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EFFECTS OF LIQUID CULTURE MEDIA CONTENT ON THE ANTIOXIDANT PROPERTIES OF MYCELIAL EXTRACTS FROM *CORDYCEPS MILITARIS* (STRAINS AG-1 AND PSJ-1)

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Abstract– The antioxidant properties of *Cordycceps militaris* mycelial extract from two strains AG-1 and PSJ-1 (obtained by submerged cultivation) was based on inorganic, organic nitrogen sources in different types: MYPS, PVC, PD, ME, and Czapek-Dox media. Dried mycelial extract was obtained by freeze-drying (FD), and oven drying (OD) and was extracted with pure ethanol, hot water, and methanol. The antioxidant content of methanol extracts from these two strains mycelial were investigated, and their antioxidant potential were determsined by the DPPH, ferrous ions, hydroxyl radical, ABTS, and lipid peroxidation assays. The results were confirmed by the low values of the ascertained IC_{50} and minimum inhibitory concentration. Moreover, the abundance of the compounds with antioxidant effects substantiated the data obtained from the extracts. Mycelial cultured in containing higher contents of MYPS static and PVC static media, resulted in higher values of total phenolic and total flavonoid contents as well as high efficiency of DPPH, $ABTS^{\cdot+}$. The methanol extracts and FD of strains AG-1 and PSJ-1mycelial showed much efficiency in improving TPC as well as antioxidant activities and except for the effect on lipid peroxidation. Antioxidant properties and antioxidant content were assessed and the composition of extracts in different submerged cultures from the mycelial two strains AG-1 and PSJ-1 depicted a significant pharmacological potential as well as the possibility of usage in the development of pharmaccutical drugs.

Keywords: antioxidant properties, *Cordyceps militaris*, extracts, freeze-dry, liquids media culture, oven dry

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INTRODUCTION

Cordyceps militaris (L.) Link (*Ascomycota*), otherwise called restorative caterpillar fungus, has compound segments like those of *Ophiocordyceps sinensis* and were available pharmacological activities similar to and, according to some of the study, more potent than *O. sinensis* (Hamburger, 2007). Medicinal mushrooms are effective reciprocities to the deterrence and treatment of many modern diseases. *Cordyceps militaris* a medicinal source; however, owing to their high content in therapeutic biomolecules, Cordycepin is a source of biologically active compounds. Mycelia can be easily acquired in sufficient quantities by fermentation. Owing to an increase in demand for the extraction of a bioactive molecule in order to produce biologically active supplements, the assurance of an effective and efficient method of extraction is hence required. Liquids cultivation method is an acceptable method; however, increased efficiency is very important. The extraction efficiency depends mainly on the species used, on the nutrient sources in the culture medium, and by the cultivation parameters (Dong, 2012). Previous studies have shown that *Cordyceps* species possesses liver protective effects (Liu, 2006), and antioxidative activities (Chen, 2006).

There are many reports on the optimization of *C. militaris* culture conditions, but few reports on shake and static treatment are reported. It is well known that culturing conditions regulate that affect growth, reproduction, ripening, and secondary metabolism of plants (Tang, 2015). Antioxidants act as a major precaution against intermsediate-level toxicity by protecting damage caused by free radicals. Inhibition of free radical generation may serve as a favorable system for identifying anti-cancer agents (Ahn, 2000). However, reports on the culture conditions and antioxidant activity of *C. militaris*, its mycelial commercially grown in the study, the effect of a source of inorganic vitamins (Vitamin B1) and of organic sources (yeast extract, peptone and corn extract) on the production of mycelial by

various liquid cultures and when shaking, the static, static + shaking condition was evaluated by the antioxidant activity, reduced capacity, inhibition of free radicals. According to the theory of oxidative stress in the process of aging, the delicate balance between the creation of reactive oxygen species and the antioxidant system of antiaging can lead to the shift of cellular oxygenation. Reactive oxygen species were thought to commonly produced in aerobic and aerobic organisms that are provided with antioxidant defense systems that could prevent damage from oxidative stress (Fridovich, 1978; Sies, 1985).

Major antioxidant defense systems include antioxidant and antioxidant enzymes. Previously included superoxide dismutase, Catalase, and glutathione peroxidase, and then including reduced glutathione, vitamin C, and vitamin E (Klivenyi et al., 2000). Antioxidant supplements can protect the human body from free radicals and react oxygen and slow the progression of many chronic diseases as well as lipid oxidation (Lai et al., 2001). Therefore, the aim of the study was to evaluate the antioxidant properties of mycelial at the present time as butylated hydroxyanisole, butylated hydroxyl toluene, propyl gallate, and tert-butylhydroquinone. Nonetheless, BHA and BHT were suspected of liver damage and carcinogenesis (Wichi, 1988; Sherwin, 1990). The antioxidant activities of the study of *C. militaris* two strains grown on different submerged cultivation and comparison among drying methods of mycelial. Therefore, the aim of this study is to determine the total phenolic, total flavonoid contents as well as the antioxidant activities of *C. militaris* two strains AG-1 and PSJ-1 mycelial grown on different submerged cultivation formulas and by different mycelial drying methods.

MATERIALS AND METHODS

Chemicals and source of strains. *C. militaris* two strains AG-1 and PSJ-1 used in the present study were obtained from Plant Physiology and Value Added Microorganisms Laboratory, Department of Plant Industry, National Pingtung University of Science and Technology (NPUST) in Taiwan in the Autumn-Winter season of 2017. Five mycelia discs (6 mm diam.) obtained from *C. militaris* were into Erlenmeyer flasks of MYPS media inoculated (6 mm diam.) and covered with mycelial. After 18 days of incubation in the dark, the liquid media was filtered and the mycelial separated from the liquid.

The chemical agents. Peptone, yeast extract, and glucose were purchased from Himedia. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Phenazine Methosulphate (PMS), Nicotinamide Adenine Dinucletide (NADH) and Thiobarbituric acid (TBA) were purchased from Sigma Chemicals Co. (St. Louis, USA). Methanol, ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), Folin-Ciocalteu Phenolic reagent were purchased from Merck (Darmstall, Germany). Ferrozine, Sodium carbonate, Potassium ferricyanide, L-Ascorbic acid, disodium salt were purchased from Japan. Hydrogen peroxide (H_2O_2), and Sodium hydroxide (NaOH) were purchased from Himedia, Mumbai were of analytical grade *C. militaris* (strains AG-1 and PSJ-1), purchased. For each strain, the carbon source was estable lished, selected between the glucose. The liquids mycelia was isolated through centrifuging, with a Hitachi SCR20BA centrifuge and it was submitted to freeze-drying in an Alpha 1-2 LD freeze-dryer and DK-600 oven drying (Ya-jie, 2007).

