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Antioxidant and cytotoxicity activity of *Cordyceps militaris* extracts against human colorectal cancer cell line

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ABSTRACT

Cordyceps militaris is famous for its medicinal effects and variety of bioactivities including antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, or antitumor properties. The research's objective is to look into the antioxidant and cytotoxic effects of *C. militaris* extract (CME) against normal human colorectal HT-29 cancer cell line. The effects of CME and fresh Cordyceps militaris (CM) on the antioxidant activities were determined using total phenolic content (TPC), total flavonoid content (TFC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) analysis. The cytotoxic effects of various concentrations of CME on HT-29 cells were evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide test. From the results, CME displayed strong activity of DPPH (83.8%, inhibitory concentration = 0.60 mg/ml), TPC (160 ± 0.74 mg gallic acid equivalent/100 g), and TFC (6.6 ± 1.13 mg rutin equivalent/100 g) relative to fresh CM. CME was found to be significantly more cytotoxic toward HT-29 cells with $p < 0.001$ in a dose-dependent manner with a cell growth inhibitory concentration of 50% of *t*1.53 mg/ml in contrast to cisplatin (3.11 mg/ml). The high antioxidant activities and cytotoxic effects of CME are probably due to the extract's high phenolic and flavonoid content. According to this report, CME's growth inhibitory activity on human HT-29 cells is driven by an apoptotic mechanism involved in it.

INTRODUCTION

There are various types of mushrooms possessing high medicinal value for humans and one of them is known as *Cordyceps*. *Cordyceps* are classified as macrofungi due to their parasitic characteristic of insect larvae and pupae. The genus *Cordyceps* is an important group of medicinal fungi, a member of Ascomycota, Pyrenomycetes, Hypocreales, and Clavicipitaceae (Ng and Wang, 2005; Shrestha and Sung, 2005; Wong *et al*., 2007; Xiao *et al*., 2013; Yu *et al*., 2006). *Cordyceps militaris*, used in traditional Chinese remedies, contain several forms of phytochemicals such as cordycepin, cordycepic acid, sterols, nucleosides, and polysaccharides, which were proven to be beneficial for medicinal purposes (Tuli *et al*., 2013; Yue *et al*., 2013). *Cordyceps militaris* has been documented to enhance several pharmacological properties, including antioxidant, immunomodulatory, anti-inflammatory, antimicrobial, and antitumor properties. However, the potency of each property is significantly different based on the specific extract ingredients (Lee *et al*., 2006; Park *et al*., 2005; Yue *et al*., 2013; Zhou *et al*., 2009).

The abundance of polyphenolic content found in this mushroom might have played an important role in the observed antioxidant capacity due to the ability of single electron transfer to scavenge free radical atoms (Joshi and Sagar, 2014; Palacios *et al*., 2011). *Cordyceps militaris* extract (CME) also was reported to have a potential impact on cytotoxic against numerous human cancer cells, such as lung carcinoma cells (Lim *et al*., 2009; Park *et al*.,

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2009). Another research recorded that extraction of Ergosterol peroxide compound from *C. militaris* has a high potential effect on the Korean gastric cancer cell line (*in vitro*) (Kim *et al*., 2011). The water extract of phytochemicals from *C. militaris* showed positive activity on the human cancer cell lines such as adenocarcinoma, colorectal adenocarcinoma, and hepatocellular carcinoma, respectively. Cordycepin was one of the active compounds in CME that was thought to have an impact on human cancer cell lines (Lim *et al*., 2004). As far as we are aware, the antioxidant and anticancer properties of CME on human colorectal cancer cells have not been extensively studied. Therefore, the study's objective is to examine the potential of antioxidant and anticancer effects of CME on colon cancer using human cancer cell lines.

MATERIALS AND METHODS

Plant materials

Cordyceps militaris fungus was produced by Ganofarm R&D SDN BHD research laboratory (Puchong, Selangor, Malaysia). The isolate of *C. militaris* (strain CMRU-1) used in the present study was collected from the Department of Plant Protection, Can Tho University, Vietnam.

