
MMM	357.3±54.5	56.5±4.2	0.2
LMM	>1000	n.d.	n.a.
HMM-P	107.8±5.9	79.4±13.0	0.7
HMM-NP	213.3±37.5	95.3±14.1	0.5
γ(paclitaxel)/(ng/mL)	7.1±0.9	7.6±0.5	1.1

Abbreviations: HMM=high molecular mass, MMM=medium molecular mass, LMM=low molecular mass, P=protein, NP=non-protein, A549=human lung adenocarcinoma, NL20=human normal lung, n.d.=not determined, n.a.=not available. Selectivity index was determined by dividing IC_{50} of NL20 normal lung cells with the IC_{50} of the A549 adenocarcinoma cells. Selectivity index above 1.0 revealed that the treatment was more cytotoxic (selective) towards A549 adenocarcinoma cells

Table 3. List of OCS02® CWE-HMW proteins identified by LC-MS/MS using NCBI non-redundant (*Ophiocordycipitaceae* family) as search database

Gel section	Spectra (no.)	Distinct peptides (no.)	Distinct summed MS/MS search score	AA coverage/%	Protein pI	Mean spectral intensity·10^5	Protein relative/%	Database accession no.	Protein name
S1	2	2	31.80	2.1	6.26	1.23	0.65	799247974	Hypothetical protein HIM_04044
S1	2	2	30.12	2.2	5.91	5.10	2.70	908394288	Alpha-mannosidase
S1	2	2	28.97	3.1	6.30	1.05	0.56	531866672	Glutaminase GtaA
S1	2	2	25.45	2.2	4.97	0.93	0.49	1032877594	WSC domain-containing protein
S2	2	2	37.54	2.8	5.63	3.28	13.07	1261512171	Hypothetical protein XA68_12018
S2	2	2	34.08	1.9	5.58	4.11	16.37	799246137	Putative beta-glucosidase A
S3	3	2	37.75	3.1	5.91	1.46	17.86	908394288	Alpha-mannosidase
S4	4	3	54.08	3.8	6.26	2.12	0.49	799247974	Hypothetical protein HIM_04044
S4	3	3	47.12	7.1	5.00	11.3	2.62	799246399	Hypothetical protein HIM_05392
S4	3	2	37.00	6.9	5.06	2.99	0.69	1008934073	Beta-1,3-glucanosyltransferase
S4	2	2	31.96	3.9	7.27	7.30	1.70	1008936229	N-acetylglucosaminidase
S4	2	2	28.87	3.0	5.39	2.51	0.58	1335267264	Alpha-1,2-Mannosidase
S4	2	2	28.30	2.3	4.46	21.6	5.01	908387070	Hypothetical protein TOPH_07589
S5	6	6	100.47	24.1	6.79	7.52	1.44	908389224	Transaldolase
S5	6	5	70.55	6.3	5.75	3.48	0.67	531863817	Peptidase M49, dipeptidyl-peptidase III
S5	5	4	69.02	4.6	5.91	6.02	1.15	908394288	Alpha-mannosidase
S5	3	3	47.81	8.2	6.48	2.34	0.45	1261512568	Hypothetical protein XA68_11515
S5	3	2	35.49	5.9	6.20	2.36	0.45	799247347	Oxalate decarboxylase oxdC
S5	2	2	32.15	8.8	5.70	4.93	0.94	531867008	Peptidase A1
S5	2	2	28.71	4.2	8.94	6.10	1.17	799249484	Hypothetical protein HIM_02208
S5	2	2	28.45	2.3	4.46	26.9	5.13	908387070	Hypothetical protein TOPH_07589
S5	2	2	26.58	5.1	6.27	11.9	2.27	799247067	Subtilisin-like proteinase Spm1
S6	4	4	59.88	9.2	6.87	3.45	1.72	799247099	Transaldolase

S6	4	4	56.08	4.8	5.99	3.85	1.92	1339424435	Alpha-mannosidase
S6	4	3	46.12	3.4	6.04	2.42	1.21	531865527	Glycoside hydrolase family 38 protein
S6	3	2	34.14	5.1	6.48	2.79	1.39	1261512568	Hypothetical protein XA68_11515
S6	2	2	33.76	5.9	6.2	1.55	0.77	799247347	Oxalate decarboxylase oxdC
S6	2	2	29.11	1.8	5.65	5.75	2.87	1335262293	Dipeptidyl peptidase 3
S7	2	2	22.19	1.3	6	5.80	1.21	1032877677	Alpha-mannosidase
S8*	-	-	-	-	-	-	3.93	-	-
S9*	-	-	-	-	-	-	5.11	-	-
S10	3	3	42.77	2.8	5.91	3.87	3.43	908394288	Alpha-mannosidase

* No protein was identified.