Trials on effect of different liquids culture media and culture methods on the biomass of mycelial growth. The submerged liquid cultures media consisted of the following: (1) MYPS media: 4g/L malt extract powder; 4 g/L yeast extract; 6 g/L peptone; 10 g/L sucrose; 0.3 g/L Vitamin B1, and 1.0 mL distilled water; (2) PVC media: 30 g/l glucose; 10 g/L corn powder; 0.25 g/L $FeSO_4 \cdot 7H_2O$; 0.5 g/L vitamin B1, 0.6 g/L K_2HPO_4; 0.7 g/L $MgSO_4 \cdot 7H_2O$; 1 g/L KH_2PO_4; 6 g/L peptone (Himedia), and 1.0 mL distilled water; (3) PD media 200 g/L potatoes; 30 g/L sucrose; 20 g/L dextrose; 0.5 g/L Vitamin B1, and 1,000 mL distilled water; (4) Malt-extract media (ME): 0.5 g/L Vitamin B1, 5g/L malt extract powder; 5g/L peptone; 20g/L glucose, and 1.0 mL distilled water (Atlas, 1993); (5) Czapek-Dox media (CD): 0.3 g/L Vitamin B1, 30 g/L Sucrose; 2 g/L $NaNO_3$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl; 1.0 mL distilled water (Stevens, 1981). The mycelia disc of *C. militaris* were transferred to the liquids culture medium from the culture grown on MYPS plates; used to inoculate 250 ml liquids media. The seed culture was incubated in a 500-ml Erlenmeyer flask at 24°C, initial pH 7.0 on a rotary shake, static, static + shak conditions incubator at 95 rpm for 18 days (shake, static) and 5 days static as 13 days shake (static + shake) (Park, 2001; Park, 2002). The mycelia were separated from the filtrate by filtration with pre-weighed filter paper (Whatman Ltd.), washed and then vacuum dried. Biomass concentration was determsined in termss of dry weight per unit volume. The filtrate was concentrated under reduced pressure (rotary evaporator) at 4°C. Dried filtrates and mycelia were ground into fine powders (20 mesh) in a mill grinder before being subjected to methanol, hot water, and ethanol extraction.

Trials on evaluation of the antioxidant properties of *C. militaris*. Preparation of mycelial extracts: Three different solvent extracts (methanol, hot water and ethanol) of mycelia of *C. militaris* two strains AG-1 and PSJ-1 were investigated for their free radical scavenging activity using DPPH radical as a substrate, chelating on ferrous ions, hydroxyl radical scavenging assay, scavenging activity of $ABTS^+$ radical cation, lipid peroxidation (LPO) inhibitory activity, the total phenolic contents (TPC) and total flavonoid contents (TFC). A dose-response curve was plotted for determsining IC_{50} values to express the results of inhibitory activity, respectively.

The drying method. Fresh mycelia of *C. militaris* two strains (AG-1 and PSJ-1) were dried by oven drying (OD) method (40°C) and freeze drying (FD) method (−40°C) for 72 hr. Freeze drying (FD) is famous for manufacturing higher quality dried products (Huang, 2011). The samples were ground to powder with a grinder (Yuqi, DM-6, Taiwan), and stored at −20°C freeze. The extracts were filtered through Whatman filter paper. The residues were then extracted with one additional 100 mL of methanol, hot water or ethanol. The combined extracts were then evaporated using a vacuum concentrator (Heidolph2, Laborta 4000) at 40°C to dryness. The dried extracts thus obtained were re-dissolved in methanol to a concentration of 100 mg/mL and stored at 4°C prior to analyses of antioxidant attributes.

Methanol extraction of mycelia. The *C. militaris* mycelial of both strains (AG-1 and PSJ-1) was cultivated, freeze dried and powdered. Approximately 2 g of powdered mycelia were extracted with 200 mL of 95% methanol using a bath/circulators and filtered through muslin cloth, at 75°C for 2 hours extraction and filtering through filter paper 110 mm. Each extraction was repeated twice and combined filtrates were concentrated in a rotary evaporator at 40°C to dryness under reduced pressure. Finally, methanol extracts were prepared from the dried material (1 mg/mL) for further experiments. The filtered extract was evaporated under reduced pressure and vacuum dried at 40°C to get the viscous residue and used for the estimation of antioxidant activities.

Hot water extraction of mycelia. Two grams (2 g) mycelia of *C. militaris* two strains (AG-1 and PSJ-1) was cultivated, freeze dried in shade and powdered were extracted with 200 mL of boiling distilled water for 2 h. The extract was filtered with filter paper (Advantec 1, Japan) while the residue was re-extracted under the same conditions twice. The filtrates obtained from three separate extractions were combined, concentrated and then lyophilized. The dried mycelial of *C. militaris* two strains extracts (HWECM) was collected, weighed and stored at 4°C until use.

Ethanol extraction of mycelium. Two grams (2 g) mycelia of (AG-1 and PSJ-1) was freeze dried and powdered were soaked with 200 mL of ethanol (95%) at room temperature for 6 days. After filtering the extract with filter paper (Advantec 1, Japan), the residue was further extracted under the same conditions. The filtrates collected from three separate extractions were combined, concentrated and lyophilized. The dried ethanol *C. militaris* two strains (AG-1 and PSJ-1) extract (EECM) was collected weighed and stored at 4°C until use.

Scavenging on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The antioxidant activity of the methanolic extract of *C. militaris* both strains AG-1 and PSJ-1 samples was determsined in termss of hydrogen donating or radical scavenging ability using the stable radical DPPH (Sigma) carried out by using the method of Shimada (Shimada, 1992). About 1 ml of 0.001M DPPH solution in methanol, an equal volume of the extract in methanol of different concentrations (0.5, 1, 2.5, 5, 7.5, and 10 mg/mL) of the extract in methanol was added and incubated in dark for 30 min and 1ml of methanol served as control. The change in color was observed in termss of absorbance using a spectrophotometer (Hitachi U2800) at 517 nm and the concentration in μg/mL required to scavenge 50% of the radical inhibitory concentration 50 (IC50) was obtained from the regression equation. The scavenging ability on DPPH radicals was calculated as the inhibition percentage according to the formula: Scavenging activity (%) = [(A517 of control − A517 of sample)/A 517 of control] ×100, where A517 of control means the absorbance of the control solution (containing all reagents except the test extract), A517 of sample means the absorbance of the test extract. All test analyses were run in at least three replicates and averaged.

