Evaluation of Anticancer Activities of *Cordyceps militaris* Grown on Fluorinated Medium

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Abstract

Cancer, a group of diseases caused by unconstrained cell proliferation which finally turns into tumor tissue, is becoming a main threaten to human health. Countless academics and researchers have been focused on developing the high efficiency cancer treatments with less side effects for over years. Nowadays, mainstream cancer treatments involve surgery, radiation therapy, and chemotherapy.

As a traditional medicine material and nutritious food resource, *Cordyceps militaris* (*C. militaris*) exhibits a good potential of anti-proliferation activities to cancer cells with almost no harm to human body. It also possesses many other health benefits functions including antioxidative, anti-inflammatory, antibacterial, anticancer, and lung protection due to its natural effective substances such as cordycepin, cordyceps polysaccharides, and cordyceps acid et al. However, the natural source of this wild edible fungi decreased sharply and cannot satisfy the huge demands of daily market. Therefore, the research of *C. militaris* with its promotion of production and bioactivities development, is of great value and has been focused on by worldwide for many years. The most common strategy for promoting *C. militaris* fruiting bodies growth is basically to adjust cultural conditions (temperature, light condition, air condition) and cultural medium. Despite low dose of fluoride exhibits some positive stimulation on some plants or algae with a biomass promotion, this stimulation effect on *C. militaris* has not been studied yet.

This study was aimed to investigate the effect of low dose fluoride on the growth of *C. militaris*. The fruiting bodies of *C. militaris* cultivated in normal and fluorinated medium were harvested for comparing their bioactive compounds and anticancer activities. For the first time, this study revealed the interaction between fluoride and the growth of *C. militaris*. Moreover, the molecular mechanism of anticancer activities of their extracts *in vitro* was also elucidated in this study.

(1) Fruiting bodies of *C. militaris* were successfully obtained from normal and fluorinated medium (CM_{fb} and F-CM_{fb}) and used for evaluation of biomass properties, potential antioxidant abilities, and anticancer activities. Dry weight of fruiting bodies obtained from normal and fluorinated medium (0, 0.001, 0.01, and 0.1 mM of fluoride content ratio) indicated that *C. militaris* cultivated in the 0.01 mM F-CM_{fb} (1.55 ± 0.14 g/bottle) led to a 44.86% biomass promotion compared with CM_{fb} (1.07 ± 0.07 g/bottle). In addition, the maximum promotion of total carotenoid content obtained from 0.01 mM F-CM_{fb} was increased to 23.43% (3161.38 ± 35.71 μg/g). *C. militaris* exhibited a growth stimulation at low dose of fluorine-supplemented medium condition.

(2) Aqueous extracts from fruiting bodies of *C. militaris* cultivated in normal and fluorinated medium (CM_{aq} and F-CM_{aq}) were used for antioxidant activity assays. A significant increase in superoxide dismutase-like activity (SOD = 84.75 U/mg) and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (IC_{50} = 2.59 mg/mL) were detected in 0.01 mM F-CM_{aq} for the evaluation of antioxidant activities, which indicated that antioxidant activities of fruiting bodies cultivated in fluorinated medium were promoted.

(3) ^{19}F nuclear magnetic resonance spectra of CM_{fb} and F-CM_{fb} were analyzed to confirm the status of existed fluoride in fruiting bodies. The fluoride contents in 0.001, 0.01, and 0.1 mM F-CM_{fb} were detected as 15.09, 33.81, and 54.38 ppm, respectively. Moreover, the detected fluoride was close to potassium fluoride, indicating the fluoride acted as inorganic ions.

(4) CM_{aq} and F-CM_{aq} were further used for anticancer activity *in vitro*. According to the results, F-CM_{aq} treatment exhibited a significantly higher antiproliferation ability to U2OS human osteosarcoma cell and A549 human non-small cell lung cancer cells as compared to CM_{aq} treatment, with no cytotoxicity effects on TIG-3 human normal lung fibroblasts. On the other hand, F-CM_{aq} treatment also exhibited higher anti-migration of the cancer cells. F-CM_{aq} treatment caused stronger apoptosis in U2OS cells (41.86%) and A549 cells (15.20%) as compared to CM_{aq} treatment (24.36% in U2OS cells, 10.26% in A549 cells). U2OS cells exhibited stronger cell arrest in G2/M phase (31.25%) when treated with F- CM_{aq} as compared to CM_{aq} treatment (21.54%). In A549 cells, F-CM_{aq} treatment caused 78.70% of cell cycle arrest in G1 phase when compared with CM_{aq} treatment (64.34%). F-CM_{aq} induced stronger apoptosis/growth arrest of cancer cells through the activation of Bax, caspase 3 and p53, and inhibition of PARP-1, Bcl2, cyclin D1/Cdk 4 and pRb.

In conclusion, this is the first time to explore the utilization of fluoride in culture medium of *C. militaris* and demonstrate that a low dose of fluoride could stimulate the growth of *C. militaris* fruiting bodies and enhance the production of bioactive ingredients that possess useful antioxidant and anticancer activities. This will provide a new strategy of the cultural application of *C. militaris* and a new option for anticancer therapeutics.

Key words: *Cordyceps militaris*; Fluoride; Anticancer activities.

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Chapter 1 Introduction

1.1. Cancer

1.1.1. Cancer and its therapy

Cancer is a group of disease caused when cells divide unrestrainedly and spread into around tissues. When normal cells grow old or become damaged, they usually die. However, sometimes abnormal, or damaged cells grow and multiply may form tumors. Without good treatments, those malignant tumors may cause serious symptoms or be life threatening. Nowadays, cancer is one of the main reasons of morbidity and decease worldwide (Sung et al., 2021), accounting for nearly 10 million deaths in 2020 (Figure 1-1). Cancer occurs from the variation of normal cells into tumor cells in a multi-phase process. The transformation was the result of the interaction between a person's genetic factors and categories of external agents including biological carcinogens (such as genetic defect and infections), physical carcinogens (such as radionuclide and radiation), chemical carcinogens (such as formaldehyde, alcohol, and tobacco). The occurrence rate of cancer rises dramatically with the trend for cellular repair mechanisms to be less effective as a person becomes older. Every cancer type requires a specific treatment regimen, most common types of cancer treatments including surgery, radiation therapy, and chemotherapy. Surgery usually offers the greatest opportunity for therapy, especially in the status of cancer has not overspread to other parts of the body. Radiation therapy applies high-energy particles or waves to destroy or injure cancer cells. Chemotherapy is mainly directed at enhancing the patient's quality by using medicines or drugs for cancer treatment.

1.1.2. Cancer prevention by *Cordyceps militaris*

The process of cancer cells invades abutting parts in body and extend to other organs is consulted to as metastasis, which is primary cause of death. A good prevention at early stage of rapid creation of abnormal cells will inhibit occurrence of metastasis. Various traditional medicinal fungi have been used by many countries for health maintenance including cancer prevention strategy. There have been many studies reported that *Cordyceps militaris* possessed anticancer ability to several kinds of cancer including colorectal carcinoma (Lee et al., 2015), lung cancer (Bizarro et al., 2015), breast cancer (Jin et al., 2008), oral cancer (Lin et al., 2018) and so on. In these studies, potential anticancer abilities of *Cordyceps militaris* were verified from *in vitro* and *in vivo* level, some mechanisms of *Cordyceps militaris* anticancer activities have also been explained from molecular and protein level. All these findings indicates that as a traditional food resource and natural anti-tumor material, *Cordyceps militaris* possessed good prevention activities to cancer as well as exhibits fewer side effects.

1.2. *Cordyceps militaris*

1.2.1. *Cordyceps*

Cordyceps, one of the most popular medicinal mushrooms widely used in Asia for centuries, is a genus belongs to the ascomycete fungi that contains near 400 species (Sung et al., 2007). Most *Cordyceps* species belong to endoparasites which are parasitic on insects, hexapods, or other invertebrates, some of them are parasitic on fungi. When these fungi invade their host, they substitute its tissue and germinate long, slim stalks that develop outside the host's body. The genus has a worldwide distribution while most of described are from Asia including China, Thailand, Japan, Vietnam, Korea, Mongolia, and Nepal. Especially in damp environment and tropical jungle, *Cordyceps* species are multiple. The remains of the insect and fungi part need to be collected and dried before they can be used to improve our health. There are two species that are mostly of interest for the scientist for their medicinal benefits for the human body – *Cordyceps sinensis* and *Cordyceps militaris*.

1.2.2. Medicinal uses of *Cordyceps militaris*

Cordyceps militaris (Figure 1-2) is a species of fungus belongs to the family *Cordycipitaceae*, and the type species of the genus *Cordyceps*. It is an entomopathogenic fungus that forms fruiting bodies mainly on pupa or larva. The dry fruiting bodies are accustomed utilized as a therapeutic herbal drugs or food material in eastern countries, with functions like revitalization of various systems of the body (Das et al., 2010). The entomopathogenic mycelia naturally grows in the host body in the autumn, killing the host. After hibernation period, the fungus bursts the host body, forming fruiting body structure connected to dead host and protrude above the ground the next summer. (Nam et al., 2001). Many active components contained in *C. militaris* (including cordycepin (Ni et al., 2009), polysaccharides (Kim et al., 2003), ergosterol (Nallathamby et al., 2015), mannitol (Yang et al., 2014), etc.) providing various physiological activities, which make it could be used for multiple medicinal purposes including modulation of immune response (Bi et al., 2018; Lee and Hong, 2011; Wang et al., 2013; Li et al., 2006), induction of cell apoptosis (Park et al., 2005; Lee et al., 2006; Lui et al., 2007), down-regulation of apoptotic and inflammatory genes (Won and Park, 2005; Ng and Wang, 2005; Han et al., 2020), inhibition of tumor growth (Ruma et al., 2014; Yoo et al., 2004; Jin et al., 2018). Nowadays, *C. militaris* is attacking more attention and becoming important in medical communities.

1.3. Cultivation strategies for *Cordyceps militaris*

As an extremely scarce resource, the market price of those uncultivatable natural *Cordyceps sinensis* fruiting body had ever been over than $10,000/kg (Stone, 2008). Therefore, artificial cultivation strategy was able to help reducing the pressure of huge market demands. *C. militaris* has been chosen due to its readily performs production from artificial medium (Zheng et al., 2011; Shao et al., 2019; Li et al., 2019). Many studies have been interested in

optimizing cultural medium to obtain more fruiting body production or bioactivities contents promotion. Normal culture process was three stages including seed culture, mycelium proliferation and fruiting body formation on solid medium (Liu et al., 2016; Ghatnur et al., 2015). There are many kinds of solid and liquid medium for *C. militaris* cultivation. Through adjusting carbon/nitrogen ratio in solid medium to achieve optimal cultural condition (Mao et al., 2005), solid material could be rice bran (Xu et al., 2019), wheat bran (Xie et al., 2010), husked rice (Das et al., 2010), and wheat grains (Dang et al., 2018) which were loaded in culture bag or bottles. For liquid medium, enriched with some benefits additives like NH_4^+ (Mao and Zhong, 2006), vegetable oils (Tang et al., 2018), or initial pH value, modes of propagation (shake-flask and static culture) (Shih et al., 2007) have also been investigated for *C. militaris* promotion. In addition, there have been other studies enhanced growth and ingredients synthesis efficiency of *C. militaris* through optimizing light condition (Yang et al., 2016; Yi et al., 2014; Chiang et al., 2017; Wang et al., 2018), temperature (Zhang et al., 2018; Tang et al., 2014; Lin et al., 2011; Nurmamat et al., 2018), and humidity condition *in vitro*, or by adding some heavy metals (Chen et al., 2011). Developments of cultivation strategies is necessary for solving contradiction between supply and demand in market, as well as environment protection.

1.4. Hormesis effect and fluoride chemistry

1.4.1. Hormesis effect strategy in cultivation

Hormesis is a dose–response phenomenon in which opposite effects are observed at low, compared to high, doses for the same measure system (Calabrese and Blain, 2011). The study of hormesis has generated considerable attentions among widely area including toxicological, pharmacological, general biomedical and biological in the past decades (Calabrese and Baldwin, 2001a, 2003a; Calabrese, 2008; Calabrese and Baldwin, 2001b, 2003b). These relative studies, demonstrated that hormetic dose responses were observed in a wide scale of biological models, occurring over a multiple group of biological endpoints, and involving a wide range of chemical classes. The stimulation in the low-dose condition was basically modest with the maximum promotion around 30–60% (Calabrese and Blain 2011). These studies demonstrated that hormetic dose responses were reproducible and broadly generalized, they are not only important for environmental risk assessment, but also providing a theory support for stimulation strategy (Kaiser, 2003). Therefore, hormetic stimulation is considerable to be utilized as cultivation strategy.

1.4.2. Fluoride chemistry

The field of fluorine chemistry was born in the latter 19th century when Henri Moissan's first isolated of fluorine in 1886. As the most electronegative element with small size and strong electron-withdrawing property, fluorine has played a particular role in major technological developments in multiple fields like fluoropolymers (Desimone et al., 1992; Ameduri, 2018), pharmaceutical/agrochemical products (Muller et al., 2007; Wang et al., 2014; Silva et al., 2020), and material science (Ilardi et al., 2014; Giornal et al., 2013). The importance of fluorine is

expected to grow more in near future.

1.4.3. Hormesis effect of fluoride

Burgstahlert supposed that fluoride possesses hormesis effect (Burgstahler, 2002), which is that biological would be stimulate under low concentration of fluorine content environment. In mammals experiments, high fluoride concentration has been found to cause adverse effects on both male (Chaithra et al., 2020a, 2020b; Gupta et al., 2007) and female (Wang et al., 2017) mice reproductive capacity while some other studies indicate that no effects on reproductive function and development in low dose of fluoride condition (Tao and Suttie, 1976; Aulerich et al., 1987; Heindel et al., 1996) . In plants, low dose of fluoride, in contrast to toxic effects usually found in mammals, may sometimes cause growth stimulations. In fact, there are plenty of interesting discoveries relating to hormesis effect of fluoride have been studied not only limited to plants, algae, animals, but also including silkworm (Chen, 2003). According to these studies, the mechanism of stimulations' effects induced by fluorine could be associated with coordination/cooperation through photosynthesis activity (Keller, 1980), dark respiration (McLaughlin and Barnes, 1975), and some bio enzyme activities (Wu et al., 2007). However, the concentration range to trigger hormetic effect of different species could be quietly difference (Elloumi et al., 2017; Rodrigues et al., 2020; Li et al., 2018; Li, 2003). According to above mentioned features of fluoride hormesis effect, it provided an idea about new cultivation strategy for *C. militaris* by using fluoride.