Extraction of the sample

Fresh fruiting bodies or mycelia of *C. militaris* were extracted using the maceration technique. 100 g of the sample was macerated in 1,000 ml of water (stirred at 200 rpm) at 90°C for 1 hour (Azrie *et al*., 2014; Morales *et al*., 2019). The crude CME was then filtered. With some modification, the CME was mixed with 10% of maltodextrin and blended until homogeneous (Chankana *et al*., 2013; Chong and Wong, 2015). The mixture was spray-dried with the inlet and exit air temperatures were 170and 80°C (Chankana *et al*., 2013). The mixture was sprayed through a 1.5 bar atomizer pressure nozzle during the spray-drying process (Chankana *et al*., 2013). The spray-dried CME was collected and weighed and the percentage yield was determined. The extracts were stored in the desiccator before further analysis. The yield of the CME was calculated using the following equation:

$$\text{Yield of crude extract } (\%\frac{w}{w}) = \frac{\text{mass of crude extract (g)}}{\text{mass of sample (g)}} \times 100 \quad (1)$$

Total phenolic and total flavonoid content (TFC)

The total phenolic content (TPC) of CME was analyzed using the Folin–Ciocalteu's method. 1 ml of CME (12.5 mg/ml) was mixed with 50% Folin–Ciocalteu reagent (50 µl) and 2% sodium carbonate (2 ml). The solution was thoroughly mixed before being incubated for 30 minutes at room temperature. Using a UV-Vis spectrophotometer (UV1800, Kyoto, Japan), the solution's absorbance was measured at 720 nm. Gallic acid was used as a standard and a calibration curve was constructed (6.55–32.79 mg/l). The TPC was measured as a milligram of gallic acid equivalent (GAE) in a gram of dry weight extract. Each experiment was carried out in triplicate, unless otherwise mentioned. The flavonoid–aluminum complex formation was used to assess the TFC of CME. 1 ml of CME (12.5 mg/ml) was combined with 1 ml of methanol and 2% aluminum chloride. After 15 minutes of incubation, the complex was formed and spectrophotometrically analyzed at 430 nm. A standard calibration curve of rutin (6.67–33.33 mg/l) was constructed. TFC was described as a milligram of rutin equivalent (RE) in a gram of dry weight extract.

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay

1 ml of sample was thoroughly mixed with 2 ml of DPPH solution (0.1 mM) at concentrations ranging from 100 to 500 µg/ml. After 30 minutes of incubation, the absorption was measured at 520 nm. In this study, rutin was used since it is commonly used as a positive control in all previous antioxidant assays. The potential to scavenge the DPPH was measured using equation (2), where the regulation and the sample absorbance are A control and A sample, respectively, as follows:

$$\text{DPPH scavenging activity } (\%) = \frac{Acontrol - Asample}{Acontrol} \times 100 \quad (2)$$

Cell culture

Human colorectal cancer cell lines HT-29 were grown in RPMI-1640 (Gibco, Waltham, MA) containing 10% fetal bovine serum (FBS) and 1% of penicillin–streptomycin mixed solution. 0.05% of trypsin-Ethylenediaminetetraacetic acid (EDTA) (GIBCO, Waltham, MA) was used to harvest the confluent cells, which was neutralized with RPMI-1640 supplemented with 10% of FBS (1:1). The cells were routinely cultured in 25 cm^2 plastic corning flasks (T-25) and kept at 37°C in a humidified atmosphere with 5% of carbon dioxide supply (CO_2) maintained at 37°C as monolayer cultures.