Chelating on ferrous ions. The chelating of ferrous ions by methanol extract of both strains AG-1 and PSJ-1 samples was estimated by the method of Dinis (Dinis, 1994). Each 1 mL of extract of different concentration (0.5, 1, 2.5, 5, 7.5, and 10 mg/mL) in methanol was blended with 3.7 ml of methanol and 0.1 mL of 2 mM ferrous chloride ($FeCl_2.4H_20$). The reaction is initiated by way of the addition of 0.2 mL of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determsined at 562 nm against a control. The control sample was also prepared as above without methanol was used for the baseline correlation. Calculated and the regression equation was obtained from which inhibitory concentration 50 (IC_{50}) was calculated. The chelating activity of the extracts was evaluated using EDTA as standard and the results were expressed as μg EDTA equivalent/g extract. The chelating ability ferrous of ions is calculated according to the formula: Inhibition (%) = ([(AC–AS)/AC]) × 100, where AC is the absorbance of control and AS is the absorbance of solution containing sample extracts.

Hydroxyl radical scavenging assay. Hydroxyl radicals (OH^-) are generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535 nm. The hydroxyl radical scavenging activity of *C. militaris* methanol extract was measured according to the method of (Halliwell, 1992). Stock solutions of EDTA (1 mM) were prepared in DMSO and $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. The method was carried out by adding 100 μl of EDTA, 10 μl of $FeCl_3$, 100 μl of H_2O_2, 360 μl of deoxyribose, 1000 μl of the extract of different concentration (0.5, 1, 2.5, 5, 7.5, and 10 mg/mL in methanol) dissolved in distilled water, 330 μl of phosphate buffer (50 mM, pH 7.5) and 100 μl of ascorbic acid. This mixture was

then incubated at 37°C for 1 h. About 1 ml of the incubated mixture was mixed with 1 ml of 10% TCA and 1 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% butylated hydroxyl anisole) and the development of pink chromogenic was measured spectrophotometric ally at 532 nm. The hydroxyl radical scavenging activity of the mushroom extract was reported as percentage inhibition of deoxyribose degradation and was calculated according to the formula: Scavenged (%) = (A0–A1) × 100/A0, where A0 was the control absorbance and A1 was the mixture containing the extract absorbance or the standard absorbance.

Scavenging activity of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or $ABTS^+$-radical cation. The scavenging activity of the extracts was estimated using the $ABTS^+$ decolorization method (Arumagam, 2006; Re, 1999). ABTS with potassium persulphate generates blue/green $ABTS^+$. The radical formed shows a maximum absorbance at 734 nm. The antioxidants cause discoloration by transferring a hydrogen atom to the radical cation. In this experiment, 5 ml of 7 mM ABTS and 88 µl of 140 mM $K_2S_2O_8$ were mixed and allowed to complete radical generation for 12–16 h in the dark at room temperature. The stock solution was diluted with ethanol and PBS (pH 7.4) to give an absorbance of 0.75 at 734 nm. Then, 1 ml of the extract samples of different concentrations (0.5, 1, 2.5, 5, 7.5, and 10 mg/mL) in methanol was added to 1 ml of diluted stock solution and the absorbance measured at 734 nm, 5 min after the initial mixing, using ethanol as the blank. All determsinations were performed in triplicate. The total antioxidant activity (TAA) percentage was calculated using the formula: TAA% = (Ac–As/Ac) × 100, where Ac means absorbance of stock solution and As means absorbance of the extract.

Antioxidant activity against lipid peroxidation. The antioxidant activity of all of the extracts was determsined by a 1,3-diethyl-2-thiobarbituric acid (DETBA) method (Furuta, 1997). Methanolic extracts samples of different concentrations (0.5, 1, 2.5, 5, 7.5, and 10 mg/mL) in methanol were added to 50 mL of linoleic acid emulsion (2 mg/mL in 95% ethanol). The mixture was incubated at 80°C for 60 min and then cooled in an ice bath. The mixture was then mixed with 200 mL of 20 mM butylated hydroxytoluene (BHT), 200 mL of 8% sodium dodecyl sulfate (SDS), 400 mL of deionized water, and 3.2 mL of 12.5 mM DETBA (Aldrich Chemical Co., WI) in sodium phosphate buffer (pH 3.0). It was thoroughly mixed and allowed to stand at 95°C for 15 min and then cooled in an ice bath. Ethyl acetate (4.0 mL) was then added to the mixture, the mixture was centrifuged at 1000 g (20°C) for 15 min and the fluorescence of the ethyl acetate layer was measured in a spectrofluorometer with excitation at 515 nm and emission at 555 nm. Each value was expressed by the mean of triplicate measurements with standard deviation. Percentage of lipid peroxidation was determsined against a blank with no sample added (100%). The antioxidant activity was expressed as the inhibition of lipid peroxidation by using the formula: Antioxidant activity (%) = [1-percentage of lipid peroxidation] × 100.

IC_{50} values in antioxidant properties. The results of antioxidant activity, DPPH free radical scavenging activity, chelating effect on ferrous ion, Hydroxyl radicals, scavenging activity of $ABTS^+$ radical cation, lipid peroxidation, respectively were normalized and expressed as IC_{50} (mg extract/ml) values which are the effective concentration of mushroom extract that are required to show 50% antioxidant properties. A lower IC_{50} value (mg extract/ml) corresponds to the higher antioxidant activity of *C. militaris* mycelia (obtained by submerged cultivation) extract.

Determination of antioxidant contents. The total phenolic contents were analyzed using gallic acid as a standard (Sato, 1996). Each 0.2 mL of extract samples with concentration of 10 mg/mL in methanol was mixed with 2 mL of Folin-Ciocalteu's phenol reagent, 2N (Sigma). The mixture was incubated at room temperature for 5 min. After that, 1.8 mL of 20% sodium carbonate (Na_2CO_3, Nihon Shiyaku) are added, the mixture was centrifuged at 3000 rpm for 10 min. After that, the mixture was kept for 90 min at room temperature. The absorbance of each reaction mixture was measured at 735 nm by a spectrophotometer (Hitachi U-2800, Japan). Gallic acid was used as a standard for a calibration curve. The TPC was obtained by interpolation from linear regression analysis in mg gallic acid equivalents (mg GAE)/g dry extract. All tests are performed in triplicate. The CMF concentration in the sample solution was determsined based on a standard curve regression equation: $A = 0.8533C + 0.0211$, $r^2 = 0.997$, where A is the absorbance and C is the concentration. Then, the extraction rate of total phenolic contents (TPC) in the *C. militaris* sample was calculated.