1.5. Fluoride

1.5.1. Utilization of fluoride in medicinal field

As described in above, fluorine plays an important character in medicinal chemistry (Ojima, 2017; Lu et al., 2017; Ojima, 2004), biochemical (Ojima, 2013, 2004), and drug development (Rennen et al., 2001; Jacob, 1999), with supported by the fact that many fluorine-supplemented productions have been certified through the U.S. Food and Drug Administration (FDA) for medical and agricultural application. In fact, about 20–25% of drugs in the medicinal pipeline involve at least one fluorine atom (Figure 1-3). The unique properties that cause fluorine and organofluoride groups popular in medicinal and biochemical involve small atomic radius with high electronegativity, and low polarizability of the carbon-fluorine bond. In fact, organofluoride compounds are essentially absent as natural products, in the current most applications of fluoride in medicinal were focused on stage of artificial synthesis. Fluorine substitution technology is generally adopted in present medicinal industry for metabolic stability developing, bioavailability enhancement, and protein-ligand interactions improvement.

1.5.2. Organofluoride in natural products

Despite the achievement of fluoride utilization in devise of synthetic medicinal field, there seems to be extremely limited of biogenic organofluorides exists in nature, according to the

investigation, fluoroacetate was the first confirmed fluorinated natural product discovered in the leaves of the *Dichapetalum cymosum*, a South African plant (David and David, 1999). Besides fluoroacetate, for the metabolites, nucleocidin, 4'-fluor deoxyadenosine derivative, was also isolated from *Streptomyces calvus* (Fukuda et al., 2009). Moreover, tea leave is commonly known as plants which possesses a fluoride accumulation ability, fluoride content raises significantly with the maturity of the leaves (Fung et al., 2003; Shu et al., 2003). Although the mechanism of organofluorides synthesis and its metabolic process in organism still need further investigations, the bioavailability of fluoride for natural environment was still very low and rely on different conditions such as fluoride administration (Aasenden et al., 1968; Carey, 2014; Weyant et al., 2013) and fluoride formulation (Heijnsbroek et al., 2006; Nazzal et al., 2016; Parkinson et al., 2021). Simultaneously, fluoride has also been proved to be essential to animal life due to imparts caries resistance to dental cavities or prevents anemia and infertility caused by iron deficiency in mice within a narrow range of safety threshold (Nielsen, 1991).

1.6. Purpose and construction

This experimental study was performed for the first time to explore the hormesis effect of fluoride on *C. militaris* by fluorine addition to its culture media, and then analyzed the growth situation of *C. militaris* fruiting bodies while the signal and form of fluoride in *C. militaris* production was also detected. We also made a comparison of the antioxidant abilities and anticancer activities between *C. militaris* raised from normal and fluorine-supplemented media. Furthermore, we investigated the difference of anticancer activities between normal and fluoride *C. militaris* from protein express level. To achieve these purposes, this thesis was separated into four chapters in which the primary instructs are shown in Figure 1-4.

Chapter 1 Introduction

In this chapter, the relating objects and their background of this research was presented, including *C. militaris*, hormesis effect, fluorine, cancer, and cancer prevention by *C. militaris*. Intention and the structure of this thesis were also discussed.

Chapter 2 Bioactivities evaluation of *Cordyceps militaris* raised in normal and fluoride-supplemented medium

In this chapter, *C. militaris* fruiting bodies were successively obtained from normal (CM_{fb}) and fluorine supplemented (F-CM_{fb}) medium and used for the evaluation of potential antioxidant activities *in vitro* while the exist form of fluorine was also detected.

Chapter 3 Comparative study on anticancer activities of aqueous extract of *Cordyceps militaris* fruiting bodies obtained from normal and fluorine-supplemented media

In this chapter, aqueous extract of *C. militaris* harvested from normal (CM_{aq}) and fluorine-supplemented (F-CM_{aq}) medium were subjected to test the growth inhibition, cell cycle distribution and apoptotic induction effects in human osteosarcoma (U2OS) cells, human non-small cell lung cancer cell (A549) and normal fibroblast (TIG-3) cells. Related protein expression levels were explored by western blotting analysis and immunofluorescence staining assays.

Chapter 4 Conclusions and future research

In this chapter, the major achievements are summarized, and the expected utilization of hormesis effect on biological was proposed. In addition, the future research about utilization of fluoride-supplemented cultivation technology was also discussed.

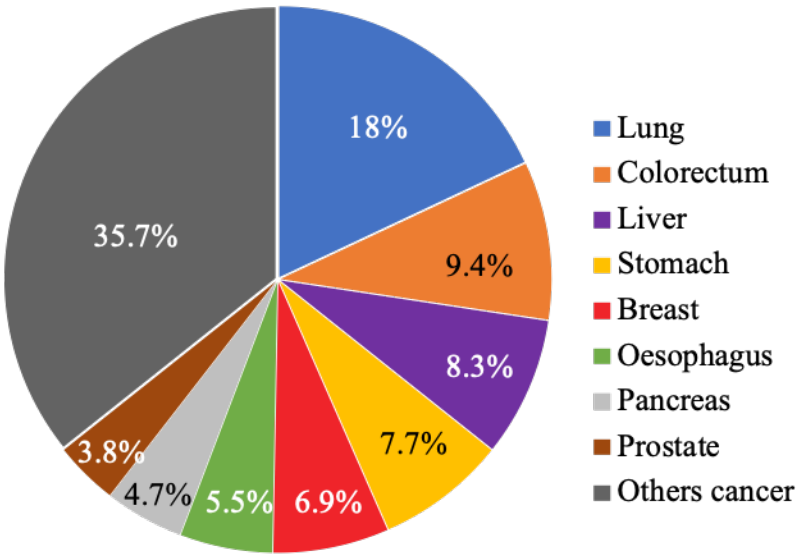

Figure 1-1. Number of deaths in 2020, both sexes, all ages.
Source: Global Cancer Observatory: Cancer Today. (https://gco.iarc.fr/today/online-analysis-pie?v=2020&mode=cancer&mode_population=continents&population=900&populations=900&key=total&sex=0&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=7&group_cancer=1&include_nmsc=1&include_nmsc_other=1&half_pie=0&donut=0)

Figure 1-2. Image of *Cordyceps militaris*.

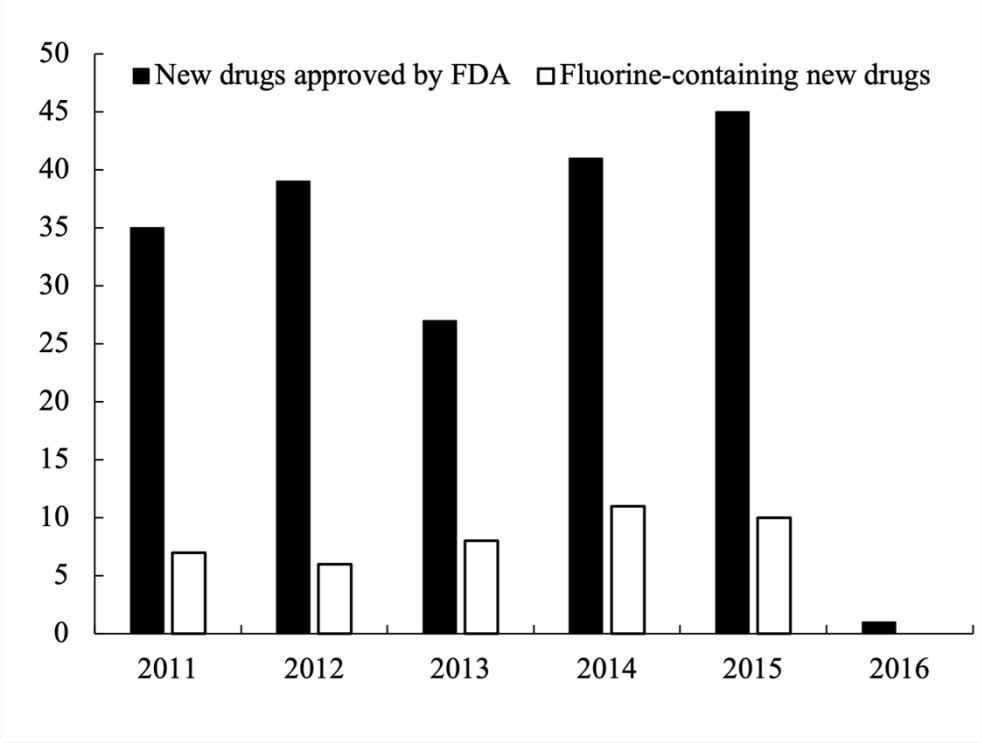

Figure 1-3. Quantity of drugs approved by FDA of fluorine-containing drugs.

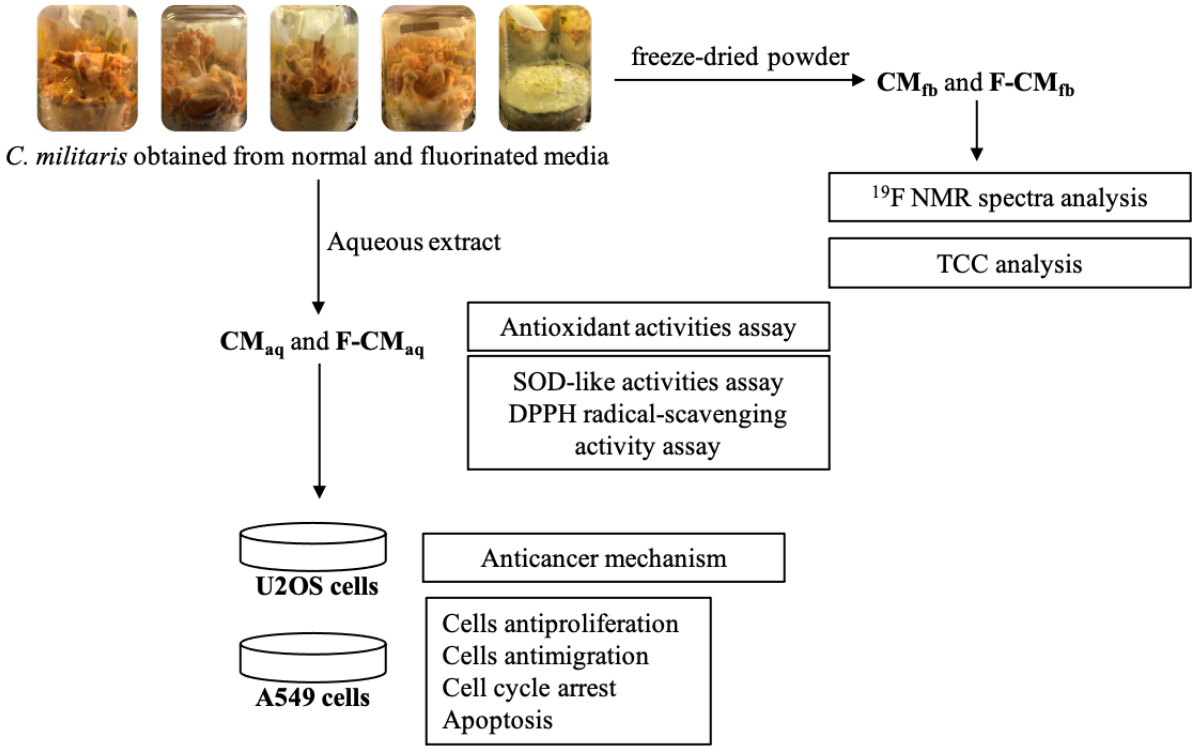

Figure 1-4. Main structure of this study.

Chapter 2 Bioactivities evaluation of *Cordyceps militaris* raised in normal and fluoride-supplemented medium

2.1. Introduction

Cordyceps militaris (*C. militaris*), a traditional Chinese herbal fungus, has been employed as an alternative medicine with functions including antimicrobial activities, antioxidant properties, and hyperglycemic inhibitory activities. On the other hand, the resources of natural edible fungus reduced rapidly which cannot supply the huge demands of market. Due to this situation, any efficiency method benefits for promoting *C. militaris* bioproduction or enhancing their bioactive substance was worthy to be explored.

Hormesis effect has been attracting generated interests in recent years, the discussions of hormesis were broadly based in toxicological, pharmacological, and general biomedical science fields, among these discussions. Burgstachlert proposed that fluoride possessed hormesis effect. As the most electronegative element, fluorine now plays an important role in almost all aspects with their applications thriving in industry, agriculture, and medicine. It is also widely applied in medicinal manufacturer for development of molecules potency and permeability, because of the small size and strong electron-withdrawing character. There was a wild range of discoveries relating to hormesis effect of fluoride to plants, algae, animals, and human being. To our best knowledge, report about fluoride hormesis effect on *C. militaris* is still almost blank until now.

In this study, potassium fluoride（KF), which was widely used in the field of fertilizer relating to agricultural including the raising and growing of animals and crops, was added as the fluoride source into *C. militaris* culture medium. The stimulation induced by fluoride to growth of *C. militaris* was recorded, while the content of bioactive compounds and antioxidant activities analysis to their fruiting body from normal (CM_{fb}) and fluoride-supplemented (F-CM_{fb}) media were also investigated and has been compared. Moreover, we detected the exist of fluoride and its form in F-CM_{fb}, this could be helping to speculate the mechanism of hormetic induced by fluoride in cultivation process of *C. militaris*.

2.2. Materials and methods

2.2.1. Chemicals and reagents

The *C. militaris* strain KCTC 6064 was purchased from the Wuhan Academy of Agricultural Sciences in China (Wuhan, China). Glucose, peptone, KH_2PO_4, $MgSO_4$, KF, and acetone were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Rice was purchased from the National Federation of Agricultural Co-operative Associations (Ibaraki, Japan). Superoxide dismutase (SOD) assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Tabaru, Kumamoto, Japan). 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Thermo Fisher Scientific K.K. (Tokyo, Japan).

2.2.2. Cultivation of *Cordyceps militaris*

The stock culture of *C. militaris* strain KCTC 6064 was maintained on agar slants containing 2% glucose, 2% peptone, 0.2% KH_2PO_4, and 0.3% $MgSO_4$. The inoculated slants were incubated at 21–23 °C in the dark for 10 days and then stored at 4°C for mycelial growth. The normal solid medium comprised 36 g rice with 63 mL nutrient solution (2% glucose, 2% peptone, 0.2% KH_2PO_4, and 0.3% $MgSO_4$) in a 300 mL cylindrical glass bottle. The KF content added to the nutrient solution was adjusted to 0.001, 0.01, 0.1, and 1 mM, to set different concentrations of KF-supplemented media. All media were then sterilized for the cultivation of *C. militaris* on a solid medium. Each solid media was prepared for 5 parallel bottles. After mycelial formation completed, they were transferred to normal and KF-supplemented solid culture media and incubated in the cultivation shed, where the temperature was maintained at 21-23 °C with air humidity above 70% and kept in the dark for 20 days for base cultivation. After *C. militaris* mycelia spread completely on the medium surface, they were subjected to alternating light-dark cycle at 21–23 °C in 12 h light and 16–20 °C in 12 h darkness for 30 days to stimulate *C. militaris* fruiting body growth. The cultivation environment and growth status were examined regularly. After 30 days, *C. militaris* fruiting bodies from normal (CM_{fb}) and fluorinated medium (F-CM_{fb}) were harvested, vacuum freeze-dried, weighed, collected, and ground into powder, stored at -20 °C for further analysis.