Cytotoxicity assay

To dilute the human cell lines to a concentration of 5×10^3 cells ml^{-1}, serum-free RPMI-1640 (GIBCO, Waltham, MA) was used. A total of 0.1 ml of cell suspension was pipetted into each of the 96-well microtiter plate's allocated wells. In this study, the blank control group consisted of three wells containing a culture medium. In a 5% CO_2 incubator at 37°C, the plate was incubated for 24 hours. The culture medium was pipetted out after incubation, and 0.1 ml of serum-free culture medium containing CME with different concentrations from 0.625 to 10.000 µg/ml was distributed in triplicate into specified wells. The positive control (cisplatin) was used in this study with a concentration of 10 µg/ml (Sigma, Cream Ridge, NJ). For 48 hours, the plate was incubated at 37°C in a 5% CO_2 incubator. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagent was then applied to each well in a volume of 20 µl. This plate was then incubated in a CO_2 incubator at 37°C for another 4 hours before the dye was clear. The supernatant was then was pipetted out, and each well was filled with 0.1 ml of Dimethyl Sulfoxide (DMSO). At 540 nm, the microplate Enzyme-linked immunosorbent Assay (ELISA) reader (Corona Microplate Reader SH1000, Hitachi) was used to measure the absorbance. Equation (3) was used to calculate the concentration inhibition (CI, %) as follows:

$$CI\ (\%) = 1 - \{(As - Ab)/(Ac - Ab)\}100 \quad (3)$$

where the absorbance of blank (*A*b), negative control (*A*c), and sample (*A*s) were denoted in the equation. Determination of the half-maximal inhibitory concentration (IC_{50}) of each extract was

made by interpolating the linear regression of percentage of mortality versus the concentration of extract. The experiment was carried out in triplicate.

Acridine orange (AO) and propidium iodide (PI) double staining

The cell lines at the concentration of 10^6 cells/ml were treated with CME (at the concentration of the IC_{50}. On the other hand, cells with no application of CME were used as the negative control. Both of the treated and untreated cells were incubated in a 25 cm^2 tissue culture flask for 48 hours at 37°C. A 20 µl of AO/PI mixture in PBS at 1:1 (*v/v*) ratio was used to stain the cells and was visualized using a Leica fluorescence microscope DM 2500 (Leica Microsystem, Wetzlar, Germany) at 100× magnification. Alpha Imager (AlphaInnotech, San Leandro, CA) was used to capture the images of the cells.

RESULT AND DISCUSSION

Antioxidant activity on the DPPH, TPC, and TFC

The percentage yield of spray-dried CME obtained was 10.2 *w/w*%. Based on the C analysis of variance analysis in Table 1, all the antioxidant activities which were DPPH, TPC, and TFC of CME were significantly ($p < 0.05$) higher than fresh CM. The higher percentage of inhibition of the antioxidant activity presence and lowest half-maximal IC_{50} in CME is due to the high content of TPC (160 ± 0.74 mg GAE/100 g) and TFC (6.6 ± 1.13 mg RE/100 g) present in the extract. While CM samples only showed a moderate inhibition on the DPPH activity which was 54.3% (IC_{50} = 2.95 mg/ml) compared to CME 83.8% (IC_{50} = 0.60 mg/ml). The CME demonstrated strong antioxidant efficacy by reducing the initial violet color of the DPPH solution to subtle violet and consequently brighter yellow. The DPPH (a stable free radical) is converted to 1,1-diphenyl-2-picrylhydrazyl due to the reaction with antioxidants (Iqbal *et al*., 2017). The level of discoloration suggests the antioxidant's radical-scavenging potential. In this step, the DPPH reacts with compounds that convert it to a solid diamagnetic molecule by contributing hydrogen (H) atoms. The antioxidant activity of CME was comparable to *Agaricus blazei* (97.1% at 2.5 mg/ml), *Ramaria botrytis* polysaccharides (82.67% at 1.4 mg/ml), and *Coprinus comatus* (84.5% at 5 mg/ml) (Huang *et al*., 1999; Li *et al*., 2017; Tsai *et al*., 2007).

