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# Potential of Chinese Herb "*Cordyceps militaris*" as a Medicinal Food

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**Abstract**

*Cordyceps militaris* is a parasite fungus which contains abundant nutritional bioactive compounds and extensively used as a traditional Chinese medicine (TCM) as well as tonic food. Many researchers reported that *Cordyceps militaris* possessed various pharmacological activities that benefit human health. In the context of culinary medicine which blends art with medicinal science, this paper reports *Cordyceps militaris* as in vitro scientifically proved as a potential source of therapeutic agent and a functional ingredient for health or supplement food with ethnomedicinal claims.

***Keywords***: *Cordyceps militaris, crude extract, pharmacological properties, antioxidant, antimicrobial, health food*

## 1. Rationale and Background of the Study

*Cordyceps militaris,* a pleomorphic parasite fungus, has been widely known for their ability to synthesize various kinds of bioactive compounds (Xiao & Xiong, 2013; Wong et al., 2007; Yu et al., 2006; Ng & Wang, 2005; Shrestha & Sung, 2005). It was categorized into more than 400 species and many of them, i.e., *Cordyceps sinensis*, *Cordyceps militaris*, *Cordyceps cicadae* and *Cordyceps ohioglossides* have been well recognized as sources of functional food and folk medicine due to their pharmacological efficacy (Xiao et al., 2009; Wong et al., 2007; Yu et al., 2006; Ying et al., 1987). *Cordyceps sinensis* is considered the most popular one that has been widely used as a traditional Chinese medicine (TCM) for centuries. However, since its high specific growth environment, *C. sinensis* is rare to find in nature and annual harvests are decreasing which lead to its incredibly high price (Lin & Li 2011; Feng et al., 2008).

Due to rarity and expensiveness of *C. sinensis*, other Cordyceps, such as *Cordyceps militaris* has been used for substitution (Yang et al., 2009). There were many reports revealing that *C. militaris* even possessed a higher level of some bio metabolic substances than those found in *C. sinensis* especially cordycepin (Zhong et al., 2017). Cordycepin, an important bioactive component of *Cordyceps*, is well known for promoting human health and scientifically proved for its broad spectrum of therapeutic potentials including anti-inflammatory,

anti-oxidant, neuroprotective, cardioprotective, antidiabetes, renal interstitial fibrosis, sexual dysfunction, anti-asthmatic and anti-cancer (Patel & Goyal, 2012; Yue et al., 2012; Zhang et al., 2012 a, b; Lee et al., 2011a, b; Wang et al., 2011; Das et al., 2010; Zhou et al., 2008; Ng & Wang, 2005; Mizuno, 1999). With its less expensive and remarkable efficacy, a number of studies aimed to investigate its roles on pharmacological action and potentiality in various disease treatments in order to develop new drugs for modern medication (Hwang et al., 2016; Ruma et al., 2014; Park et al., 2012; Oh et al., 2011; Park et al., 2009 a, b; Won & Park 2005).

According to the advancement on cultivation techniques, it is possible to produce *C. militaris* commercially with high yield and high productivity of bioactive compounds as well as isolated and concentrated via extraction process. The extract obtained from *C. militaris* has been extensively investigated for its therapeutic properties and numerous clinical activities (Park et al., 2009 a, b; Rao et al., 2010; Won & Park, 2005; Yu et al., 2007). However, to our best knowledge, drug action and dose consumption of the extracts were varied due to differentiation of cultivation protocol, extraction technique and active ingredients embedded in it.

In this study, the researchers aimed to (1) investigate the in vitro pharmacological effect of crude extract received from *C. militaris* TOLA00 which was cultivated by solid state fermentation using a proprietary medium formulation, and (2) explore the possibility of developing this variety as an ingredient in health/medical food.

## 2. Material and Method

### 2.1. Raw Material

*Preparation of C. militaris TOLA00 (CMTOL) crude extract*

*C. militaris* TOLA00 (CMTOL) was a gift from Thai Orchids Lab Co.,Ltd., Sampran, Nakhon Pathom, Thailand. The culture was cultivated using solid state fermentation until certain period of time to reach its harvest. The sample was dried and ground into small particles and sent to Origin Plant Co., Ltd., Thailand, for extraction. Ethanol extraction was conducted with the ratio of CMTOL: ethanol equal to 1:10 under 50°C for 5 days. The solid was discarded by filtration and the filtrate was subjected to rotary evaporator for ethanol removal for crude extract of CMTOL being obtained. Analysis of cordycepin and adenosine contained in the crude extract was done using HPLC method. The crude extract was kept in a glass bottle under -28°C in this experiment.