Total flavonoid content (TFC) is determsined according to the method of Jia (1999). Each extract sample (0.2 mL) with the concentration of 100 mg/mL in methanol was mixed with 1.5 mL of distilled water. Thus 0.1 mL of 10% Aluminium nitrate [$Al(NO)_3$] and 0.1 mL of 1M Potassium acetate (CH_3COOK) was added with the solution. After 40 min at room temperature, the absorbance of the mixture was measured at 415 nm. Quercetin was used as a standard for a calibration curve. The flavonoid contents were obtained by interpolation from linear regression analysis in mg quercetin equivalents (QE)/g dry extract. All tests are performed in triplicate. The CMF concentration in the sample solution was determsined based on a standard curve regression equation: $A = 3.2173A + 0.0618$, $r = 0.997$, where A is the absorbance and C is the concentration. Then, the extraction rate of total flavonoids contents (TFC) in the *C. militaris* sample was calculated.

Experimental design and data analysis. The experiment was arranged in a randomized complete block de-

sign with three replications and twenty-four culture bags per treatment. One-way analysis of variance (Anova) was conducted with Duncan's multiple range tests to compare the mean significant differences ($p \leq \leq 0.05$) among treatments by using computer software SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA). Each value is expressed as mean ±SE ($n = 3$).

RESULTS AND DISCUSSION

Antioxidants properties

Effect of different liquids culture media and culture methods on the biomass of mycelial growth of *C. militaris*. The results showed that the mycelial maximum of biomass mycelial product in the fermentation broth depends on the product of dries mycelial biomass PVC per liter (g/L) of strains AG-1 on five different medium significantly differed ($p < 0.05$) from those strain PSJ-1 on five medium did not significantly differ. When the mycelial was grown in the MYPS media static culture, the mycelial biomass reached its highest level of two strains ranged from 15.03 ± 0.71 g/L (PSJ-1) to 17.88 ± ± 0.87 g/L (AG-1) was obtained at 18 days of cultivation in the static culture; while when grown in the PVC by static culture method, the mycelia fresh biomass reached a maximal value: PSJ-1: 18.20 ± 1.84 g/L; AG-1: 21.85 ± 1.00 g/L. The mycelia biomass production was over PSG-1: 12.96 ± 1.99 g/l; AG-1: 15.65 ± ± 0.60 g/L (shake culture) and PSG-1: 13.24 ± ± 1.45 g/L; AG-1: 17.12 ± 1.49 g/L (static + shake) in comparison with those of the static and static+shake cultivation, respectively. In general, static of two strains AG-1 and PSJ-1 in present research was far lower in comparison to other studies. (Cui, 2010) observed that static of *C. minitaris* grown on different methods culture from 15.8.g/L was obtained at 7 days of cultivation in the shake culture, static 4.8 g/L; static + shake 13.6 g/L cultivation, respectively. Similar results were obtained in the study of (Jiapeng Tang, 2015). However, the results of this study were similar to some previous studies, for example, 6.79 g/L during the first three days of fermentation and maximum mycelial dry weight was about 23.78 g/L on day 5 of *C. militaris* strain 14014 was grown on the shake condition. Obviously, the production of biomass was higher in static culture than in shake culture, although the yield of yeast was slightly lower in shake cultivation compared to static + shake culture. The two-stage fermentation process significantly enhances the production of hyphae. Thus, the two-stage fermentation process by combining the globular fermentation with static culture was proposed and significantly enhanced GA production (Fang, 2002). Previous studies on EPS production from various *Cordyceps* species were limited to conventional culture. However, the biomass of solid culture is insufficient compared to submerged culture. In addition, biological toxins, which was synergistic with hyphae, can be produced simultaneously in submerged culture (Bae, 1998). The basic information obtained in this work is complementary to previous studies on *C. militaris* culture for the production of biological metabolites, and the optimal two-stage culture method obtained in This work to improve the biomass of *C. militaris* exopolysaccharides (EPS) has many values applied in the industry.

The effect of different media and drying methods on the mycelial dry weight of *C. militaris*. The experimental data of the process variables for biomass of mycelia under different dryer and extraction conditions are presented. Two strains AG-1 and PSJ-1 of mycelia grown on nine different culture media showed the significant difference in terms of mycelial weight in Fig. 1. While air circulate during the OD, the mycelial dry weight increased swiftly at the beginning of OD. The mycelial weight of two strains AG-1 and PSJ-1 ranged from AG-1: 21.49 ± 0.68 to 25.95 ± 0.84 mg/g extracts; and PSJ-1: 18.31 ± 0.75 to 24.02 ± 0.04 mg/g extracts. The liquid media PVC culture formulas gave the highest weight (AG-1: 25.95 ± 0.84 mg/g; PSJ-1: 23.55 ± ± 0.69 mg/g) followed by medium PVC culture formulas of two strains AG-1: 27.79 ± 0.59 mg/g; PSJ-1: 24.56 ± 0.64 mg/g extracts, respectively. Among methanol extracts from commercial fruiting bodies and fermented mycelia of *C. militaris* reported that *C. militaris* produced (fruiting body: 21.025 ± 1.38 g; fermented mycelia: 26.82 ± 0.58 g in 100 g of dry sample, respectively) (Dong, 2014). Formulas containing PVC liquid media and MYPS media culture of the two strains AG-1 and PSJ-1 had higher values of the (FD) weight of almost formulas media culture, and thus the weight of the ethanol extracts AG-1 by PVC culture media was higher than that of other mycelial on MYPS, PD, ME, CD media by ethanol and water extracts formulas. In terms of extracts, strain AG-1 had the highest value than strain PSJ-1 at all medium of methanol, ethanol, and hot water extracts. PVC media gave the highest weight on (FD) also gave the higher value of extracts whereas, formulas CD media showed the lowest mycelial weight, as well as the lowest extracts of both strains AG-1 and PSJ-1. The highest mycelial on methanol extracts of strain AG-1 was obtained from PVC media and MYPS media formulas (AG-1: 9.73 ± 0.13 mg/g; AG-1: 8.78 ± 0.75 mg/g, respectively). The second higher extracts were observed from hot water extracts containing PVC media and MYPS media (AG-1: 7.07 ± 0.15 mg/g; AG-1: 7.53 ± 0.32 mg/g extracts, respectively). Strain PSJ-1 of extracts ranged from methanol and hot water extracts from 3.68 ± 0.46 mg/g to 8.84 ± 0.27 mg/g and 2.80 ± 0.51 mg/g to 7.39 ± ± 0.61 mg/g. Media culture formulas PVC, MYPS; PD; ME; CD media were more suitable for both strains AG-1 and PSJ-1 in termss of weight biomass and extracts compared to other media culture formulas (Fig. 2) (B). On the whole, the dryer and extracts methods of both AG-1 and PSJ-1 in this study were lower or equal to those of other studies. Additionally, other studies on *C. militaris* BCC2816 reported that *C. militaris* BCC2816 produced 36 mg in total 5 litters