2.2.3. Analytical methods

(1) Fluoride

^{19}F nuclear magnetic resonance (NMR) spectra of CM_{fb} and F-CM_{fb} were recorded on a Bruker AVANCE 400 (400 MHz) NMR (PS751) (Bruker Corporation, Japan) with trichlorofluoromethane (CFC-11) (0.5 wt%) as an internal standard in MeOD-d4 at 25 °C. Fluoride was quantitated by using ^{19}F-NMR and compared with 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) as follows:

The sample m (x) g and the internal standard 1,1,1,3,3,3-hexafluoroisopropanol n(std) mol were mixed in 1 mL of MeOD-d4.

The above mixture (600 μl) was analyzed using ^{19}F NMR, and the ratio of the area of the sample and the internal standard was obtained, which was equal to the molar ratio of fluorine in the sample and 1,1,1,3,3,3-hexafluoroisopropanol according to the equation (2-1):

$$Area(std)/Area(x) = 6 \times n(std)/n(x) \quad (2\text{-}1)$$

The quantification of fluoride in *C. militaris* was estimated as equation (2-2):

$$Fluoride\ (ppm) = 100\% \times 19 \times n(x)/\ m(x) \quad (2\text{-}2)$$

where Area(std) is peak intensity value of HFIP, Area(x) is peak intensity value of sample, n(std) is amount of HFIP (mol), n(x) is amount of sample (mol), m(x) is weight of sample (g).

(2) Total carotenoids

Total carotenoids content (TCC) was evaluated as follows: each group of *C. militaris* fruiting body powder (1.0 g) was mixed with 20 mL of acetone evenly. The mixture was then heated by microwave oven for 3 minutes at 200 W. After that, samples were shaking at ambient

temperature (25 °C) for 30 minutes and through centrifuged at 4000 rpm for 15 minutes and collect the supernatant. To detect the exist of carotenoids, take supernatant liquid, and further extracted by petroleum ether, removed the water in sample by eluted with anhydrous sodium sulfate, the anhydrous petroleum ether extract was then mixed with 2 mL of concentrated sulfuric acid and check the color of stratification area. For TCC quantification, take supernatant and diluted properly, the absorbance was tested at 475 nm with a spectrophotometer (Lambda35, Prekin Elmer Co. Ltd., USA). The total carotenoids content (TCC) was calculated by the following equation (2-3):

$$TCC\ (\mu g/g) = As \times V \times D/\ (0.16 \times W) \qquad (2\text{-}3)$$

where As is absorbance of each sample, V is dosage of acetone (mL), D is dilution factor, 0.16 is molecular extinction coefficient of carotenoids, W is mass of *C. militaris* powder (g).

2.2.4. Preparation for *C. militaris* aqueous extracts

CM_{fb} and F-CM_{fb} powder (2.0 g) was extracted with 40 mL deionized water at 100 ℃ for 45 minutes, this operation was repeated for 3 times. After cooling down to room temperature (25 °C), all these aqueous extracts were collected and centrifuged at 7500 rpm for 15 minuets at ambient temperature (25 °C), each supernatant was then filtered through a 0.2 μm sterile filter membrane and concentrated in a rotary evaporator at 60 ℃. Finally, the condensed solutions were lyophilized to obtain extract powder after vacuuming freeze-dried treatment. These aqueous extracts powders (CM_{aq} and F-CM_{aq}) were stored at -20 ºC for further analysis.

2.2.5. Bioactivity assays

(1) SOD-like activity assay of CM_{aq} and F-CM_{aq}

SOD-like activity assay of CM_{aq} and F-CM_{aq} was measured by the SOD Assay Kit-WST according to the instruction manual supplied by Dojindo Molecular Technologies, Inc. First, 20 μl of sample liquid was added into each sample and blank 2 well, and 20 μl of deionized water was added to each blank 1 and blank 3 well. Secondly, 200 μl of WST working solution was added to each well and mixed softly. 20 μl of dilution buffer was then mixed with blank 2 and blank 3 well, respectively. 20 μl of enzyme working solution was then mixed properly to each sample and blank 1 well, respectively. After that, the 96 well plate was keeped at 37 ºC for 20 minutes incubation. Finally, the absorbance of each well was read by microplate reader at 450 nm. The inhibition rate (%) of SOD-like activity was calculated according to the following equation (2-4):

$$\text{SOD activity (inhibition rate \%)} = 100 \times [(A_1 - A_2) - (A_s - A_2)]/\ (A_1 - A_3) \qquad (2\text{-}4)$$

where A_1, A_2, A_3, A_s were the absorbance value of blank 1, blank 2, blank 3, and sample wells. 1 unit of SOD activity was determined as the estimate of enzyme with 50% inhibitory action to WST-1.

IC_{50} value (mg/mL) was valid density at which SOD activity was scavenged by 50%, the value was calculated according to a linear regression analysis.

(2) DPPH radical-scavenging activity assay of CM_{aq} and F-CM_{aq}

DPPH radical-scavenging activities analysis of CM_{aq} and F-CM_{aq} were measured as followed: 1mL of various concentrations of each sample (CM_{aq} and F-CM_{aq}) were blended with 3 mL (40μg/mL) of a MeOH solution of DPPH, respectively. The mixtures were shaken vigorously and keep in dark for 30 minutes at room temperature (25 °C). The optimal density (OD) of each sample and blank were measured by a spectrophotometer at 517 nm. Reduction of the DPPH solution absorbance demonstrated the enhancement of the DPPH radical-scavenging activity. The calculation of DPPH free radical-scavenging activity was following the equation (2-5):

$$\text{DPPH free radical-scavenging activity (\%)} = 100\% \times [(A_0-A_1)/A_0] \quad (2\text{-}5)$$

where A_0 is the absorbance of control group, and A_1 is the absorbance of the samples.

IC_{50} value (mg/mL) was the effectual density at which DPPH radicals were scavenged by 50 %, it was calculated according to a linear regression analysis.

2.2.6. Statistical analysis

All experiments were performed in triplicate, and all results were expressed as mean ± standard deviation (SD). The degrees of statistical significance among the control and sample groups were analyzed using an unpaired t-test (GraphPad Prism 6 Software, San Diego, CA, USA). Significant values are represented as * p <0.05, ** p <0.01, and *** p <0.001.

2.3. Results and discussion

2.3.1. Dry weight and total carotenoids content of CM_{fb} and F-CM_{fb}

Several studies in the past have focused on optimization of culture conditions (Shih et al., 2007) and media composition (Xie et al., 2009) for mycelia liquid culture. However, only a few studies have investigated the conditions for solid-state cultivation of fruiting bodies. In this study, we successfully obtained *C. militaris* fruiting bodies from normal and 0.001, 0.01, and 0.1 mM potassium fluoride supplemented media. We found that in the 1 mM FM group, growth process was completely stopped after mycelia covered the surface of solid media in all 5 bottles. This was in line with earlier studies that have reported inhibitory effect of high dose of fluoride on the growth of organisms (Chae et al., 2018). Notably, as shown in Table 2-1, 1.55 ± 0.14 g/bottle dry weight of fruiting bodies was obtained from 0.01 mM F-CM_{fb}. The mass of freeze-dried fruiting bodies from CM_{fb}, 0.001 mM, and 0.1 mM F-CM_{fb} was 1.07 ± 0.07, 1.28 ± 0.13, and 1.06 ± 0.12 g/bottle, respectively. We found that the biomass of *C. militaris* fruiting bodies was promoted in the solid-state media containing with low dose of fluoride. The optimization of fluoride-addition (0.01 mM) resulted in increase of fruiting bodies to 44.86% (1.55 ± 0.14 g/bottle). We next analyzed the total carotenoids content (TCC) in *C. militaris* raised in CM_{fb} and F-CM_{fb}. “Carotenoids” are the important natural pigments with many bioactive functions including antioxidation, anti-inflammation and anticancer (Yang et al., 2014), they are commonly found in plants, fungi, and algae (Milani et al., 2017). *C. militaris* is a well-known potential source for this nature carotenoids (Zheng et al., 2019). As one of the primary active substances, the TCC of *C. militaris* was evaluated. As shown in Table 2-1, the TCC of *C.*

militaris raised in CM_{fb}, 0.001 mM, 0.01 mM, and 0.1 mM F-CM_{fb} was 2561.27±26.56, 2751.00±21.36, 3161.38±35.71, and 3035.96±53.30 μg/g, respectively. The TCC of 0.01 mM FM group was about increased by 23.43% as compared with CM_{fb} group. This was better than the *Rhodotorula glutinis*, a fungal elicitor, which stimulated the accumulation of carotenoids at 13.72% promotion (Tang et al., 2019). According to previous study, TCC of *C. militaris* fruiting bodies was significantly increased with stimulation of light (Dong et al., 2012). On the other hand, low concentration of fluoride has no effect (Reddy and Kaur, 2008) or a positive stimulation on photosynthetic pigments has also been reported (Doley, 1989). In present study, we considered that carotenoids accumulation *C. militaris* was enhanced at low dose of fluoride condition which resulted in light reaction activity promotion. These results indicated that low concentration of fluoride addition in solid media was able to promote the production of *C. militaris* fruiting bodies as well as accumulation of carotenoid.

2.3.2. ^{19}F NMR spectra of CM_{fb} and F-CM_{fb}

Fluoride was commonly accumulated in plants through airborne deposition or direct uptake from soil (Janiszewska and Balcerzak, 2013; Gago et al., 2014). Despite the achievements of fluoride in the devise of synthetic bio compounds, nature environment seems to have developed only few kind of biogenic organofluorides, like fluoroacetate discovered from the leaves of the South African plant *Dichapetalum cymosum* (David and David, 1999). A possible explanation for the few of biologically-produced organofluorides is the finite bioavailability of fluorine (Walker and Chang, 2014). To determine whether fluorine participates in the metabolic processes in *C. militaris* fruiting bodies and to identify the form in which fluorine existed, we detected and quantified the fluorine status by using ^{19}F NMR spectra. As shown in Figure 2-1A, there was no fluoride signal in CM_{fb}. The results of 0.001, 0.01, and 0.1 mM F-CM_{fb} are shown in Figure 2-1B, 2-1C, and 2-1D, respectively; the three peaks (from left to right) represent the internal standard of CFC-11, quantitation standard of 1,1,1,3,3,3-HFip, and fluorine signal of each sample. We observed that the strength of the fluorine signal in F-CM_{fb} gradually increased from 0.001 mM to 0.1 mM by comparing the peak value with that of 1,1,1,3,3,3-HFip. The deviation value between F-CM_{fb} (approximately 118 ppm) and internal standard CFC-11 (0 ppm) was very close to the deviation value of KF (Shamsipur et al., 2011). Therefore, we assumed that fluoride detected from F-CM_{fb} existed as inorganic ions. The quantification of total fluoride content (TFC) is summarized in Table 2-2. The TFC of CM_{fb}, 0.001, 0.01, and 0.1 mM F-CM_{fb} groups were 0, 15.09, 33.81 and 54.38 ppm, respectively. For fluorosis risk evaluation, according to Huimei Cai's reports of Chinses tea, a beverage with a similar cooking method to *C. militaris*, possess an average of 81.7 ppm of fluoride concentration in infusions of 115 commercially available teas from Chinese tea markets, which was much higher than accumulated fluoride in this study, has been proved that no risk of fluorosis through the hazard quotient (Cai et al., 2016). Furthermore, organic fluorine signal was unable to be detected out indicating that fluoride ions are unlikely to participate in the synthesis of organic active substances but acted as inorganic form in the metabolism of *C. militaris*.

2.3.3. CM_{aq} exhibited a stronger antioxidant activity

Superoxide dismutase (SOD) are enzymes that catalyze the transformation of superoxide into oxygen and hydrogen peroxide, it possesses functions in immune response of organisms, defense against toxic superoxide radicals (Dong et al., 2012). SOD-like activity assay is widely applied for evaluation of antioxidant capability. As shown in Figure 2-2, the SOD-like activity of all samples increased constantly in a dose-dependent manner. The SOD-like scavenging ratio of 0.01 mM F-CM_{aq} was significantly higher than CM_{aq} at the range of 0.08 to 0.4 mg/mL. The half maximal inhibitory concentration (IC_{50}) values (Table 2-3) of CM_{aq} and F-CM_{aq} is: 0.001, 0.01 and 0.1 mM were 1.13, 0.81, 0.59, and 0.87 mg/mL, respectively. The IC_{50} of SOD in CM_{fb} (1.13 mg/mL) is very close to Li's previous result on *C. militaris* (SOD IC_{50} around 1 mg/mL) (Li et al., 2021). SOD activity of CM_{aq} (44.25 U/mg), 0.001 mM F-CM_{aq} (61.37 U/mg), 0.01 mM F-CM_{fb} (84.75 U/mg), and 0.1 mM F-CM_{aq} (57.47 U/mg) was also calculated for evaluation. Although several reports have reported the effect of fluoride stress on SOD activity in various organs of plants (Yadu et al., 2016) or animals (Shivarajashankara et al., 2001). However, in present study, SOD-like activity of 0.01 mM F-CM_{aq} was increased (84.75 U/mg) remarkably. A similar enhancement of SOD activity in edible crops of India with increasing concentration of fluoride has also been reported by Chakrabarti and Patra (Chakrabarti and Patra, 2015). The fluoride stimulation possibly cause an increase in SOD activity through enhanced SOD biosynthesis or metabolism activities as a positive feedback mechanism (Chakrabarti and Patra, 2013).

Figure 2-3 shows the DPPH radical scavenging activities of the four samples. All samples showed dose-dependent inhibition of DPPH radical activity. The scavenging ratio of samples at 10 mg/mL on DPPH radicals were 89.32%, 91.66%, 91.20% and 90.30% for CM_{aq}, 0.001 mM, 0.01 mM, and 0.1 mM F-CM_{aq}, respectively. Notably, 0.01 mM F-CM_{aq} exhibited the strongest DPPH radical activity from 2.5 to 5 mg/mL. The IC_{50} value (Table 3) of DPPH for CM_{aq} (4.16 mg/mL) was consistent with an earlier report on *C. militaris* (4.62 mg/mL). IC_{50} value for 0.001 mM, 0.01 mM, and 0.1 mM F-CM_{aq} were 2.92 mg/mL, 2.59 mg/mL, and 2.89 mg/mL respectively. 0.01 mM F-CM_{aq} exhibited conspicuous free radical scavenging ability. This finding combined with the results of SOD-like scavenging activity suggests that antioxidant ability of F-CM_{aq} was significantly improved.