*Microorganism and chemical*

Microorganisms used in this study were both gram positive and negative, e.g., *Acinetobacter baumanii* MDR I, *Acinetobacter baumanii* MDR II, *Enterococcus faecalis* VRE, *Escherichia coli* ESBL, *Escherichia coli* P174 ESBL, *Klebsiella pneumoniae* ESBL, *Pseudomonas aeruginosa* MDRI, *Pseudomonas aeruginosa* MDR II, *Staphylococcus aureus* MRSA I, and *Staphylococcus aureus* MRSA II. All chemicals used were of analytical grading.

### 2.2. Anti-inflammatory Assay of CMTOL Crude Extract

Anti-inflammatory was determined as the inhibition effect upon protein denaturation using the modified method of Mizushima & Kobayashi (1968). The crude extract CMTOL was diluted to the concentration of 625, 1,250, 2,500, 5,000 and 10,000 µg/mL. Pipette 100 µL of each concentration mixed with 500 µL of 1% bovine serum albumin then let the

mixtures to stand at ambient temperature for 10 minutes. The mixtures were heated at 51°C for 10 minutes and let cooled. The absorbance was measured at 660 nm by using acetyl salicylic acid as positive control.

**2.3. Anti-oxidant Assay of CMTOL Crude Extract**

*Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay* (Blois, 1958)

100 µL of 0.2 mMol DPPH solution was pipetted to microtiter plate, then an aliquot (100 µL) of CMTOAL crude extract was added. The mixtures were left for 30 minutes in a dark place and the absorption was measured at 517 nm. The calibration curve was also established using vitamin c equivalent antioxidant capacity (VCEAC) by solution of L-ascorbic acid in methanol was analyzed in the same test protocol as a respective sample.

*Folin-Ciocalteu colorimetric assay* (Singleton et al., 1999)

100 µL of the CMTOL crude extract was mixed with Folin-Ciocalteu's reagent (100 µL, previously diluted with water 1:10, v/v) and 7.5% sodium carbonate (80 µL), respectively. The mixture was allowed to stand for 30 minutes at ambient temperature for color development and the absorbance was then measured at 765 nm. Gallic acid was used to conduct the calibration curve.

*Hydrogen peroxide scavenging activity* (Halliwell et al., 1987)

An aliquot (850 µL) of the CMTOL crude extract at different dilution (625, 1,250, 2,500, 5,000 and 10,000 µg/mL) was added to 150 µL of 4 mM Hydrogen peroxide solution in phosphate buffer (0.1 M, pH 7.4). The solution was incubated for 10 minutes and the absorbance measurement was performed at 230 nm where butylatedhydroxytoluene (BHT) was used as positive control.

*Anti-lipid peroxidation (LPO)*

Thiobarbituric acid reactant substances (TBARS) assay was used to analyze the inhibition of lipid peroxidase as described by Ruberto et al. (2000) with some modification. Briefly, egg yolk was diluted to 10% (w/v) with KCL solution being added to 50 µL of different concentration CMTOL crude extract. Addition of 20% acetic acid (pH 3.5) 300 µL and thiobarbituric acid (TBA) 300 µL were put into the previous solution, then being mixed with a vortex mixer. Incubation of the mixtures was at 95°C for 1 h, and left cool at ambient temperature. Added was 750 µl of butanol, followed by centrifugation at 3,000 rpm for 10 minutes. Supernatant was absorbance measurement using ELISA reader at 532 nm.

Anti-oxidative low density lipoprotein (LDL) ability was also determined. Mixed was 9 µL of human low density lipoprotein (LDL) with 10 mM ferrous sulphate 191 µL and 100 µL of CMTOL crude extract at different dilutions (625, 1,250, 2,500, 5,000, 10,000 µg/mL). After that, 15% trichloroacetic acid (TCA) 500 µL and 1% thiobarbituric acid (TBA) 1 mL were added. The mixtures were incubated at 100°C for 10 minutes, then cooled by letting them stand at ambient temperature. Pipette 300 µL of the mixtures was put into microtiter plate and measured for the absorbance at 532 nm using ELISA reader and $CuSO_4$ (pH 7.4) applied as blank.