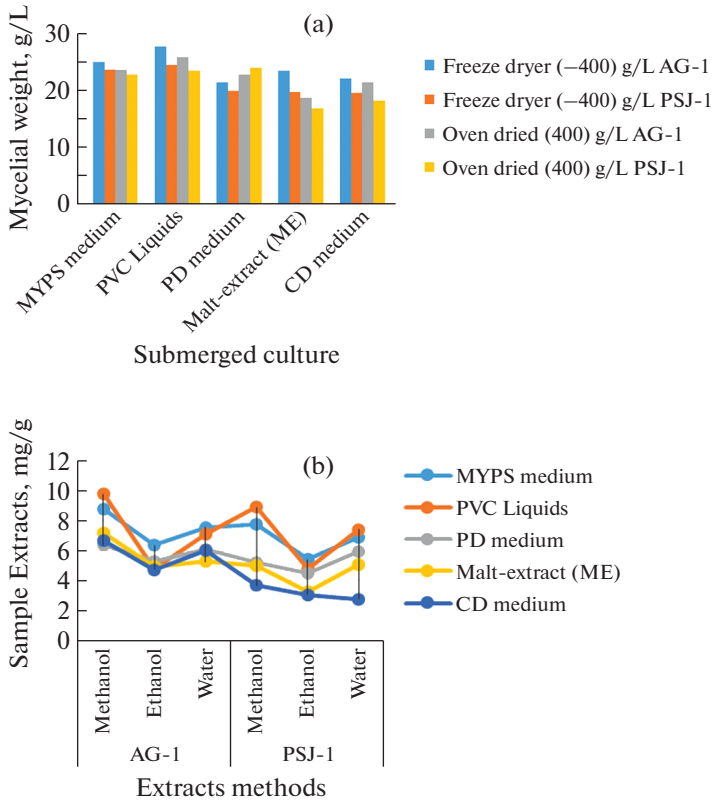

Fig. 1. Different media and drying methods used (a) and different extracts techniques (b) for *Cordyceps militaris* material cultured during 18 days. Each value is expressed mean ± standard error (n = 3). Means within the same capital letters followed by the same row are not significantly different ($p \leq 0.05$).

of potato dextrose broth medium at 25°C (Rukachaisirikul, 2004; Chunyan, 2009) also reported that *C. militaris* dry mycelia weight (DMW) (19.1 g/L) varied with the transformation of culture conditions, the condition of fermentation temperature 28°C, pH 6.2 and medium capacity 57 mL. The result was in agreement with (Ing-Lung Shih, 2007) who stated that the cell growth and with shake culture (15.5 g/L) at 36 days, static culture (14.0 g/L) at 30 days. The mycelial strains AG-1 at liquids medium culture on methanol extracts showed much higher extraction yields than the strain PSJ-1 mycelial.

Scavenging activity on DPPH radicals. The primary reaction which takes place is the formation of free radical R. and the reduced form of DPPH. The parameter used to measure the radical scavenging activity of extracts and fractions evaluated is IC_{50} value, defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in this specified time period. The smaller IC_{50} value, the higher antioxidant activity of the plant extract/fraction (Maisuthisakul, 2007). The IC_{50} value of the various liquids medium culture of mycelial *C. militaris* two strains AG-1 and PSJ-1extracts are shown in Fig. 2.

The results in terms of strain AG-1 grown on different submerged culture method, IC_{50} values varied was: 3.13–7.50 mg/mL (FD); 2.96–5.04 mg/mL (OD) and IC_{50} values of strain PSJ-1 also differently ranged from: 2.77–5.07 mg/mL (FD); 2.69–4.85 mg/mL (OD), formula PD shake of both drying method also indicated the most effective with the lowest value of IC_{50}: 2.77 mg/mL (FD) and 2.69 mg/mL (OD). On the other hand, with the oven drying method, mycelial strain PSJ-1 extracts grown on formula PD shake showed the lowest value of IC_{50} (2.69 mg/mL) and was not significantly different with IC_{50} values of *C. militaris* mycelial extracts grown on formula MYPS shake (2.70 mg/mL), PVC shake (3.10 mg/mL), MYPS static (3.22 mg/mL), PD static (3.06 mg/mL), MYPS static + shake (3.46 mg/mL), PVC static + shake (3.34 mg/L); PD static + shake (3.20 mg/mL), respectively in Fig 2. The report that the mycelial biomass was subjected to extraction with boiling water for exopolysaccharides and intracellular polysaccharides production was tested under different factors from *C. cicadae* showed high DPPH scavenging activity (EC_{50}: 7.32 mg/mL EPS and 6.79 mg/mL IPS) at ranged from 60–100 mg/mL concentration, respectively), (Sapan Kumar Sharma, 2015). However, the extraction in hot water at 120°C for 30 min and 95% ethanol (1 : 10) for 7 day each at room temperature, and their results showed: IC_{50} = = 7.34 (hot water) and 3.81 mg/ml (95% ethanol) of