2.4. Summary

In summary, results suggest that hormesis effect of fluorine occurred on *C. militaris* was possible. However, fluoride concentration greater than 1 mM would inhibit the formation of fruiting bodies. In this study, the optimal stimulation concentration should be around 0.01mM, and the production could be increased from 1.07 ± 0.07 g/bottle (CM_{fb}) to 1.55 ± 0.14 g/bottle (0.01mM F-CM_{fb}). There was no evidence to certificate that fluorine participated the bioactive substance synthesis but simulated *C. militaris* in form of inorganic ions. Besides the higher productivity, 0.01mM F-CM_{fb} exhibited a better antioxidant activity in SOD-like activity and DPPH radical scavenging activity when compared with CM_{fb}.

Table 2-1. Dry weight and total carotenoid content (TCC) of fruiting body from each medium.

Sample	Dry weight (g/bottle)	TCC (μg/g)
CM_{fb}	1.07 ± 0.07	2561.27 ± 26.56
0.001 mM F-CM_{fb}	1.16 ± 0.11	2751.00 ± 21.36
0.01 mM F-CM_{fb}	1.55 ± 0.17	3161.38 ± 35.71
0.1 mM F-CM_{fb}	1.28 ± 0.14	3035.96 ± 53.30

Table 2-2. Quantification of fluoride in *C. militaris* fruiting body.

Sample	m (x) (g)	n(std) (mol)	A(std)/A(x)	Fluoride (ppm)
0.001 mM F-CM_{fb}	0.13	5.7×10^{-4}	33129.81	15.09
0.01 mM F-CM_{fb}	0.09	5.7×10^{-4}	21350.02	33.81
0.1 mM F-CM_{fb}	0.09	5.7×10^{-4}	13277.53	54.38

Table 2-3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and superoxide dismutase (SOD)-like activity of four fruiting bodies extract.

Sample	IC_{50} value (mg/mL)		SOD activitiy (U/mg)
	DPPH	SOD	
CM_{aq}	4.16	1.13	44.25
0.001 mM F-CM_{aq}	2.92	0.81	61.37
0.01 mM F-CM_{aq}	2.59	0.59	84.75
0.1 mM F-CM_{aq}	2.89	0.87	57.47

Figure 2-1. *C. militaris* growth in normal and fluoride-supplemented medium.

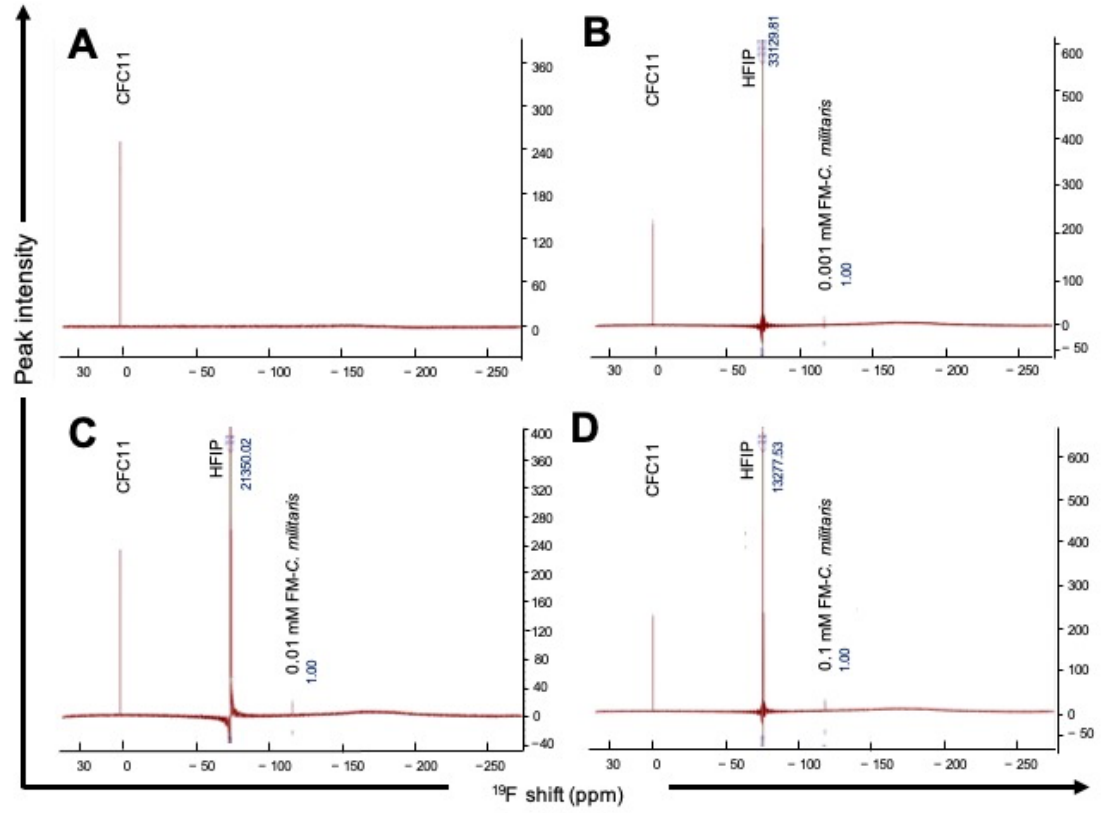

Figure 2-2. ^{19}F NMR spectra of CM_{fb} and F-CM_{fb}. (A) CM_{fb}. (B) 0.001 mM F-CM_{fb}. (C) 0.01 mM F-CM_{fb}. (D) 0.1 mM F-CM_{fb}.

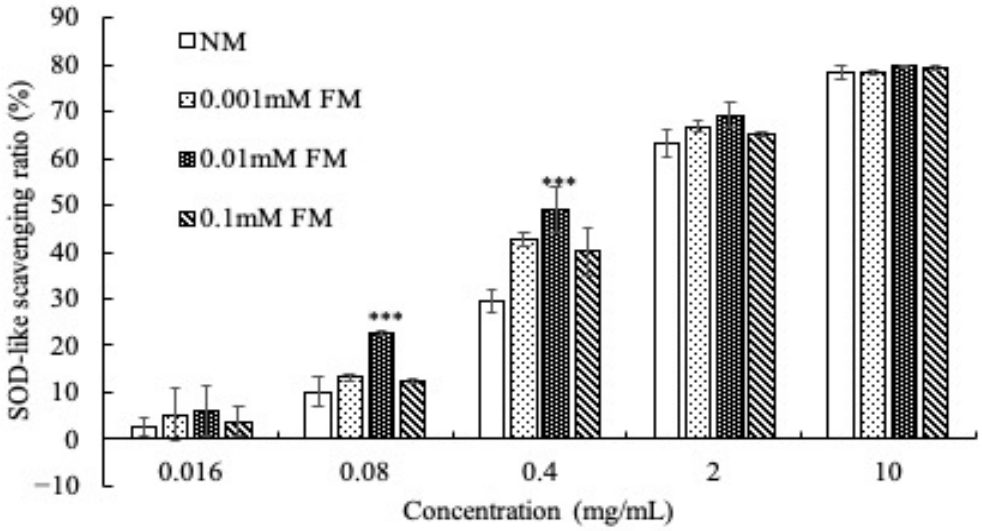

Figure 2-3. Superoxide dismutase-like activity of aqueous extracts of four fruiting bodies at various concentrations. Quantitation of results shown on the right (mean ± SD, n = 3), *** $p <$ 0.001 (Student's t-test).

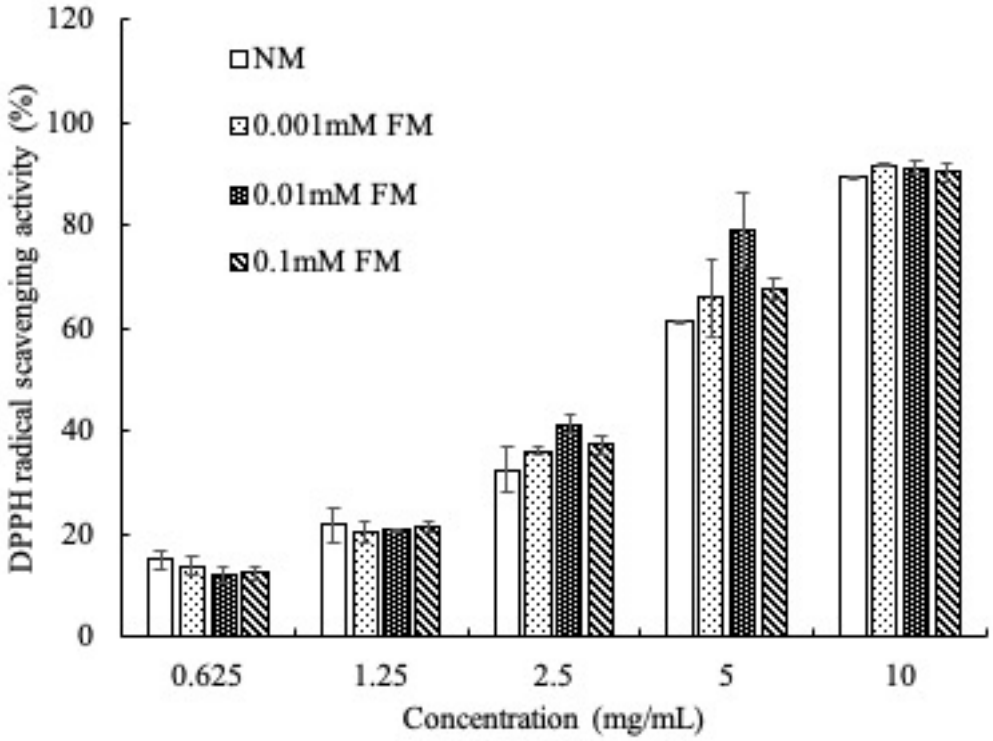

Figure 2-4. 2,2-diphenyl-1-picrylhydrazyl radical scavenging activities of aqueous extracts of four fruiting bodies at various concentrations. Quantitation of results shown on the right (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test).

Chapter 3 Comparative study on anticancer activities of aqueous extract of *C. militaris* fruiting body raised from normal and fluoride-supplemented media

3.1. Introduction

Many studies have demonstrated that *C. militaris* extract possessed excellent potential of anti-proliferation activities to cancer cell due to its active substances such as polysaccharides and cordycepin (Yue et al., 2008; Nakamura et al., 2015; Park et al., 2017). We concluded that *C. militaris* extract basically inhibit cancer cell through anti-proliferation enhancement (Rao et al., 2010), more cell apoptosis induction (Lee et al., 2015), and reduction of migration activities (Jo et al., 2020). *C. militaris* extract induced apoptosis in A549 human non-small cell lung cancer cell (Park et al., 2009) as well as anti-proliferation in U2OS human osteosarcoma (Li et al., 2021) has been reported. In chapter 2, a comparison study of total carotenoids content and antioxidant activities among *C. militaris* obtained from normal and various concentrations of fluorine-supplemented media has been conducted. The results demonstrated that 0.01mM F-CM_{aq} possessed strongest antioxidant activities as well as highest content of active substances. To evaluate if F-CM_{aq} possessed better anticancer abilities, comparative experiments on anticancer activities to A549 and U2OS cells of *C. militaris* extract from CM_{aq} and F-CM_{aq} were investigated.

In this chapter, CM_{aq} and 0.01 mM F-CM_{aq} (recorded as F-CM_{aq}) were selected for further investigations. The half maximal inhibitory concentration (IC_{50}) of CM_{aq} to three cancer cell lines (including A549 cell, U2OS cell, and HT1080 cell) were compared for verification sensitivity of cancer cells. To determine the cytotoxicity to normal cell, we also detected effect of CM_{aq} and F-CM_{aq} to TIG3 human normal lung fibroblasts cell line.

We found that F-CM_{aq} reduced colony formation viability, induced more suppression of migration activities compared to CM_{aq} treatment in A549 cell and U2OS cell, indicated its potential ability to apoptosis induction. Apoptosis assay results demonstrated that F-CM_{aq} induced more apoptosis activities in A549 cell (15.20%) and U2OS cell (41.86%) compared to CM_{aq} (10.26% in A549 cell, and 24.36% in U2OS cell). Cell apoptosis was an important process in development procedures, maintenance of physiological processes, and removal of damaged cells. Expression of key proteins results revealed the molecular mechanism for regulation of apoptosis via caspase, PARP, Bcl-2, and Bax pathways. In addition, F-CM_{aq} induced 78.70% of A549 cell cycle arrest in G1 phase, which is more than CM_{aq} treatment of 64.34%. However, in U2OS cell, F-CM_{aq} induced more cell cycle arrest in G2/M phase (31.25%) compared to CM_{aq} (21.54%). Western blots results demonstrated that F-CM_{aq} increased cell cycle arrest in G1 phase via downregulated expression of CDK4 and p53 pathways in A549 cell. For U2OS cell, F-CM_{aq} induced G2/M phase cell cycle arrest increasing was through protein Cyclin D1, Cyclin B1, CDK4, pRb, and p53 pathways.

3.2. Materials and methods

3.2.1. Chemicals and reagents

Dulbecco's Modified Eagle Medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS) and penicillin-streptomycin solution were purchased from Thermo Fisher Scientific K.K. (Tokyo, Japan).

3.2.2. Cell lines

Human fibro sarcoma cell (HT1080), human non-small cell lung cancer cell (A549), human osteosarcoma (U2OS) and normal lung fibroblast (TIG-3) were purchased from the National Institute of Physical and Chemical Research (RIKEN, Japan) and the Japanese Collection of Research Bioresources, respectively. Cells were cultured in Dulbecco's Modified Eagle Medium containing 10% FBS supplemented with 1% penicillin/streptomycin at 37 °C and 5% CO_2. Typically, cells were plated and incubated for 24 h to reach a stable adherence status and were then used for experiments.

3.2.3. MTT assay

TIG-3, A549, HT1080, and U2OS cells were seeded in 96-well plates at a concentration of 5000 cells/well and incubated at 37 °C under 5% CO_2. After 24 h of incubation, the cells were cultured in varying concentrations of extracts CM_{aq} for 24 h. MTT solution was then added into each well of a 96-well plate, and the samples were incubated for 4 h at 37 °C. After incubation, the medium was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to each well. The optical density (OD) of the wells was measured at 570 nm using a microplate reader (Infinite M200 PRO, TECAN, Switzerland).

3.2.4. Colony formation assay

U2OS cells and A549 cells (500 cells/well) were seeded in 6-well plates and allowed to adhere to substratum for overnight followed by 6 hours treatment with 0.75 mg/mL of CM_{aq} and F-CM_{aq}. The cells were then cultured in normal medium with a regular change of medium every two days until colonies were formed. Colonies were washed three times with cold PBS and fixed with methanol/acetone (1:1, v/v) at 4 ºC for 10 minutes. Fixed colonies were again washed thrice with cold PBS, stained with 0.1% crystal violet solution (Wako, Osaka, Japan) overnight, remove the stain, washed with water, and left open for air drying. The plates were then subjected to photography and colony counting.