### 2.4. Anti-microbial Test of CMTOLA00 Crude Extract

Determination of minimum inhibitory concentration (MIC) was carried out by the microdilution method (Clinical and Laboratory Standards Institute, 2008). Gram-negative and gram-positive bacteria mentioned above were used for an anti-microbial test. Those respective bacteria from freeze vial on nutrient agar were re-cultured and its turbidity was adjusted using spectrophotometer measurement at 625 nm to achieve concentration of 1.5×108 CFU/mL. Different dilutions of CMTOL crude extract were added to the wells containing 100 µL of tryptic soy broth (TSB) and 10 µL of inoculum in microtiter plate. The microtiter plate was incubated at 37 °C for 15 h and the absorbance has been measured at 620 nm. Gentamicin at successive concentration was used as control. All experiments were repeated 3 times.

## 3. Results and Discussion

The CMTOL crude extract obtained from ethanol extraction contained cordycepin and adenosine in the value of 0.94% and 0.04%, respectively and the extraction yield (% based on cordycepin content) was 35.95%. Successive dilutions were made and submitted to evaluate various therapeutic properties, i.e., anti-inflammatory, anti-oxidant and anti-microbial where all experiments were repeated 3 times.

### 3.1. Anti-inflammatory Activity

The CMTOL crude extract was subjected for its potent of protein denature inhibition. BSA was denatured by heat treatment and different doses of the extract being tested to prevent BSA denaturation as shown in Figure 1.

**Figure 1:** Effect of CMTOL Crude Extract on Preventing BSA Denaturation.

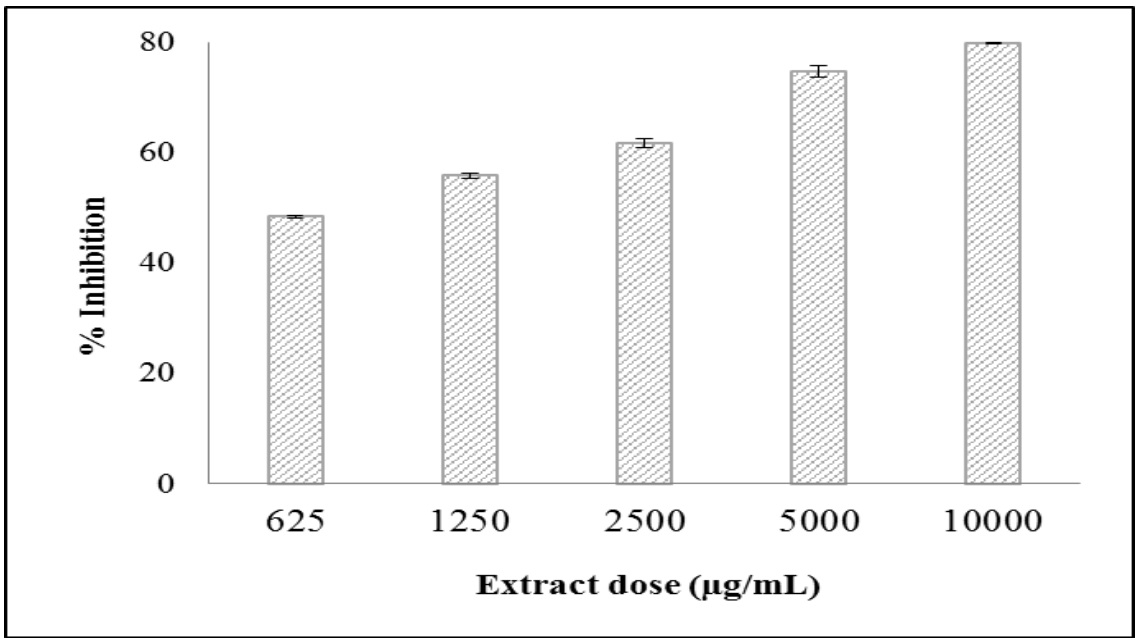



Figure 1 shows that the inhibition of BSA denaturation was dose-dependent. At 625 µg/mL, CMTOL demonstrated an inhibition effect of BSA denaturation more than 50% and increased to almost 80% when the dose reached 10,000 µg/mL. Protein denature was the cause of inflammatory (Perumal et al., 2008); therefore, the ability in preventing protein denaturation

suggested that the CMTOL crude extract undoubtedly possessed anti-inflammatory activity. Rosner et al. (2001) reported that BSA consists of two binding sites which are aromatic tyrosine rich and aliphatic threonine and lysine residue region. To be able to inhibit BSA denaturation, those therapeutic compounds existing in the extract might activate the tyrosine motif rich receptor dually with threonine that regulate signal transduction biological pathways for their overall biological action (Rosner et al., 2001; William et al., 2002b). It is also noteworthy that those compounds which react to aliphatic lysine residue on BSA may possess anti-oxidant or anti-cancer properties in the same way as polyphenol substances (Kawabata & Packer 1994; William et al., 2002a, b).