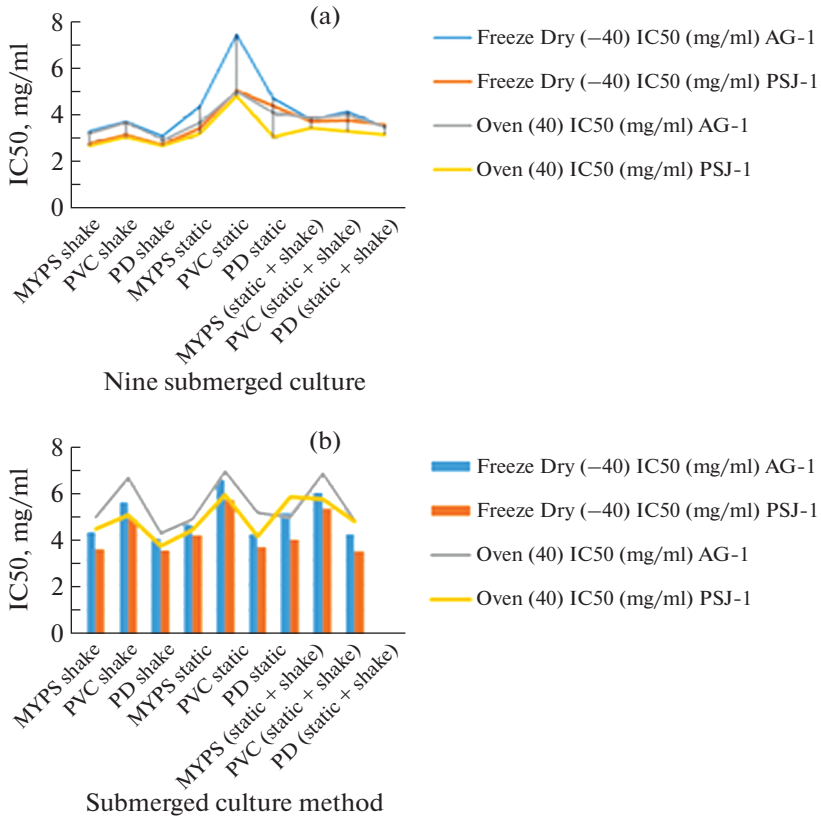

Fig. 2. IC_{50} values of DPPH (a) and ferrous ions concentration (b) scavenged by 50% methanol extracts from *Cordyceps militaris* mycelia. Each value is expressed mean ± standard error (n = 3). Means within the same capital letters followed by the same row are not significantly different ($p \leq 0.05$).

hydroxyl radicals at the final concentration of 3.67 g/L (sample/water) (Somkamol Manchun, 2017). The hot water extraction and ethanol precipitation of *C. militaris* fruiting bodies showed high scavenging of DPPH (89% at 8 mg/mL, respectively). (Wu, 2011). Some reports also available for DPPH scavenged activity (over 80%) of *C. militaris* against DPPH (Zhan, 2006). The results were similar to previous research when indicating that polysaccharides precipitated with ethanol and lyophilized with vacuum freeze-drying from *Ophiocordyceps sinensis*. This polysaccharide at 3 mg/mL scavenged DPPH radical (58.67 and 72.41%, respectively). Mycelial extracts of two strains AG-1and PSJ-1 grown on liquids medium culture showed the highest values of DPPH free radical scavenging ability with the lowest values of IC_{50} (Fig. 2). The lowest values of DPPH radical scavenging ability with the highest IC_{50} of mycelial strains PSJ-1 were recorded at liquids medium culture formula. Virtually mycelial of two strains AG-1and PSJ-1 grown on different substrates which was freeze dried (FD) showed significantly better on DPPH radical scavenging than those dried by oven drying (OD) method. The water extract of *Cordyceps militaris* mycelia showed far lower scavenging of DPPH (0.318 ± 0.021 at a 60 µl sample, respectively) (Kim, 2015). The methanol extract of natural and in vitro cultured mycelium of *Ophiocordyceps sinensis* showed scavenging activity of DPPH (2.98 mg/ml and 2.81 mg/ml sample, respectively) (Singh, 2018). The methanolic extracts of *C. militaris* strain: MCI 10304 fruiting bodies showed high scavenging of DPPH scavenging activity EC_{50}: 12.17, respectively). (Filipa, 2013). Also reported that the antioxidant activity of methanolic extract of *C. militaris* (strain: MCI 10304, species revealing EC_{50} values for DPPH radical-scavenging activity ranging from 0.68–20.02 mg/ml, and 4.58–58.14 mg GAE/g for Folin–Ciocalteu assay; therefore the EC_{50} value presented herein could be considered within the range of the results presented by the mentioned authors (Pereira, 2012). Wherefore the EC_{50} value reported herein was considered within the range of the results presented by the mentioned authors. *C. militaris* revealed the presence of some antioxidant molecules such as δ-tocopherol or phydroxybenzoic acid, which may be related to its antioxidant activity (Heleno, 2010; Pereira, 2012).

Chelating effect on ferrous ions. The effective of ferrous chelating ability is also one of the important antioxidant properties. Transition metals such as Fe^{2+} are known to play key roles in lipid peroxidation by generating hydroxyl radicals through Fenton reaction. In the assay, the ferrous ion chelating ability of the antioxi-

dants was detected by inhibiting the formation of red-colored ferrozine- Fe^{2+} complex. (Wu, 2011). The IC_{50} value of the various liquids medium culture of mycelial *C. militaris* two strains AG-1 and PSJ-1 extracts are shown on all of the formulas (Fig. 2).

The results in terms of strain AG-1 grown on different submerged culture method, IC_{50} values varied was: 3.90–6.26 mg/mL (FD); 3.52–5.65 mg/mL (OD) and IC_{50} values of strain PSJ-1 also differently ranged from: 3.36–5.42 mg/mL (FD); 3.08–4.83 mg/mL (OD), formula PD static + shake and PD shake of both drying method also indicated the most effective with the lowest value of IC_{50}: 3.36 mg/mL (PSJ-1-FD) and 3.08 mg/mL (PSJ-1-OD). In comparison between FD and OD methods of mycelial, there were not so many differences in the chelating ability of mushroom PSJ-1 and AG-1. The chelating ability of two strains AG-1 and PSJ-1 in our study was lower than that of wild *Ophiocordyceps sinensis* indicated by (Zheng, 2008) (EDTA chelated almost 87.4% of Fe^{+2} whereas the extracts chelated only 45.5% of Fe^{2+} at a concentration of 10 mg/mL, respectively). In our study, the high ferrous ion chelating ability of methanolic extracts *C. militaris* both strains AG-1 and PSJ-1 mycelial was beneficial. This fact was due to ferrous ions being the most effective pro-oxidations in food systems. (Dong, 2014) showed that the *Cordyceps militaris* a comparative study of the antimicrobial, antioxidant, and cytotoxic activities of methanol extracts from fruit bodies and fermented mycelia had stronger ferrous ion chelating ability (75%) at a concentration of 2 mg/ml, respectively). The hot-water extracts from natural and cultured mycelia of *Ophiocordyceps sinensis* showed high scavenging of the chelating abilities 1,2 and reached (41.86 and 53.86% at 8 mg/ml, respectively) (Dong, 2007).