3.2.5. Wound healing assay

Cancer cells (2.0×10^5 cells/well) were seeded in 6-well plates and allowed to adhere overnight. A wound was created by scratching the monolayer with the tip of the sterile 100 µL pipette and then cultured with CM_{aq} and F-CM_{aq} at concentration of 0.25 mg/mL for U2OS

cells, while 0.35 mg/mL for A549 cells. Cell migration to the wound area was recorded at 0 h, 24 h, and 48 h using a digital camera.

3.2.6. Apoptosis assay

Cell apoptosis was determined by Annexin-V and 7-aminoactinomtcin (7-ADD) double staining. Cancer cells (2.0×10^5 cells/well) were seeded in 6-well plate and incubated for 24 h and then treated by CM_{aq} and F-CM_{aq} (0.25 mg/mL) for 24 h. Cells were then collected and washed by PBS solutionand stained with 100 µL of Guava Nexin Reagent (Millipore) and incubate 40 minutes at ambient temperature (25 ºC) in dark. Apoptotic cells were evaluated by a Flow Cytometer (Guava PCA-96, ALT, East Lyme, Connecticut, USA).

3.2.7. Cell cycle assay

Cancer cells (2.0×10^5 cells/well) were seeded in 6-well plate and incubated for 24 h and then treated by CM_{aq} and F-CM_{aq} at 0.25 mg/mL for another 24 h. Cells were harvested and suspended in 70% ethanol for 24h incubation at -20 ºC. Treated cells were then collected by centrifugation at 500 G for 5 min at 4 ºC. Ethanol was removed followed by washing with PBS and centrifugation to collect the cell pellet that was re-suspended in 1 mL cold PBS (containing Ribonuclease of 100 µg/mL). Pellets were incubated for 2 h at 37 ºC followed by centrifugation and addition of 200 µL Guava Cell Cycle Reagent to the pellet. The number of cells in different stage of cell cycles was quantified by Flow Cytometry (Guava PCA-96, ALT, East Lyme, Connecticut, USA).

3.2.8. Western blotting assay

Cancer cells (2.0×10^5 cells/well) were seeded in 6-well plate and incubated for 24 h and then treated by CM_{aq} and F-CM_{aq} (0.25 mg/mL for U2OS cells, 24 h, 0.35 mg/mL for A549 cells, 24h). Cells were collected and then lysed in RIPA buffer (radio immunoprecipitation assay buffer; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Cell lysates with 20 µg of proteins was decomposed in SDS polyacrylamide gel and then transferred to the polyvinylidene fluoride (PVD) membrane by semi-dry transfer equipment (ATTO Corporation, Tokyo, Japan) for 75 min. The membrane was blocked with 3% bovine serum albumin (BSA) for 1 h and then treated with primary antibody at 4 ºC overnight incubation. Membrane was washed with Triton X-100 Tris-buffered saline (TTBS) three times (10 min each) and were finally treated with secondary antibodies for 1 h at room temperature (25 ºC). The membranes were then washed with TTBS and detected with ECL kit (Thermo Scientific™, Waltham, MA, USA). Their quantification of relative expression for each protein was determined through Image J 1.53 Software.

3.2.9. Immunofluorescence assay

U2OS and A549 cells (1.0×10^5 cells/well) were seeded on glass coverslips in 12-well

plate and treated with 0.25 mg/mL of CM_{aq} and F-CM_{aq} for 24 h. Cells were washed 3 times with cold PBS and fixed with methanol/acetone (1/1, v/v) at 4 ºC for 10 min and then again washed thrice with cold PBS. Coverslips containing cells were incubated with Alexa Fluor-conjugated secondary antibodies (Thermos Fisher Scientific). The nuclear was stained with 1 μg/mL Hoechst 33342 dyeing for 10 min in dark. The cells were observed under fluorescent microscope. The quantification of relative expression was determined through Image J 1.53 Software.

3.2.10. Statistical analysis

All experiments were performed in triplicate, and all results were expressed as mean ± standard deviation (SD). The degrees of statistical significance among the control and sample groups were analyzed using an unpaired t-test (GraphPad Prism 6 Software, San Diego, CA, USA). Significant values are represented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.3. Results and discussion

3.3.1. CM_{aq} exhibited stronger cytotoxicity to U2OS cells and A549 cells

Although *C. militaris* extract has been reported with anti-cancer ability to U2OS and A549 cells in previous studies, to determine if the sensitivity of cytotoxicity induced by *C. militaris* was different in multiple cancer cells, we selected U2OS, A549 and HT1080 three cancer cell lines for MTT assay. IC_{50} values were showed in Figure 3-1. As we expected, U2OS cell and A549 cell were more sensitive to CM_{aq} (IC_{50} was 0.64 mg/mL for U2OS cells, and 0.75 mg/mL for A549 cells) compared with HT1080 cells (IC_{50} = 0.94 mg/mL). We further investgated the cytotoxicity of CM_{aq} and F-CM_{aq} to TIG-3 normal cells, Figure 3-2 showed that cell viability of TIG-3 was maintained at high level from 0 to 1 mg/mL, which indicated that no increased cytotoxicity to human normal cell was found from CM_{aq} or F-CM_{aq} in this dose range. Then we checked difference of cytotoxicity between CM_{aq} and F-CM_{aq} to U2OS cell and A549 cells, respectively. In Figure 3-3, U2OS cells (Figure 3-3A) and A549 cells (Figure 3-3B) showed a dose-dependent inhibition of cell viability to *C. militaris* extract. In addition, F-CM_{aq} treatment exhibited a higher resistance to cancer cell proliferation activities in both U2OS and A549 cell lines compared to CM_{aq}.

Besides the short-term effects determined by MTT assay, long-term cytotoxicity effect of CM_{aq} and F-CM_{aq} was evaluated according to the colony formation assay. As shown in Figure 3-5A, CM_{aq} and F-CM_{aq} treated group both showed a greater reduction in A549 cells colony-forming efficiency which was same as previous study described (Luo et al., 2019) that *C. militaris* inhibits cell proliferation. A similar result also observed in U2OS cells group (Figure 3-4A). Quantification results for U2OS cell colony formation assay was showed in Figure 3-4B, colonies number of F-CM_{aq} (41.87%) treatment was obviously less than CM_{aq} (68.22%). For A549 cell group, colonies number of F-CM_{aq} was 29.72%, significantly less than CM_{aq} of 43.37%.

Take all these results, we may demonstrate that cytotoxicity to U2OS and A549 cancer cells

were both enhanced in F-CM_{aq} treatment as well as negligible cytotoxicity on TIG-3 human normal cell.

3.3.2. Comparison on anti-migration activities in cancer cells

Next, we conducted a wound healing assay to estimate the inhibitory effect of CM_{aq} and F-CM_{aq} on migratory abilities of U2OS and A549 cell, respectively. As shown in Figure 3-6A, and Figure 3-7A, scratched gaps were recorded at 0 h, and the distance of gaps were reduced gradually after either 24 or 48 h of incubation in the control group while other two treatments retarded down the speed of narrowing process, especially in F-CM_{aq} treatment. This demonstrated that CM_{aq} exhibited anti-migration activities in U2OS and A549 cells.

The quantification of open area ratio of U2OS and A549 cells was showed in Figure 3-6B, and Figure 3-7B, respectively. In U2OS cells, we noticed that recovery of open ratio in F-CM_{aq} treatment (13.38% at 24 h and 11.63% at 48h) was wider than CM_{aq} treatment (11.6% at 24 h and 9.8% at 48 h). In A549 cell group, F-CM_{aq} inhibits more wound healing of 17.47% at 24h, and 16.64% at 48 h, compared to CM_{aq} treatment of 16.20% at 24 h, and 13.95% at 48 h, respectively. Which suggesting that F-CM_{aq} performed a better resistance to U2OS and A549 cell migration activities.

To test this further, we next estimated the expression level of key metastasis-regulatory proteins and found a significant decrease in MMP-2, MMP-9, and Vimentin in CM_{aq} and F-CM_{aq} treated cancer cells (Figure 3-6C and Figure 3-7C). The downregulation in level of these proteins reveled the molecular mechanism of migrative inhibition. In U2OS cell (Figure 3-6D), F-CM_{aq} treated cells showed a greater decrease in the expression of MMP-2 (11.21%), MMP-9 (15.27%), and Vimentin (49.2%) compared to CM_{aq} which the relative expression level was 28.00% (MMP-2), 30.36% (MMP-9) and 71.32% (Vimentin), respectively. Which means these anti-migration protein pathways were inhibited more in F-CM_{aq} treatment. However, in A549 cells, F-CM_{aq} remarkably reduced the expression of MMP-9 and MMP-2, no significant difference found in Vimentin. Which means the molecular mechanism of anti-migration in A549 cell induced by F-CM_{aq} was via MMP-2 and MMP-9 pathways. All these results demonstrated that F-CM_{aq} enhanced resistance to migration in U2OS and A549 cells compared to CM_{aq}.

3.3.3. F-CM_{aq} induced stronger apoptosis in cancer cells

Apoptosis is a resolutely regulated procedure discerned by cell shrinkage, plasm membrane fall off, and chromatin condensation (Ghobrial, Witzig, and Adjei 2005). To further investigate the cytotoxic effects of CM_{aq} and F-CM_{aq} in cancer cells, we checked the difference between two extracts induced apoptosis progress in U2OS cells, and A549 cells, respectively. For apoptotic cells analysis, we employed Annexin V-/7-ADD-, Annexin V+/7-ADD+, Annexin V+/7-ADD+ represent healthy, early apoptotic, late apoptotic and debris cells, respectively. Annexin V-positive (early and late apoptosis) were considered as the apoptotic cells. After U2OS cells and A549 cells were treated with CM_{aq} and F-CM_{aq} (0.25 mg/mL for U2OS cells,

and 0.50 mg/mL for A549 cells) for 24 h, respectively. They were then collected as previous mentioned for staining. Later, the apoptotic cells were analyzed by flow cytometry immediately.

Figure 3-8A showed the images of apoptotic U2OS cells treated with normal medium, CM_{aq} and F-CM_{aq}, respectively. We could tell that U2OS cells stay in normal status clearly in control treatment. For those white spots, they were shrinking and debris cells, we could easily observe that F-CM_{aq} treatment induces more apoptosis compared to CM_{aq} treatment. Similar phenomenon also observed in A549 cells group (Figure 3-9A).

Figure 3-8B exhibited that apoptotic U2OS cells were concentrated in early apoptosis stage. According to quantification results we suggested that F-CM_{aq} treatment induced more apoptosis (41.86%) in U2OS cells compared to CM_{aq} treatment (24.36%). To determine the molecular mechanism of apoptosis, we analyzed the expression of proteins involved in apoptosis. These included polyADP-ribose polymerase-1 (PARP-1), Caspase 3, B-cell lymphoma 2 (Bcl2) and Bax which were closely related to the apoptotic signaling pathway. Western blots analysis revealed a significant downregulation of anti-apoptotic factors including PARP-1 and Bcl2 (Figure 3-8C). While the expression level of pro-apoptosis marker Bax was upregulated to a large extent. Caspase family could accelerate apoptosis and there was a significant downregulation of pro-caspase 3 indicating the increasing of cleaved-caspase 3 which means the acceleration process was activated. The quantification results (Figure 3-8D) showed that F-CM_{aq} enhanced inhibition to the anti-apoptotic proteins including PARP-1 (39.05%) and Bcl2 (46.23%) compared to CM_{aq} (64.06% in PARP-1 and 60.82% in Bcl2). In addition, relative expression of pro-caspase 3 treated with F-CM_{aq} (47.85%) was lower than CM_{aq} (83.31%) indicated that activities of caspase family were enhanced in F-CM_{aq}. Furthermore, relative expression of pro-apoptotic maker Bax was significantly promoted in F-CM_{aq} (163.77%) compared to CM_{aq} (132.65%). These results supported that stronger apoptosis of U2OS cells in F-CM_{aq} is induced by activation of Bax, caspase 3, and inhibition of PARP-1 and Bcl2.

On the other hand, in A549 cell group, we found that apoptosis was mainly concentrated in late apoptosis stage (Figure 3-9B). F-CM_{aq} induced 15.20% of apoptosis, more than CM_{aq} of 10.26%. According to western blots results (Figure 3-9C and Figure 3-9D), F-CM_{aq} treatment significant promoted the expression of caspase 3, as well as inhibited level of PARP-1 and Bcl2 compared to CM_{aq} treatment. However, the expression of Bax in CM_{aq} and F-CM_{aq} treatment almost remained at same level. We speculated this might be relating to induction of late apoptosis stage but still need more evidence.

3.3.4. F-CM_{aq} enhanced cell cycle arrest in G2/M phase in U2OS cells and G1 phase in A549 cells

Flow cytometric assay was applied to evaluate the influences of CM_{aq} and F-CM_{aq} on cell cycle distribution in U2OS cell, and A549 cells, respectively. For U2OS cell, Figure 3-10A showed that subpopulation in G2/M phase was increased from 14.72% (Control) to 21.54% in CM_{aq} and 31.25% in F-CM_{aq}, respectively. This result suggested the potential enhancement of F-CM_{aq} to cause growth arrest of U2OS cells. To further investigate the molecular mechanism of F-CM_{aq} induced growth arrest, we analyzed the expression of various protein (p53, cyclin

D1, cyclin B1, CDK4 and pRb) involved in cell cycle progression. p53, a major tumor suppressor protein was stronger up-regulated in F-CM_{aq} (Figure 3-10B). The activated cyclin/CDK complexes phosphorylate and inactivate member of the retinoblastoma (Rb) protein family induced cell cycle progress. With the upregulation of p53, there was a significant decrease in the expression of cyclin D1, cyclin B1, CDK4, and pRb. Moreover, quantification of relative expression (Fig. 3-10 C) reveled that downregulation in F-CM_{aq} treatment of cyclin D1 (18.32%), cyclin B1 (21.69%), CDK4 (64.86%), and pRb (12.39%) were all significantly lower than CM_{aq} treatment which were: 56.56% of cyclin D1, 51.22% of cyclin B1, 88.98% of CDK4 and 38.77% of pRb. These results supported that the stronger growth arrest in F-CM_{aq} treated cell is mediated be enhanced activation of p53 and inhibition of pRb signaling pathways.

For A549 cells, Figure 3-11A showed subpopulation in G1 phase was increased from 55.32% (Control) to 64.34% in CM_{aq} and 78.70% in F-CM_{aq} treatment, respectively. Figure 3-11B and Figure 3-11C reveled the molecular mechanism of F-CM_{aq} induced growth arrest, the downregulation in F-CM_{aq} treatment (Figure 3-11D) of CDK4 (47.07%) was significantly lower than CM_{aq} (62.37%), while p53 expression level is enhanced. No significant difference was found in cyclin B1 and cyclin D1 expression in two extracts treated A549 cells.