### 3.2. Anti-oxidant Activity

To determine the CMTOL crude extract for its anti-oxidant activity, numerous methods were used to yield the complete anti-oxidant profile of the respective sample. The results are shown in Table 1 and Figures 2-3.

**Table 1:** Scavenging Effect on DPPH Free Radical, Total Antioxidant and Total Phenolic Compound Presented in 0.1g/ml CMTOL Crude Extract

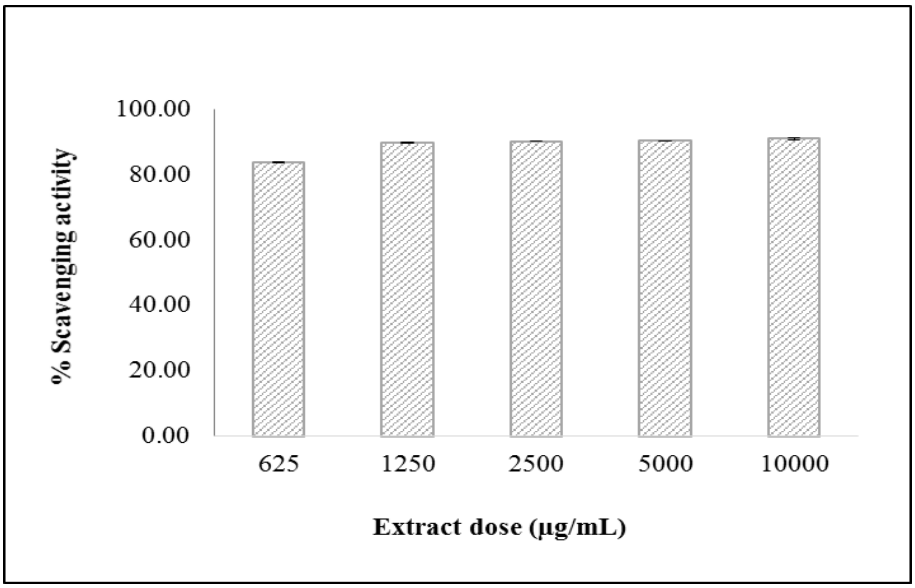
| Anti-oxidant Assay | Average±SD |
|---|---|
| Total phenolic compound (µg gallic acid equivalents) (GAE)/ 0.1g dry matter) | 715.53±0.01 |
| DPPH scavenging activity (%) | 77.76±1.87 |
| Total anti-oxidant (µg of vitamin C equivalent/0.1 g (dry matter) | 64.30±3.38 |

The total phenolic compound of the crude extract showed high value with 715.53 µg gallic acid equivalents (GAE)/ 0.1g dry matter. This result supported the same finding reported earlier by several publications and indicated that *C. militaris* might able to produce phenolic substances by itself or catalyze them from substrate (Zhang et al., 2012b; Juan & Chou, 2010). DPPH--free radicals which accept electron or proton to become stable--is a quick protocol to test free radical scavenging activity and widely used as an anti-oxidant index (Mokbel & Hashinaga, 2006; Zhan et al., 2006). The CMTOL crude extract possessed DPPH free radical scavenging activity at 77.76% which was similar to the finding reported by Zhang et al. (2012), Zhan et al. (2006), Reis et al. (2013) and Xiao et al. (2015). Some evidences also revealed that consumption of medicinal mushroom which contained anti-oxidants is a way to prevent or reduce over two-third of cancer-related deaths (Borchers et al., 2004; Zaidman et al., 2005).