Protein relative percentage was determined using a formula of (mean peptide spectral intensity of a protein/total mean peptide spectral intensity of proteins) × percentage relative intensity of each gel section in the protein lane (estimated by densitometry using Thermo Scientific™ Pierce™ myImage Analysis™ Software (USA)

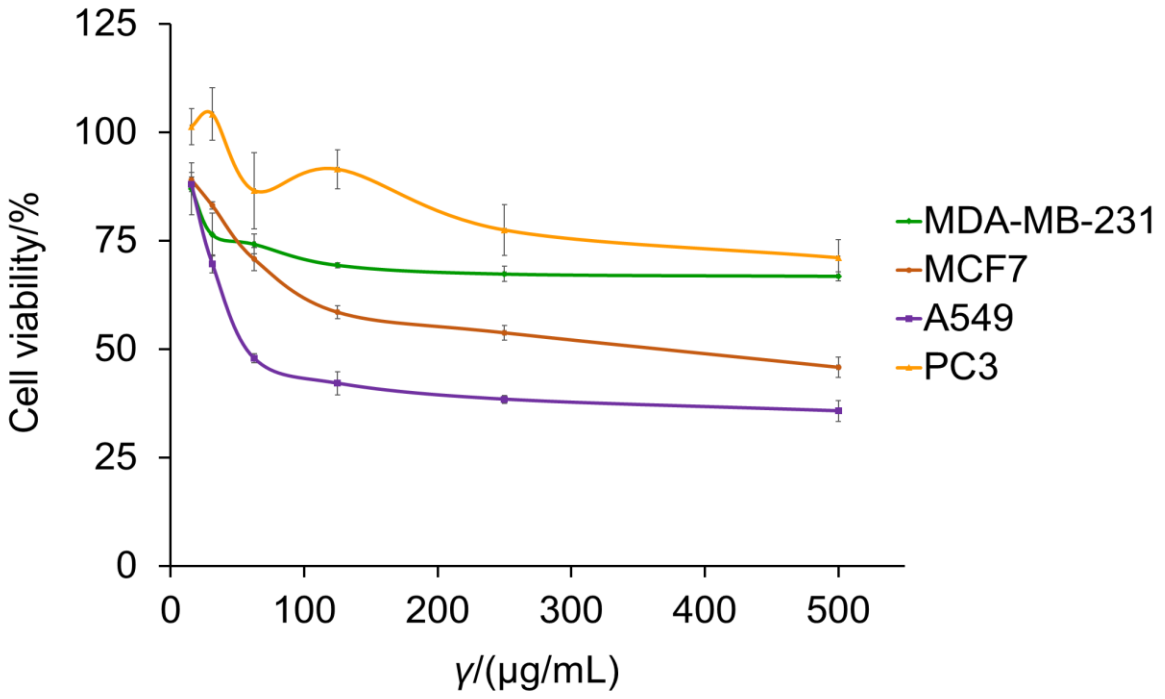

Fig. 1. Cytotoxic activity of OCS02® cold water extract at various concentration against MCF7, MDA-MB-231 (human breast adenocarcinoma), A549 (human lung adenocarcinoma) and PC3 (human prostate adenocarcinoma) cell lines. Values are expressed as mean±SD (*N*=3).

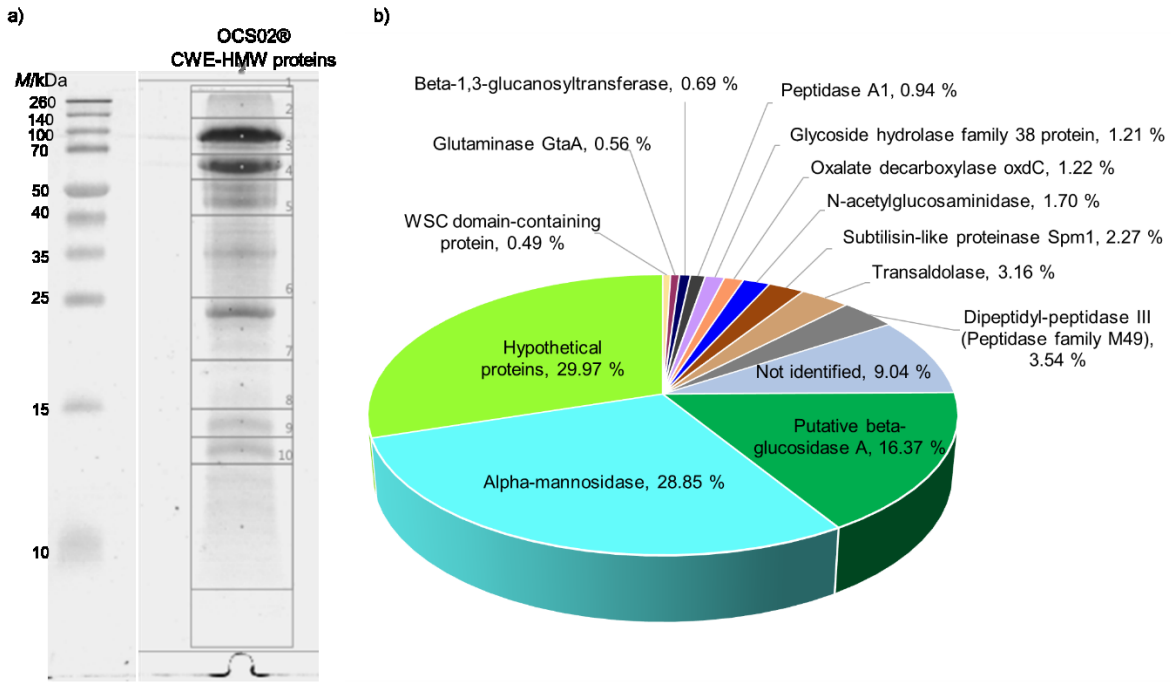

Fig. 2. Protein profiles of HMW fraction proteins of OCS02® cold water extract. a) Separation of CWE-HMW proteins on SDS-PAGE 15 % gel. b) Percentage distribution of OCS02® CWE-HMW proteins identified by shotgun LC-MS/MS based on NCBI non-redundant Ophiocordycipitaceae database.