We also used immunofluorescence staining method for further confirmation. The fluorescence intensity of antibody cyclin B1 (Figure 3-12) and CDK4 (Figure 3-13) in U2OS cells treated with F-CM_{aq} was significantly inhibited compared with CM_{aq} treatment. In A549 cells, we checked expression level of cleaved-caspse-3 (Figure 3-14) under CM_{aq} and F- CM_{aq} treatment, respectively. Quantitation of results exhibited that cleaved-caspase-3 was up-regulated higher in F-CM_{aq} treatment. All these results were consistent with the results of western blotting, further proved the accuracy of the molecular pathways mechanism.

3.4. Summary

In summary, F-CM_{aq} treatment exhibited significant stronger anti-cancer activities in two cancer cell lines (U2OS and A549 cells) compared to CM_{aq}, including stronger resistance to migration, more apoptosis induction, increased of U2OS cell cycle arrest in G2/M phase, and more A549 cell cycle arrest in G1 phase. *C. militaris* aqueous extracts have been shown to be effective in the treatment of multiple cancer cells *in vitro* through induction of cell cycle arrest and apoptosis in this study. The molecular level comparison on anticancer activities of U2OS cells between CM_{aq} and F-CM_{aq} revealed that F-CM_{aq} induced stronger cell apoptosis to U2OS was possibly caused by increasing cell cycle arrest in G2/M phase through activation of p53 and inhibition of pRb signaling pathways. For A549 cells, a similar trend of increased apoptotic induced by F-CM_{aq} was confirmed. F-CM_{aq} treatment caused more cell cycle arrest in G1 phase, which indicated that *C. militaris* extracts adapted different molecular adjusting mechanism in different cancer cell, but F-CM_{aq} performed still better. According to this comparison study on anti-cancer activities, we could further certificate that F-CM_{aq} obtained a significant promotion on anti-cancer activities.

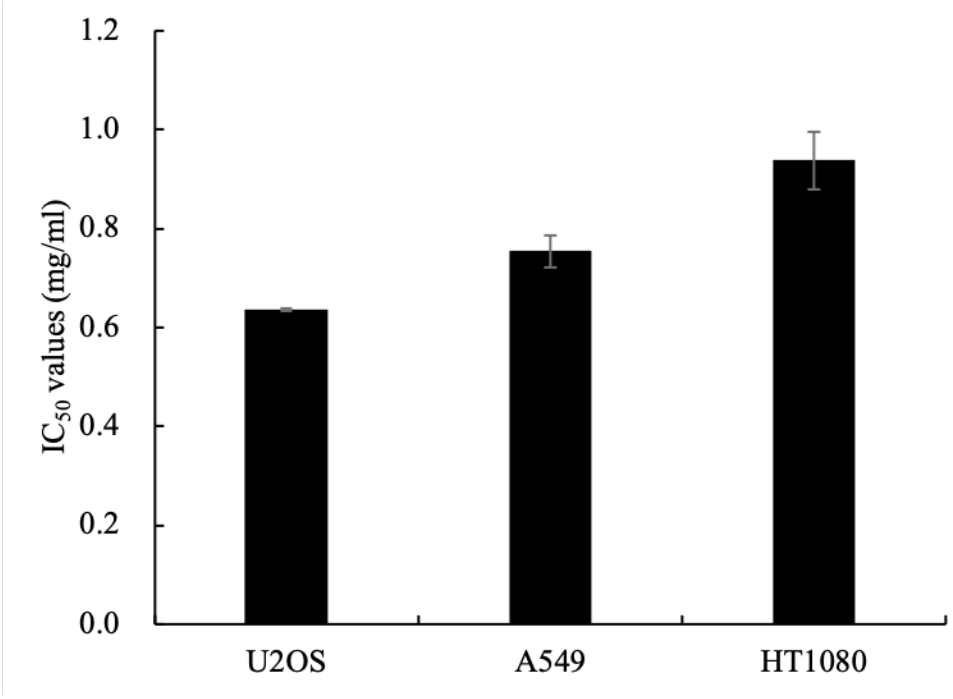

Figure 3-1. IC_{50} of U2OS, A549 and HT1080 cancer cell lines treated by CM_{aq} for 24 h.

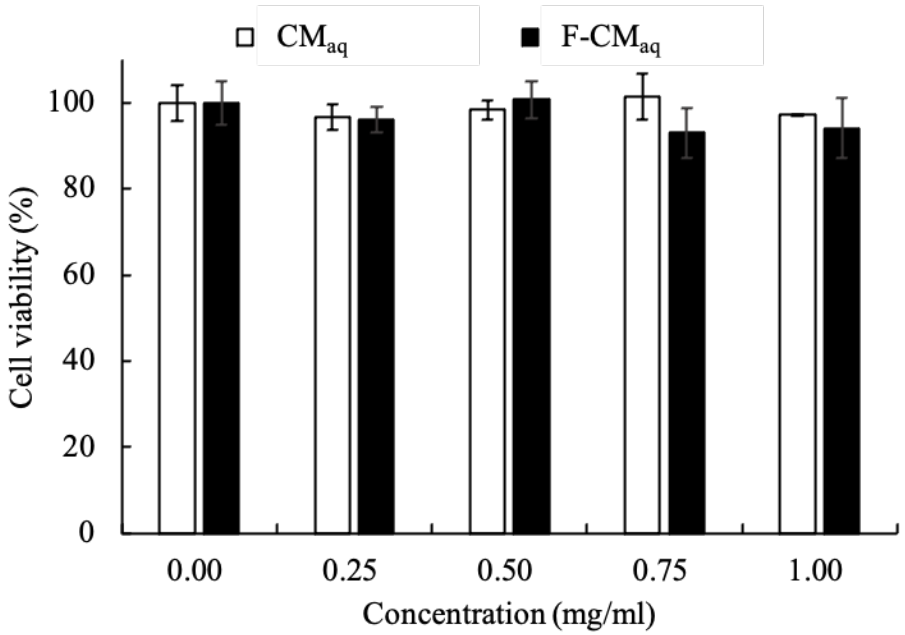

Figure 3-2. MTT assay of TIG-3 normal cell line treated with CM_{aq} and F-CM_{aq} for 24 h.

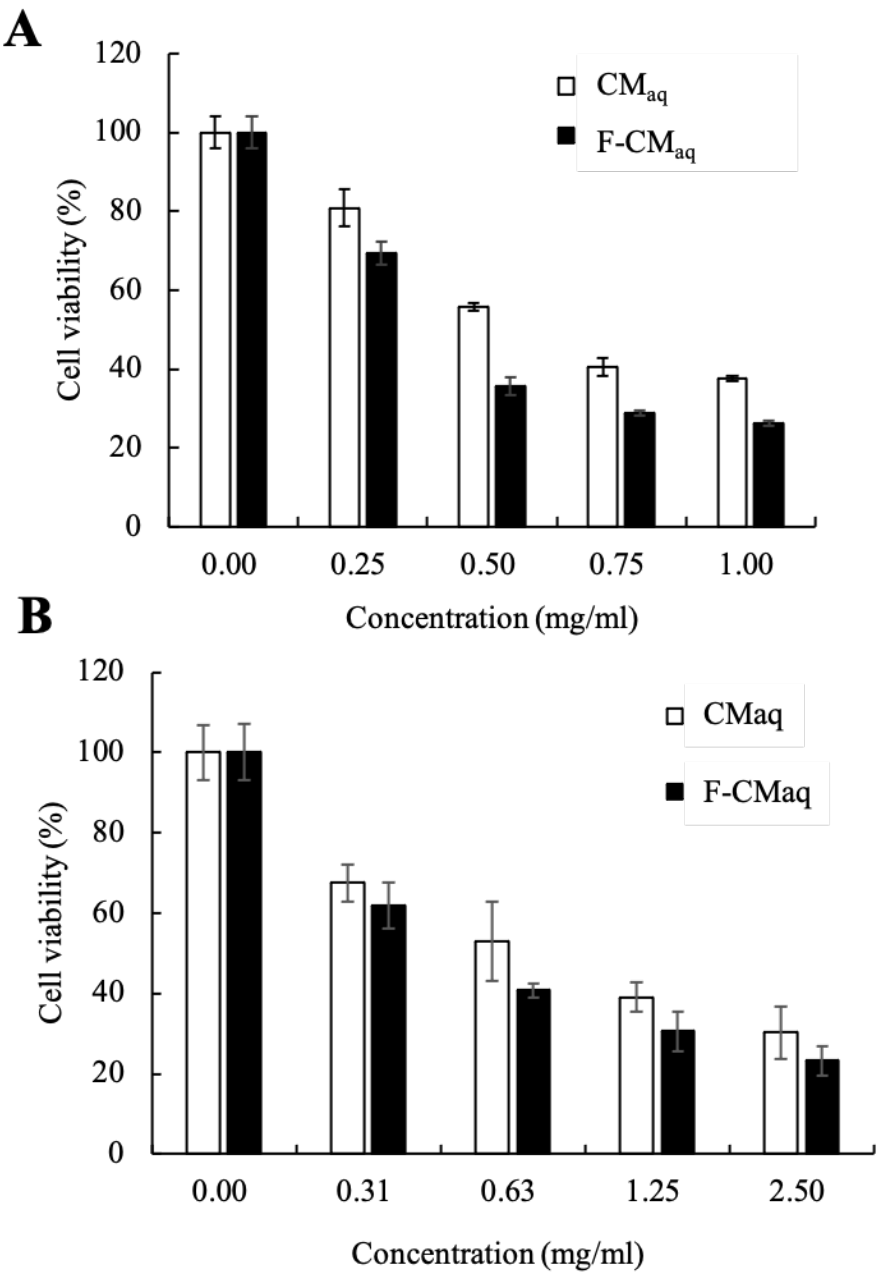

Figure 3-3. MTT assay of cancer cells treated with CM_{aq} and F-CM_{aq} for 24 h. **A,** U2OS cell line. **B,** A549 cell line.

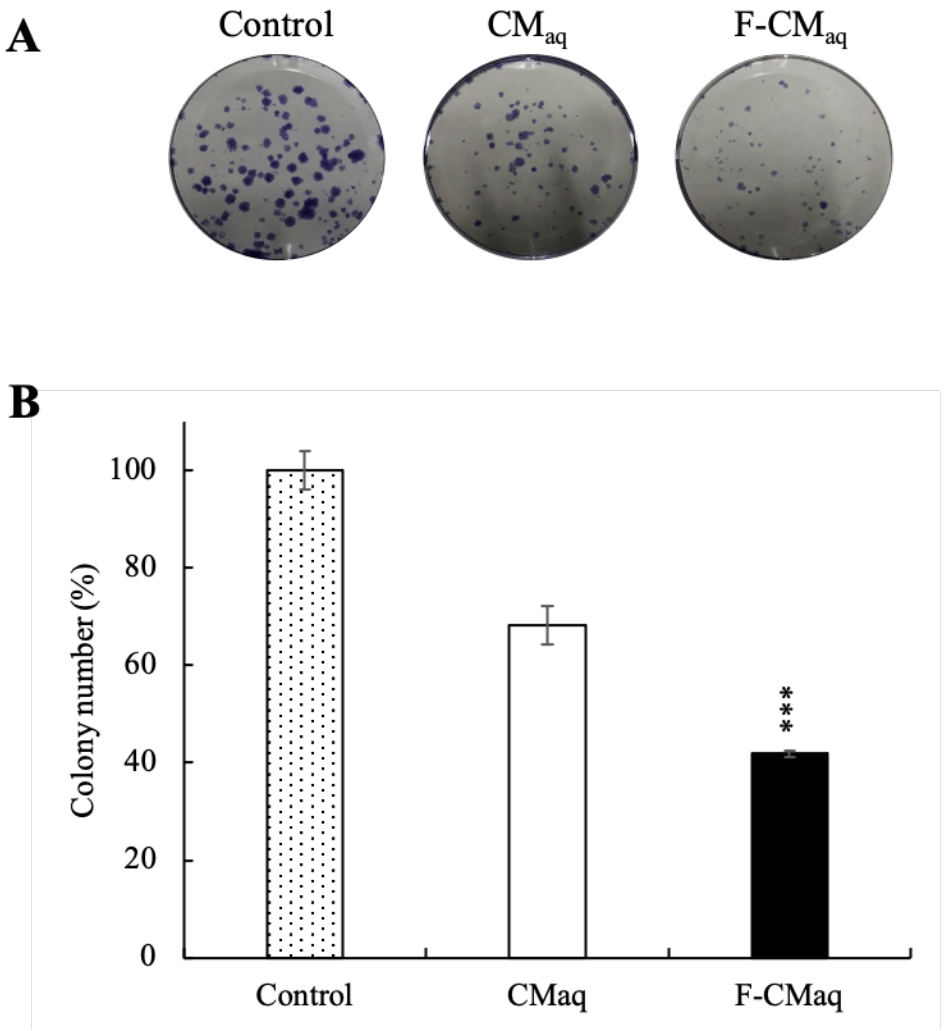

Figure 3-4. Colony assay of U2OS cells treated with CM_{aq} and F-CM_{aq}. **A,** Image of colony for each treatment. **B,** Quantitation of colony number (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs CM_{aq}).

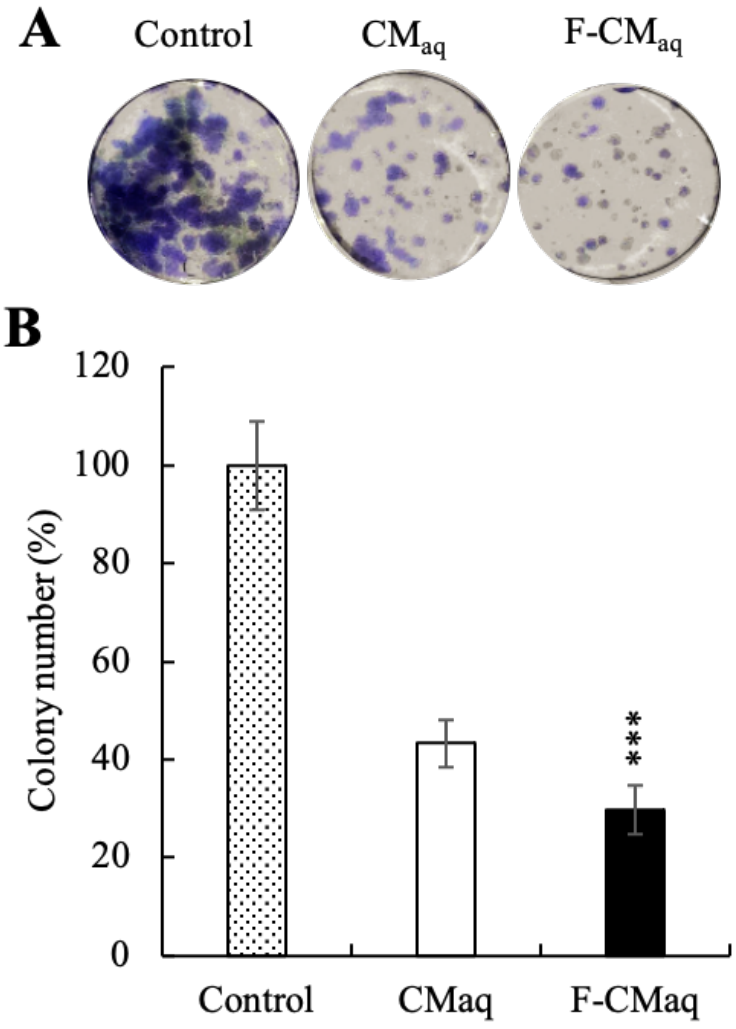

Figure 3-5. Colony assay of A549 cells treated with CM_{aq} and F-CM_{aq}. **A,** Image of colony for each treatment. **B,** Quantitation of colony number (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs CM_{aq}).