Mycelial extracts of two strains AG-1and PSJ-1 grown on PVC media culture showed the highest values of the chelating abilities of 1,2 with the lowest values of IC_{50} (Fig. 2). The lowest value of the chelating abilities on Fe^{2+} with the highest IC_{50} of mycelial strains PSJ-1 has recorded at PVC media culture formula. Virtually mycelial of two strains AG-1and PSJ-1 grown on different liquids culture which were (FD) showed significantly better on the chelating abilities 1.2 on Fe^{2+} than those dried by (OD) method. Almost mycelia of solid-state fermentation samples and grain substrates with *Cordyceps militaris* grown on different substrates which were effect of ultraviolet B (UVB) light irradiation showed significantly better on chelating ability than those dried by drying method results showed IC_{50}: hot water: 0.69, 0.68, 0.87, 0.58, 0.65, 0.68, 12.2, 8.3, 8.0, 7.6, 6.37, 6.8 mg/mL and ethanolic: 8.2, 6.9, 8.7, 5.4, 3.7, 6.8, 23.9, 15.1, 26.0, 10.9, 7.8; 7.6 mg/mL (B – buckwheat, BM – buckwheat mycelia, B-U – UVB irradiated buckwheat, CBD-U – fermented then dried and UVB irradiated buckwheat, CBF-U – fermented and UVB irradiated buckwheat, CBN-U – fermented unirradiated buckwheat, ER – embryo rice, ERM – embryo rice mycelia, ER-U – UVB irradiated embryo rice, CERD-U – fermented then dried and UVB irradiated embryo rice, CERF-U – fermented and UVB irradiated embryo rice, CERN-U – fermented embryo rice without UVB irradiation, respectively) (Shih-Jeng Huang, 2017). The water extract of *C. militaris* mycelia showed far lower the chelating abilities of 1, 2 and EDTA on Fe^{2+} (EC_{50}: 385.68 μg/mL and 107.14 μg/mL at 60 μl in the range of concentrations from 25 to 1600 μg/mL sample, respectively) (Jing, 2015).

Hydroxyl radical scavenging. The hydroxyl radical scavenging activities can easily penetrate the cell membrane and virtually damage all types of macromolecules, such as carbohydrate, protein, lipids, and DNA, resulting in necrotic cell and organic pathology (Reiter, 1997). The hydroxyl radical has a very short in vivo half-life of neighborhood 10^{-9} sec and high reactivity (Sies, 1993). Thus, removing $OH^{\bullet}$ is very important for the protection of living systems.

In our study, the results of the terms that all the mycelial extracts were able to hydroxyl radical scavenging. At each extract concentration, the different liquids culture sample of both drying methods showed different scavenging ability on hydroxyl radical scavenging (Fig. 3). In case of 10 mg/mL concentration, two strains AG-1and PSJ-1 showed the highest values of hydroxyl scavenging activities on hydroxyl radical scavenging with range from AG-1: 78.40–87.92% (freeze drying); PSJ-1: 74.91–81.65% (freeze drying) and AG-1: 77.70–84.67% (oven drying); PSJ-1: 73.29–79.79% (oven drying). The results in terms of strain AG-1 grown on different culture methods, IC_{50} values varied was: 2.69–4.12 mg/mL (FD); 2.16–3.57 mg/mL (OD) and IC values of strain PSJ-1 also differently ranged from 2.12–3.12 mg/mL (FD); 1.84–3.01 mg/mL (OD) (Fig. 3). The results were similar to previous research when increased with the increasing concentrations that the extraction in hot water of 95°C for 30 min was adopted and their results showed 76% of the highest scavenging ability of hydroxyl radicals at the final concentration of 3.67 g/L (sample/water) (Shen, 2001). However, the methanol extract from PO at 6.4 mg/ml scavenged DPPH radical by 81.8%, whereas scavenging effects of methanol extract of natural and in vitro cultured mycelium of *Ophiocordyceps sinensis* were observed 86% and 84% at a concentration of 1.4 mg/ml of radical scavenging activities whose IC_{50} values was 3.28 mg/ml and 2.95 mg/ml, respectively. (Seema Singh, 2018). The hot water extracts of cultured and natural *O. sinensis* mycelia showed lower scavenging of radical scavenging activities (IC_{50}: 0.96 ± 0.06 mg/ml and 1.03 ± 0.03 mg/ml at the concentration of 0.25–2.0 mg/ml), respectively) (Dong, 2007). The hot water and ethanol extracts of hydroxyl radicals scavenging ratio during the submerged fermentation on mycelium showed high radicals scavenging of hydroxyl scavenging (hot water extract: 37.1–74.2% and ethanol extract:

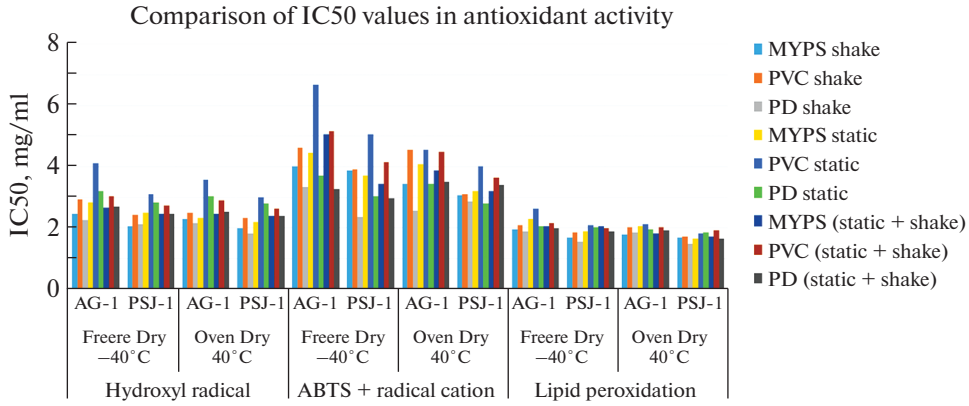

Fig. 3. Comparison of antioxidant activities of extracts from liquid cultural media of *Cordyceps militaris* mycelial submerged culture. IC_{50} values the of hydroxyl radical; $ABTS^{+}$ radical cation, lipid peroxidation concentration at which the radical scavenging assay, radical cation, inhibitory activity were scavenged by 50% methanol extracts from *C. militaris* mycelia.