The results on hydroxyl radicals scavenging of the CMTOL crude extract shown in Figure 2 revealed that scavenging activity of the extract was increased when its dose was higher. The scavenging activity of the extract at 625 µg/mL was very high with over 80%. The finding corresponded with those reported by Shen & Shen (2001) and Zhan et al. (2006) indicating *C. militaris* extract as scavenging ability of hydroxyl radicals at 76-80%. Hydroxyl radicals--the most reactive radicals generated during aerobic metabolism--is the

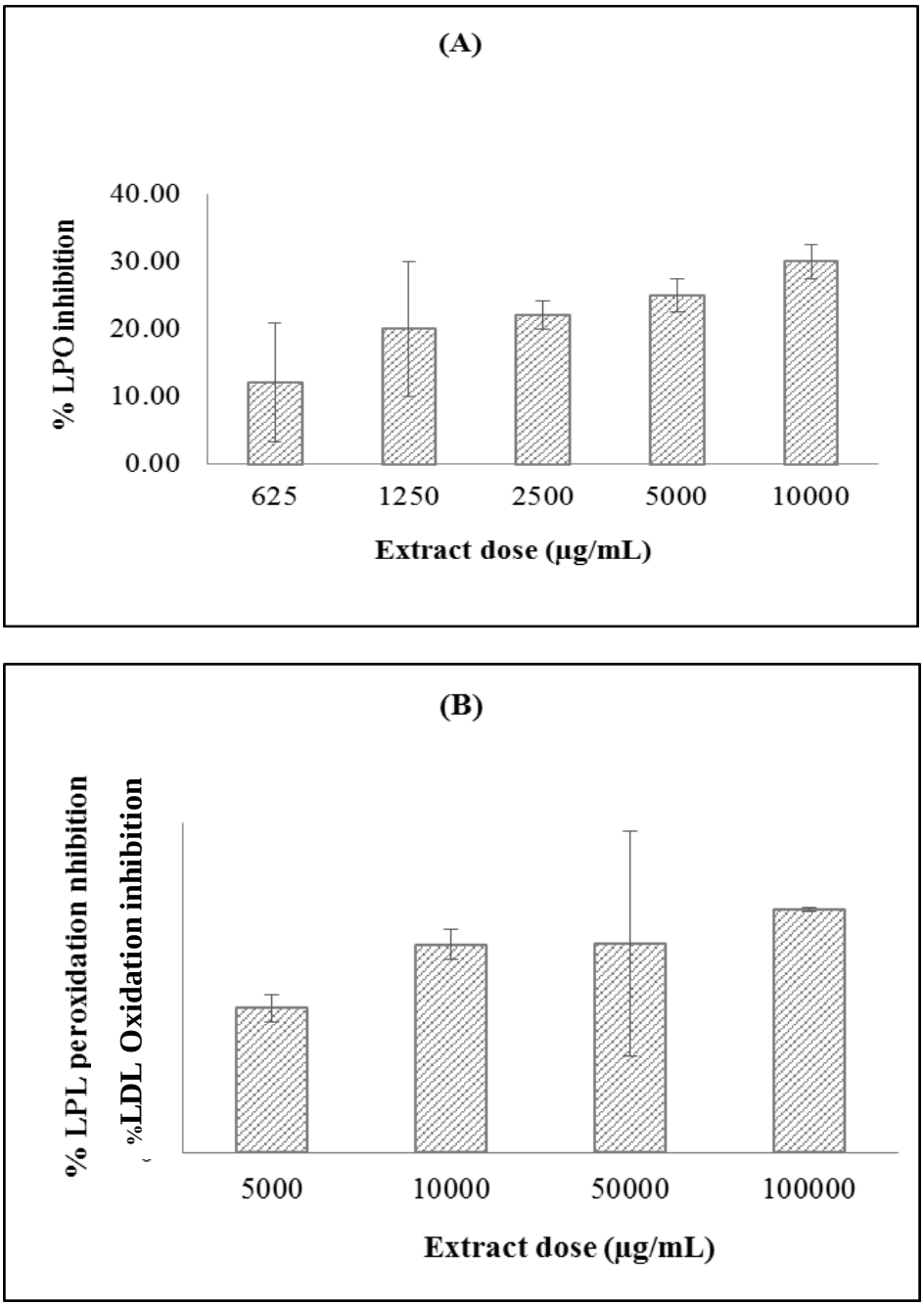
form of a number of reactive oxygen species, such as superoxide anion, hydrogen peroxide and nitric oxide which damage biological molecules (Zhang et al., 2012a, b; Badmus et al., 2011; Siddhuraju & Becker, 2007). Two mechanisms of anti-hydroxyl radicals were proposed; one was to prevent hydroxyl radicals generating via ligate to metal ions, and the other scavenged the generated hydroxyl free radical (Zhang et al., 2012a, b). This might suggest that the CMTOL crude extract also carried those mentioned anti-hydroxyl radicals activity.