Cytotoxic effect of CME on HT-29 cells

The MTT cytotoxicity results showed that CME has the highest inhibitory activities followed by synthetic anticancer drug cisplatin in a dose-dependent manner (Fig. 1). On the contrary, cisplatin expressed low inhibitory activities against HT-29 cells. The IC_{50} values for CME and cisplatin were to be 1.53 and 3.11 mg/ml, respectively. The obvious difference in the mean percentage inhibition of the five concentrations ($p < 0.0001$) of CME and cisplatin using Tukey's Honest significant difference (HSD) *post-hoc* analysis stipulated that the 10 mg/ml of extract had significantly decreased the value of the absorbance as the extract concentration increased. However, the inhibition percentage increases with an increase in CME. This result showed that the compound has a high cytotoxic effect on HT-29 cells even at the lowest concentration of the extract, which is 0.625 and 1.25 mg/ml with 64.66% and 52.14% cell inhibition as compared to cisplatin with 52.52% and 49.14% cell inhibition, considering that the CME is a crude extract.

Table 1. The antioxidant activity of CME and fresh CM.

Samples/test	DPPH	TPC (mg GAE/100 g)	TFC (mg RE/100 g)
CME	83.8 % ± 1.02 (IC_{50} = 0.60 mg/ml)	160 ± 0.74	6.6 ± 1.13
Fresh CM	54.3 ± 1.88 (IC_{50} = 2.95 mg/ml)	90 ± 2.56	4.5 ± 1.48

The chemopreventive activity of cordycepin, a major compound responsible for anticancer properties in CME against human colon, liver, bladder, and renal cancer, lung, and breast cancer cell lines, has been reported (Choi *et al*., 2011; Lee *et al*., 2009; Shao *et al*., 2016; Tao *et al*., 2016; Yamamoto *et al*., 2015; Yoon *et al*., 2018). Nevertheless, the study has been limited to its effect on mitochondrial dehydrogenase activity. It was confirmed that the crude CME exhibits powerful concentration-dependent growth inhibitory activity against HT-29 cells. Furthermore, the results showed that CME was more cytotoxic against colorectal cancer (IC_{50} of 1.53 mg/ml) than cisplatin (IC_{50} = 3.11 mg/ml). Furthermore, changes in the HT-29 cell shape to round in shape (from polygonal shape), reduction of cell adherence (due to cell death), increment of cell debris, and decrease in cell density were observed, indicating the cytotoxicity effect of CME (Fig. 2).

Disruption of cell membrane integrity results in the increment of red fluorescence and the reduction of green fluorescence image in the AO/PI assay. The fluorescent microscopy study was executed to investigate the mode of HT-29 cell death by CME. The HT-29 morphological observation demonstrates features of chromatin condensation and nuclear margination which were the main characteristics of apoptosis together with the loss of cell membrane integrity after 48 hours of incubation. Chromatin condensation and nuclear margination due to apoptotic trigger were observed in both early (indicated by the chromatin condensation and nuclear fragmentation) and late apoptosis (formation of apoptotic bodies and membrane loss) features as shown in Figure 3.

It was observed that the HT-29 cells treated with CME exhibited early apoptotic behavior with the formation of condensed

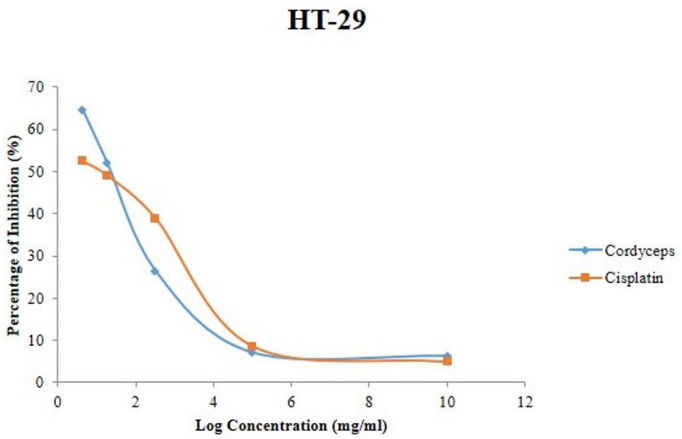

Figure 1. Viability (%) of HT-29 cells 48 hours after treatment with different concentrations of CME.

