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 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., 2013).
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:

\[
\text{Yield of crude extract (\text{g} / \text{g})} = \frac{\text{mass of crude extract (g)}}{\text{mass of sample (g)}} \times 100
\]

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 ml) 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 530 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 Acontrol and A sample, respectively, as follows:

\[
\text{DPPH scavenging activity (\%)} = \frac{A\text{control} - A\text{sample}}{A\text{control}} \times 100
\]

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 cm² plastic corning flasks (T-25) and kept at 37°C in a humidified atmosphere with 5% of carbon dioxide supply (CO₂) maintained at 37°C as monolayer cultures.

Cytotoxicity assay

To dilute the human cell lines to a concentration of 5 × 10⁴ 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% CO₂ 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% CO₂ 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 CO₂ incubator at 37°C for another 4 hours before the dye was clear. The supernatant was then 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:

\[
\text{CI (\%) = \left[1 - \left(\frac{A\text{sample} - A\text{blank}}{A\text{control} - A\text{blank}}\right)\right] \times 100}
\]
The antioxidant activity of CME and fresh CM. Nevertheless, the study has been limited to its 50.

Fig. 1 Choi 50 DPPH 50). The level of discoloration 50).

Viability (%) of HT-29 cells 48 hours after treatment with different p;

50 Iqbal 90 ± 2.56 50 50 50 50 83.8 % ± 1.02 (IC 6 54.3 ± 1.88 (IC 4.5 ± 1.48 50 50 50 50 160 ± 0.74

mean percentage inhibition of the five concentrations (42x143)

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...
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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