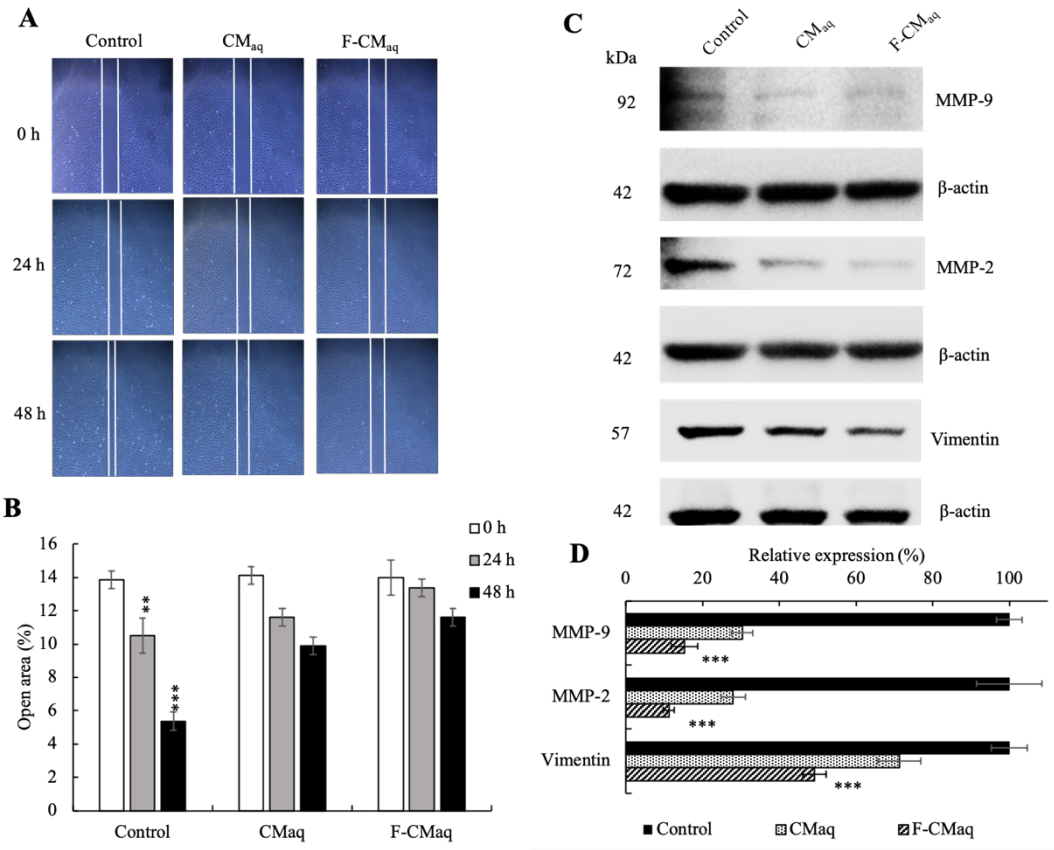

Figure 3-6. F-CM_{aq} inhibits more migration in U2OS cells. **A,** Image of wound recovery under microscope. **B,** Quantitation of open area. **C,** Expression of anti-migration proteins in U2OS cells, detected via western blot analysis. **D,** Protein expression levels were quantified by densitometry analysis and normalized to β-actin (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs CM_{aq}).

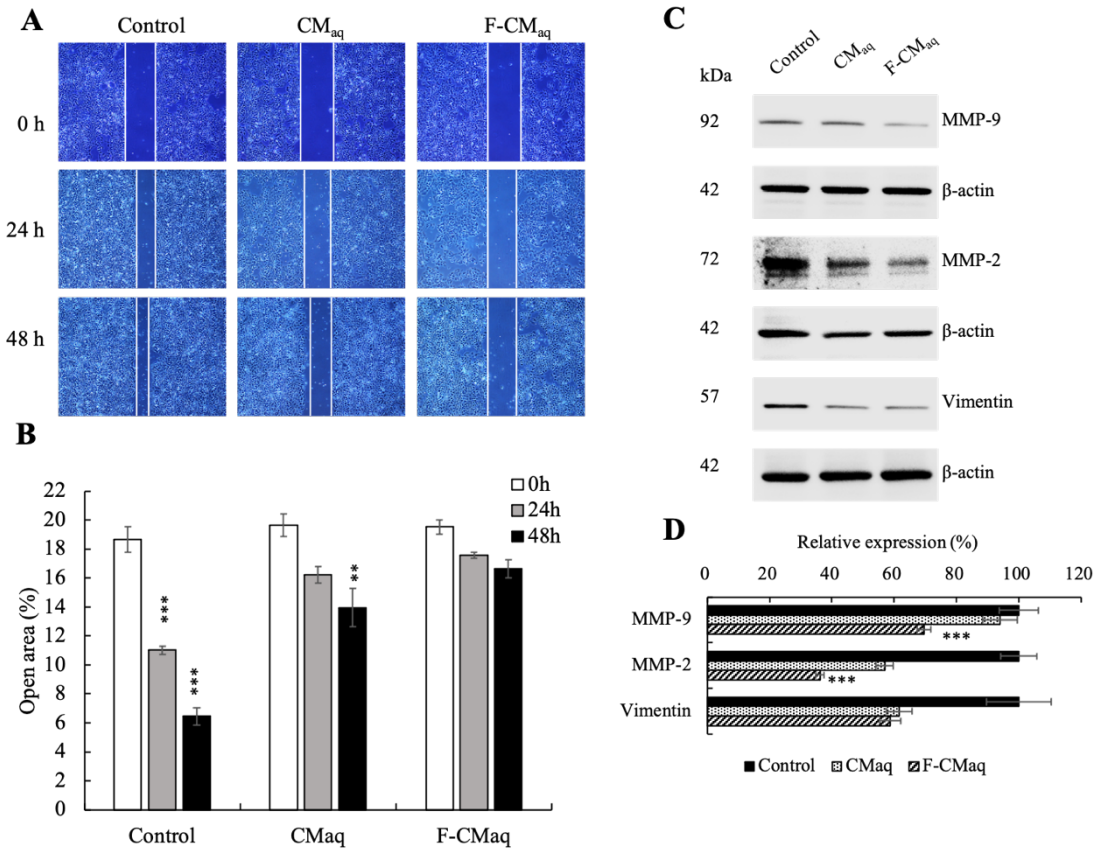

Figure 3-7. F-CM_{aq} inhibits more migration in A549 cells. **A,** Image of wound recovery under microscope. **B,** Quantitation of open area. **C,** Expression of anti-migration proteins in A549 cells, detected via western blot analysis. **D,** Protein expression levels were quantified by densitometry analysis and normalized to β-actin (mean ± SD, n = 3), ** $p < 0.01$, *** $p < 0.001$ (Student's t-test vs CM_{aq}).

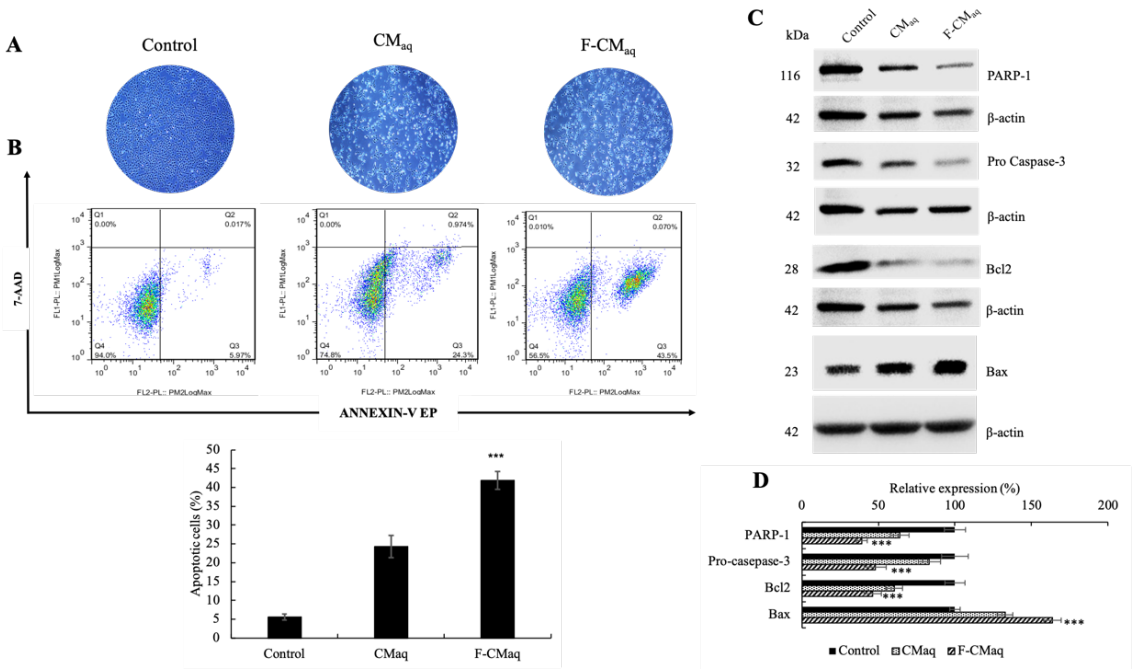

Figure 3-8. Effects of CM_{aq} and F-CM_{aq} on apoptosis progress in U2OS cells. **A,** Image of apoptotic U2OS cells under microscope. **B,** Apoptosis analysis from flow cytometry. Quantitation of the results is shown in below (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. CM_{aq}). **C,** Western blot analysis for apoptotic proteins (PARP-1, Pro-caspase 3, Bcl-2, and Bax) after 24 h. **D,** Protein expression levels were quantified by densitometry analysis and normalized to β-actin (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. CM_{aq}).

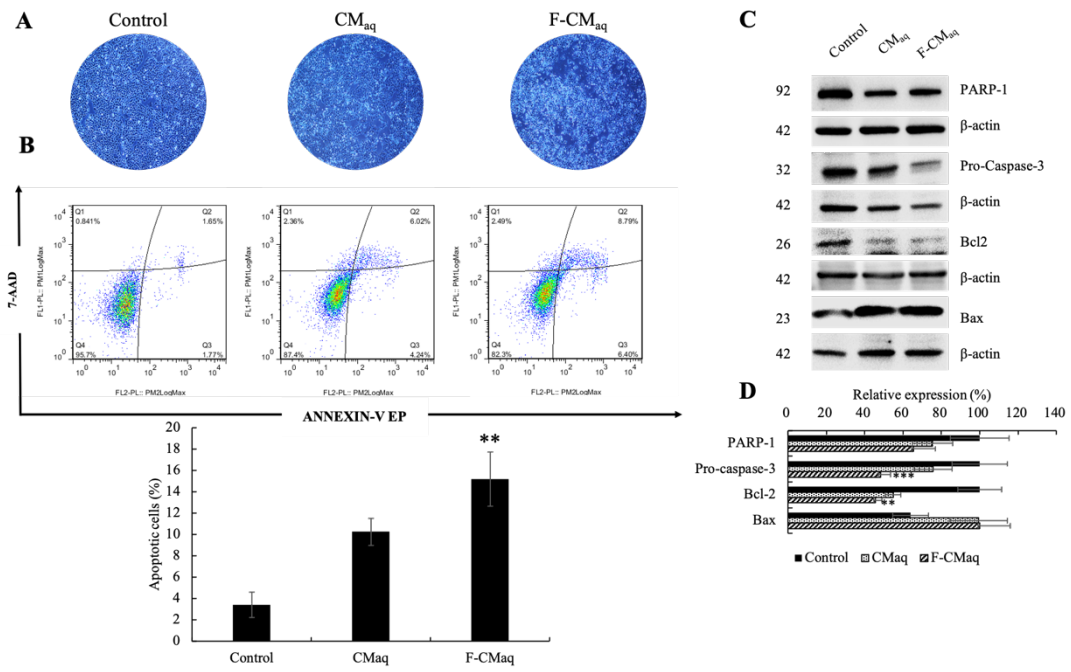

Figure 3-9. Effects of CM_{aq} and F-CM_{aq} on apoptosis progress in A549 cells. **A,** Image of apoptotic A549 cells under microscope. **B,** Apoptosis analysis from flow cytometry. Quantitation of the results is shown in below (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. CM_{aq}). **C,** Western blot analysis for apoptotic proteins (PARP-1, Pro-caspase 3, Bcl-2, and Bax) after 24 h. **D,** Protein expression levels were quantified by densitometry analysis and normalized to β-actin (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. CM_{aq})

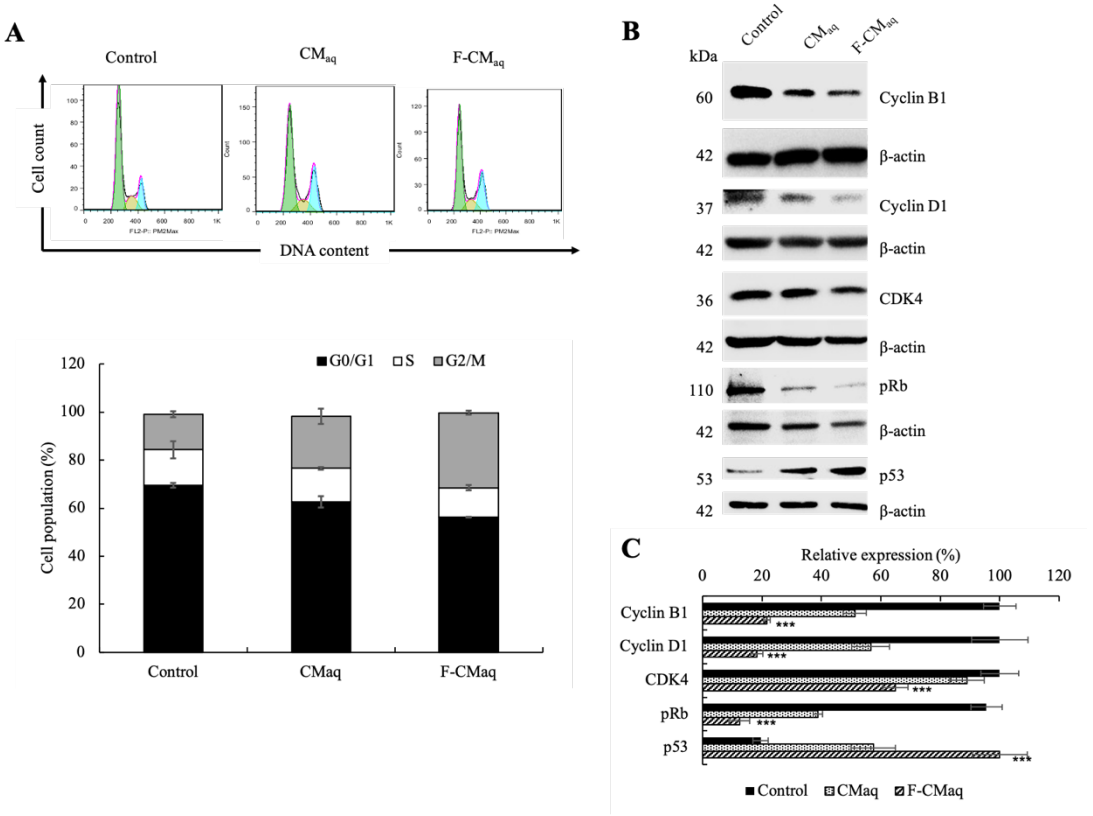

Figure 3-10. Effects of CM_{aq} and F-CM_{aq} on cell cycle arrest in U2OS cells. **A,** Cell cycle analysis from flow cytometry. Quantitation of the results is in below. **B,** Western blot analysis for cell cycle regulatory proteins (cyclin B1, cyclin D1, CDK4, pRb, and p53) after 24 h of incubation. **C,** Protein expression levels were quantified by densitometry analysis and normalized to β-actin (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. CM_{aq}).