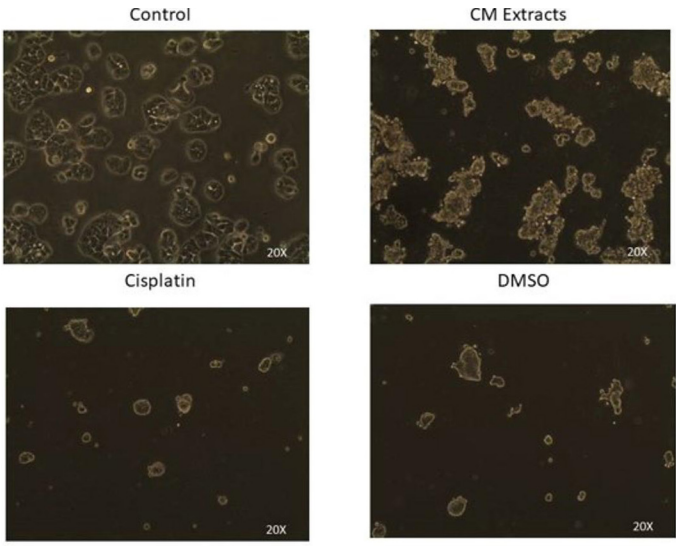

Figure 2. Morphological changes of HT-29 cancer cell. Cells are imaged by inverted phase-contrast microscope.

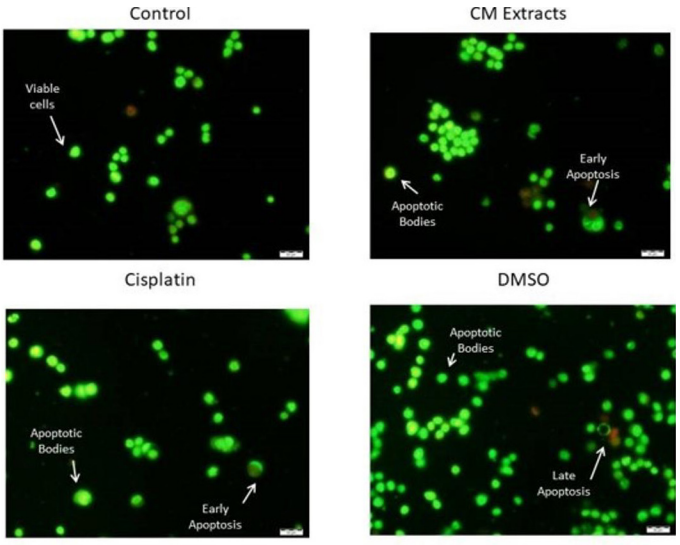

Figure 3. Morphological changes of HT-29 cancer cell detected with AO/PI staining method and imaged by fluorescence microscope.

chromatin and marginated nuclear indicated with a bright-green color stain after 48 hours of treatment together with membrane blebbing. In addition, late stages of apoptosis also appeared after the treatment as green-orange fluorescence stain was observed. Treatment with CME also denatures the deoxyribonucleic acid of the cell as observed in red in the morphological image analysis due to the binding with AO.

CONCLUSION

Overall, the CM treatment on colorectal cancer HT-29 cell lines possesses a strong cytotoxic effect in which it has the highest percentage of cytotoxicity with the lowest IC_{50} value. The high antioxidant content in CM extract shows the parallel agreement with the results of cytotoxic and apoptotic activities of CM extract against HT-29 cells. In conclusion, the results of the present study indicate that CM extract reduces the malignancy of colorectal cancer cells and this anticancer effect of CM extract may present a novel method of treating colorectal cancer and provide evidence on the pharmaceutical potential of CM crude extract as a chemotherapeutic agent against colorectal cancer.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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