**Figure 2:** Effect of CMTOL Crude Extract on Hydroxyl Radicals Scavenging Ability




Not only in aqueous matrix, oxidative reaction also occurs in lipid layer in which lipid peroxidation is regarded as the main cause of damaging cellular component and fragmentation of connective tissues (Halliwell & Gutteridge, 1999; Chance et al., 1979). As shown in Figure 3, the CMTOL crude extract appeared to contain LPO and LDL inhibition property in the dose-dependent manner. The extract dose at 10,000 µg/mL performed approximately 30% of LPO inhibition. Many researches also demonstrated the LPO inhibition activity of *C. militaris* extract in a similar manner in support of this finding (Gu et al., 2006; Hui et al., 2006; Zhan et al., 2006).

**Figure 3:** Effect of CMTOL Crude Extract as an Anti-lipid Oxidation: (A) Inhibition Ability on LPO, and (B) Inhibition Ability on LDL.




Moreover, the CMTOL crude extract also expressed high potential of LDL oxidative inhibition property which over 60% of LDL inhibition was received at the extract dose of 10,000 µg/mL. Oxidized LDL is recognized as the cause of lipid accumulated in arterial wall and leads to atherosclerosis related disease (Steinberg et al., 1989; Cross et al., 1987). Many earlier reports confirmed that treated coronary artery disease patients with anti-oxidant substances have positive effect on cardiovascular condition (Milman et al., 2008; Lee et al., 2005; Tepel et al., 2003; Boaz et al., 2000; Stephen et al., 1996).

### 3.3. Anti-microbial Property

Anti-microbial property of CMTOL has been test against both gram negative and gram positive microorganism where gentamicin was used as positive control and the results were shown in Table 2.

**Table 2:** Minimum Inhibitory Concentration (MIC) of CMTOL Compared with Gentamicin

| Microorganism | (MIC µg/ml) | |
|---|---|---|
| | CMTOL crude extract | Gentamicin |
| *Staphylococcus aureus* MRSA 1 | G | 0.47 |
| *Staphylococcus aureus* MRSA2 | 10,000 (NG) | 1.88 |
| *Acinetobacter baumannii* MDR1 | G | >120 |
| *Acinetobacter baumannii* MDR2 | G | >120 |
| *Pseudomonas aeruginosa* MDR1 | 10,000 (NG) | 0.12 |
| *Pseudomonas aeruginosa* MDR2 | G | 0.12 |
| *Escherichia coli* P174 ESBL | G | 0.12 |
| *Escherichia coli* ESBL | 10,000 (NG) | 1.88 |
| *Klebsiella pneumonia* ESBL | G | 7.5 |
| *Enterococcus faecalis* VRE | G | 0.12 |

Note: G = Growth; NG = No Growth

The results in this study showed that the CMTOL crude extract at 10,000 ug/mL demonstrated itself as the least concentration to inhibit *Staphylococcus aureus* MRSA2 (gram positive), *Pseudomonas aeruginosa* MDR1 (gram negative) and *Escherichia coli* ESBL (gram negative) with no growth detected (MIC). It should be noted that the preliminary test using agar well-diffused method [data not shown in this paper], the CMTOL crude extract also presented the inhibition effect against *Acinetobacter baumannii* MDR2. For *Staphylococcus aureus* MRSA2, the extract even possessed a higher inhibition zone (11 mm) than that found in commercial antibiotics, gentamicin (9 mm). Ahn et al. (2000) reported that *C. militaris* extract using methanol was greatly inhibited toward *Clostridium* species; however, adverse effects on the growth of lactic acid bacteria were not detected. Therefore, *C. militaris* has been considered an interesting rich source of bioactive compound associated with its anti-pathogenic bacteria activity.

## 4. Conclusion

In the context of culinary entrepreneurship and innovation, this research provided the information concerning some clinical efficacy of *Cordyceps militaris* TOLA00. The results revealed that the CMTOL crude extract possessed various pharmacological properties (in vitro evaluation), e.g., anti-microbial, anti-inflammatory and anti-oxidant which expressed against both free radicals in aqueous and lipid matrix. The finding of the study could support the possibility of using *C. militaris* TOLA00 as a potential natural ingredient to improve the therapeutic effects of *medical food* for some diseases. It could also be commercially developed as a supplement food for health after meticulous retesting for consumers' safety.

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