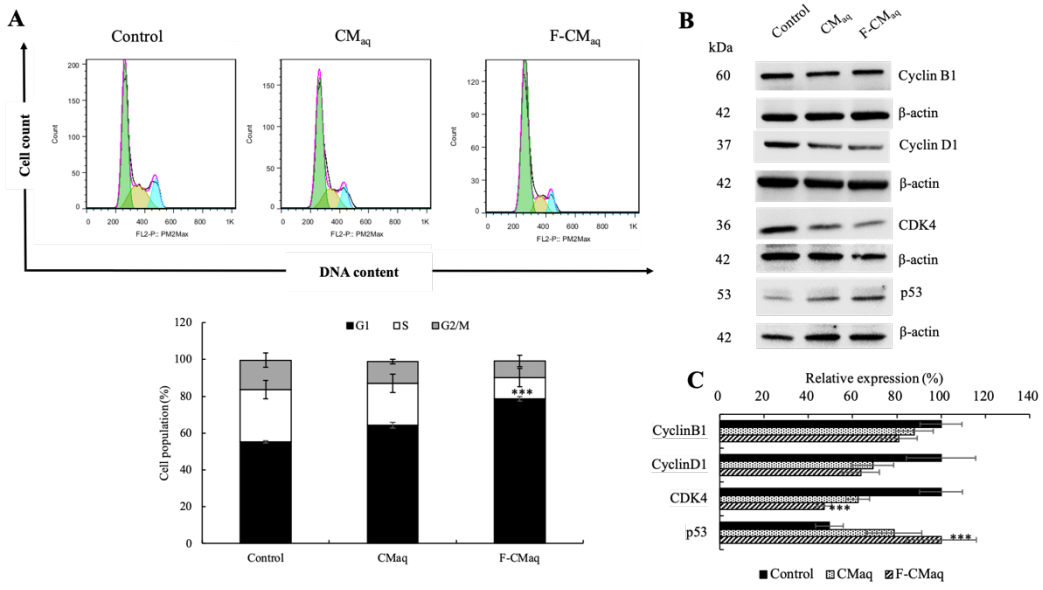

Figure 3-11. Effects of CM_{aq} and F-CM_{aq} on cell cycle arrest in A549 cells. **A,** Cell cycle analysis from flow cytometry. Quantitation of the results is in below. **B,** Western blot analysis for cell cycle regulatory proteins (cyclin B1, cyclin D1, CDK4, and p53) after 24 h of incubation. **C,** Protein expression levels were quantified by densitometry analysis and normalized to β-actin (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. CM_{aq}).

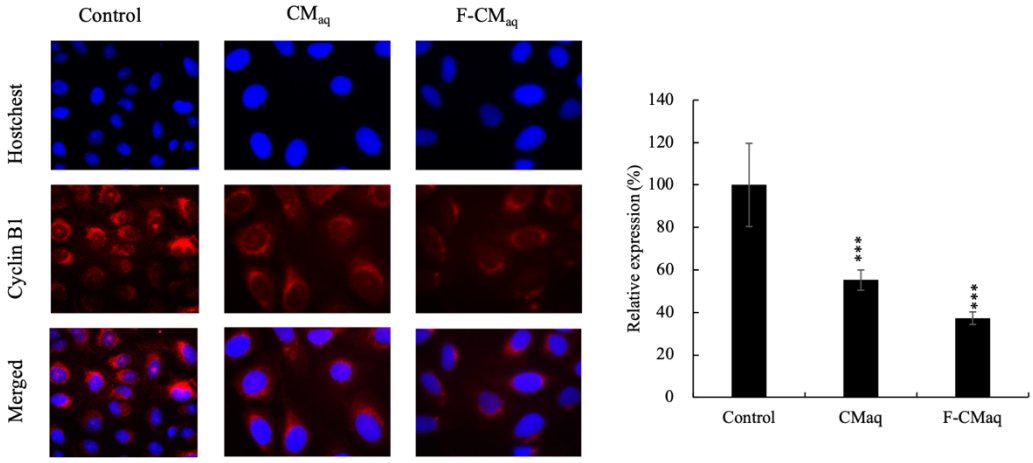

Figure 3-12. Immunofluorescence staining results of cyclinB1 (U2OS cells) treated with CM_{aq} and F-CM_{aq} Hoechst staining showing the location and size of nuclei. Top line, nucleus; Middle line, cyclin B1; Bottom line, merge. Quantitation of the results is shown on the right (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. control).

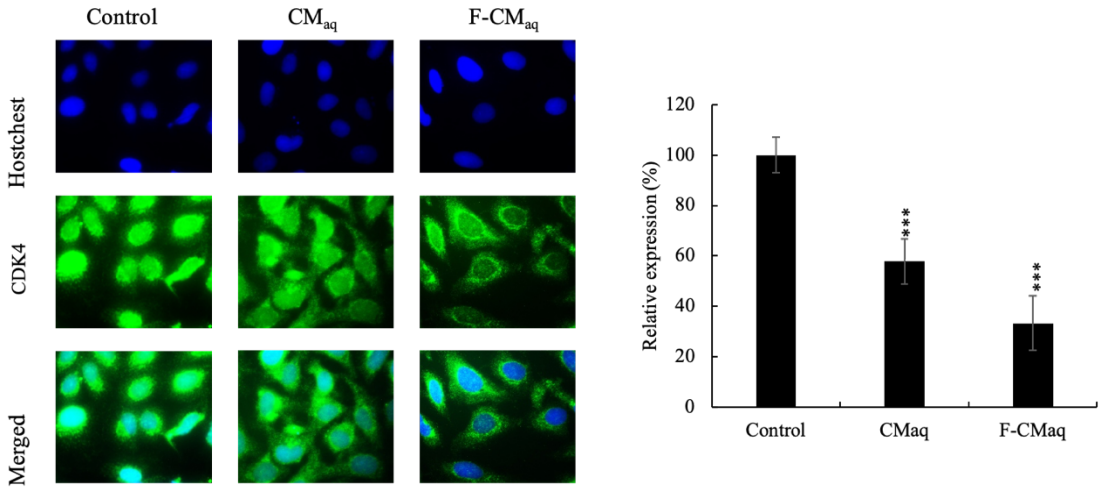

Figure 3-13. Immunofluorescence staining results of CDK4 (U2OS cells) treated with CM_{aq} and F-CM_{aq} Hoechst staining showing the location and size of nuclei. Top line, nucleus; Middle line, CDK4; Bottom line, merge. Quantitation of the results is shown on the right (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. control).

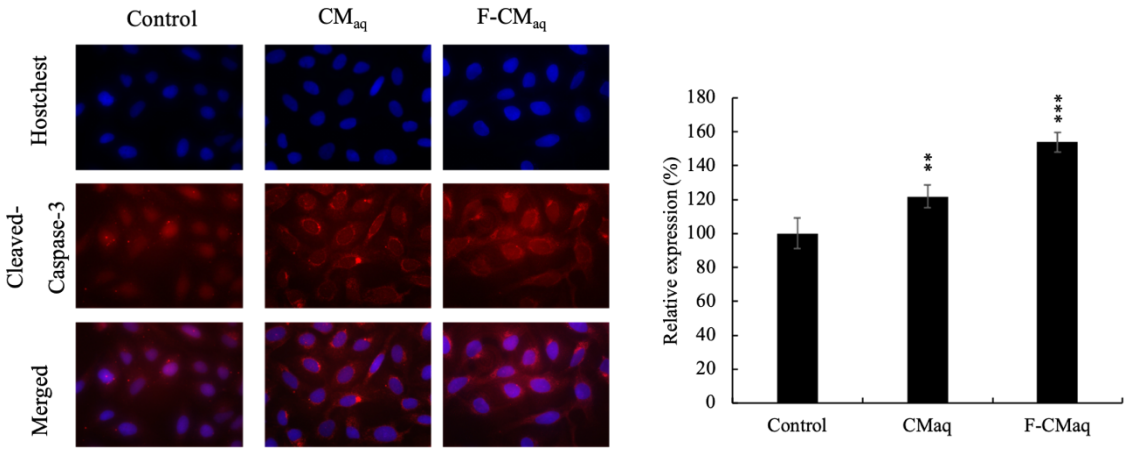

Figure 3-14. Immunofluorescence staining results of cleaved-caspase-3 (A549 cells) treated with CM_{aq} and F-CM_{aq}. Hoechst staining showing the location and size of nuclei. Top line, nucleus; Middle line, Cleaved-Caspase-3; Bottom line, merge. Quantitation of the results is shown on the right (mean ± SD, n = 3), *** $p < 0.001$ (Student's t-test vs. control).

Chapter 4 Conclusions and future research

4.1. Conclusions

As one of most famous Chinese traditional food and medicine material, *C. militaris* attracted more and more attention due to its excellent performance on pharmacodynamics. More details about the functional ingredients in *C. militaris* and their mechanisms have been reveled with the advanced research. However, due to the lack of natural *C. militaris* production and long-term of artificial culture period, the demand of market still cannot be satisfied. It is necessary to keep explore new method and technology on promoting *C. militaris* utilization. This study is the first attempt to use low dose of fluoride on stimulation of *C. militaris* production, which was originated from the hermetic response on plants and algae. According to the results, it has been proven that the stimulation effect of fluoride could be utilized on *C. militaris* fruiting bodies promotion, with active substance accumulation being enhanced, resulting in a significant improvement in anti-cancer activities. Main results can be summarized as follows.

(1) Hormesis effect of fluoride on *C. militaris* enhanced the production of fruiting bodies followed a hormetic dose response manner, the dry weight of CM_{fb}, 0.001mM F-CM_{fb}, 0.01mM F-CM_{fb}, and 0.1mM F-CM_{fb} was 1.07 ± 0.07 g/bottle, 1.16 ± 0.11 g/bottle, 1.55 ± 0.17 g/bottle, and 1.28 ± 0.14 g/bottle, respectively. TCC was 2561.27 ± 26.56 μg/g, 2751.00 ± 21.36 μg/g, 3161.38 ± 35.71 μg/g and 3035.96 ± 53.30 μg/g, respectively. 0.01mM F-CM_{fb} exhibited the best promotion. *C. militaris* growth was inhibited and stay at mycelia stage in 1mM content media.

(2) Fluoride was only detected out of fruiting bodies obtained from KF-supplemented media, the fluoride absorb efficiency of *C. militaris* was decreased with the increasing content of fluoride, quantification of total fluoride content in 0.001mM F-CM_{fb}, 0.01mM F-CM_{fb}, and 0.1mM F-CM_{fb} was 15.09 ppm, 33.81 ppm, and 54.38 ppm. Fluoride existing status was defined as inorganic ion.

(3) 0.01mM F-CM_{aq} exhibited a stronger antioxidant activity, IC_{50} of DPPH radical scavenging of CM_{aq}, 0.001mM F-CM_{aq}, 0.01mM F-CM_{aq} 0.1mM F-CM_{aq} was 4.16 mg/mL, 2.92 mg/mL, 2.59 mg/mL, and 2.89 mg/mL, respectively. For SOD vigor, they were 44.25 U/mg, 61.37 U/mg, 84.75 U/mg, and 57.47 U/mg, respectively.

(4) F-CM_{aq} treatment enhanced cell apoptosis in U2OS (41.86% in early apoptosis stage) and A549 cell (15.20% in late apoptosis stage) compared to CM_{aq} treatment (24.36% in early apoptosis stage of U2OS cells, 10.26% in late apoptosis stage of A549 cells) by activation of pro-apoptosis protein including Bax, caspase-3, and downregulate the expression of anti-apoptosis protein of PARP-1 and Bcl2. Furthermore, cell cycle arrest in G2/M phase (U2OS cells) and G1 phase (A549 cells) were increased through activation of p53 and inhibition of cyclin B1, cyclin D1, CDK4, and pRb.

(5) F-CM_{aq} possessed significant resistance to U2OS cells migration compared to CM_{aq} by downregulation of MMP-2, MMP-9, and Vimentin pathways. While in A549 cells, similar resistance was caused via MMP-2, MMP-9 signaling pathways.

Taken all these together, this study proved low dose of fluoride stimulation on *C. militaris* promotion for the first time and provided the benefits evidence from bioactive substance content, antioxidant activity and anti-cancer ability, providing an easy, economic, alternative method on promoting utilization of *C. militaris* warrant further studies.

4.2. Future works

In present study, the stimulation enhancement in growth of *C. militaris* has been proved available. However, further investigation of stimulation effects by using this technology is necessary. Therefore, the future works are proposed as follows:

(1) Even though the working style of fluoride was investigated as inorganic ion's form in this study, the possibility for it participating the synthesis of bioactive substance was still worthy to be discussed and further explored.

(2) The content of other active substance like cordycepin and cordyceps acid were remained to be detected.

(3) More accurate range of the optimal concentration for hormesis effect induction and this effect could weather to be possible happened on other similar species of *Cordyceps* family was necessary to be studied.

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