92.7–99.4% at 1st day to 6th day cultivation, respectively) (Gu, 2006).

Scavenging activity of $ABTS^{\pm}$ radical cation. The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and vitamin C (Walker, 2009). Through this reaction, the blue ABTS cation is converted back to its colorless neutral form, the reaction can be monitored by spectrophotometrically. This essay is often referred to as the Trolox equivalent antioxidant capacity (TEAC) assay. The reactivity of the various antioxidants tested is compared to that of Trolox, which is a water-soluble analog of vitamin E (Barclay, 1985). The $ABTS^{\cdot+}$ of *C. militaris* mycelial extracts grown on different liquids culture formulas and dried by freeze drying method (FD) and oven drying (OD) method is shown in Fig 3. The $ABTS^{\cdot+}$ of *C. militaris* mycelial extracts increased along with the increased concentrations. In case of 10 mg/mL concentration, the ABTS radical scavenging activity of mycelial strain AG-1 extracts ranged from 66.82 ± 1.74 to 80.23 ± 1.48 (FD), and 66.54 ± 0.93 to 77.15 ± 0.81 (OD) while the ranges of mycelial strain PSJ-1 extracts were 66.48 ± 0.88 to 74.36 ± 1.25 (FD), and 65.98 ± ± 2.40 to 76.58 ± 0.60 (OD). The significantly strongest ABTS radical scavenging activity was identified in an extract of mycelial strain AG-1 grown on the culture medium formula PVC static media in both cases of (FD) and (OD) were almost concentrations with the IC_{50} values: 3.27–6.68 mg/mL (FD), 2.58–4.57 mg/mL (OD). In case of mycelial strain PSJ-1, on both dryings were almost concentrations with the IC_{50} values: 2.36–5.07 mg/mL (FD), 2.87–4.03 mg/mL (OD), formula PD shake also indicated the most effective with the lowest value of IC_{50} (2.36 mg/mL). On the contrary, with oven drying method, mycelial strain PSJ-1 extracts grown on formula PD shake showed the lowest value of IC_{50} (2.87 mg/mL) and was not significant different with IC_{50} values of *C. militaris* mycelial extracts grown on formula MYPS shake (3.06 mg/mL), PVC shake (3.10 mg/mL), MYPS static (3.22 mg/mL), PD static (2.79 mg/mL), MYPS static + shake (3.22 mg/mL), PVC static + shake (3.65 mg/L); PD static + shake (3.42 mg/mL) (Fig. 3). For example, ABTS radical scavenging of methanolic extract of unfermented chickpea (UFC) and *C. militaris* fermented chickpea (CFC) at 4 mg/ml on ABTS radical were 59.1 and 96.8%, respectively (Fan, 2009) reported that Douchi, a traditional Chinese fermented soybean product, had stronger ABTS radical scavenging activity than unfermented steamed soybeans, and (Kim, 2011). Among freeze drier of methanolic (80%) and ethanolic (80%) extracts of UFC and CFC from commercial a solid state fermentation of chickpeas with *C. militaris* SN-18 exhibited excellent ABTS radical scavenging activity of EC_{50}: 3.61 mg/mL (methanolic 80%) and 3.34 mg/mL (ethanolic 80%), 5.25 mg/mL (hot water) at 4 mg/ml, respectively (Xiao, 2014). However, stated that the methanolic extract from natural and in vitro cultured mycelium of *Ophiocordyceps sinensis* showed a strong ABTS radical scavenging activity was found to be approx 82 and 81%, at 2.0 mg/mL concentration, respectively. The IC_{50} value of methanolic extract of natural and in vitro cultured sample was 4.94 mg/ml and 4.65 mg/ml, respectively. (Singh, 2018). According to (Sapan Kumar Sharma, 2015), stated that the hot water extract from exo-polysaccharides (EPS) and intracellular polysaccharides (IPS) extracted from the mycelial culture of *Cordyceps cicadae* incubated showed a strong the ABTS radical scavenging activity of EC_{50}: 6.38 mg/mL (EPS) and 5.23 mg/mL (IPS), at a concentration of 8.0 mg/mL. The ethanol extracts fermentation of *C. militaris* CICC-14013 mycelia showed high scavenging of ABTS· scavenging activities IC_{50} of *C. militaris* polysaccharide (CMP), *C. militaris* polysaccharide deprotonated by (CMP-D-S) and derationed polysaccharide by an enzyme (CMP-D-E) was 7.264, 7.192 and 6.966 mg/mL, at 6 mg/mL, respectively (Chen, 2017).

Inhibition of lipid peroxidation. Lipid peroxidation was the subject of extensive studies for several decades, and its mechanisms, dynamics, and products are now fairly well estable lished. It was first studied in relation to the oxidative deterioration of foods. In 1955, the oxygenase enzyme was discovered by Hayaishi (1955) and Mason (1955) independently, and since then lipid peroxidation by lipoxygenases and cyclooxygenases has been studied extensively. Lipid oxidation by cytochrome P450 has also been studied and is well documented. The free radical mediated peroxidation of lipids has received a great deal of attention in connection with oxidative stress in vivo. The oxidation hypothesis for atherosclerosis (Steinberg, 1989) has stimulated extensive studies on the oxidative modification of low-density lipoprotein. The lipid peroxidation of the two strains AG-1 and PSJ-1 of *C. militaris* mycelial extracts grown on different liquids culture formulas and dried by oven drying method and freeze drying method was shown in Fig. 3.

The lipid peroxidation of the two strains AG-1 and PSJ-1of *C. militaris* mycelial extracts increased along with the increased concentrations for 10 mg/mL concentration, two strains AG-1 and PSJ-1 of *C. militaris* mycelial extracts exhibited highest antioxidant activity comparatively than other extracts and the results were compared with butylated hydroxyl toluene (BHT). The mean values of the two strains AG-1 and PSJ-1 of *C. militaris* mycelial were not significantly ($P < 0.05$) different from BHT. In case of 10 mg/mL concentration, their activity to inhibit lipid peroxidation of strain AG-1 extracts ranged and the overall results were decreased in the order of BHT > PVC static > MYPS static > PVC static + shake > PVC shake > MYPS static + + shake > MYPS shake > PD static > PD static + shake > > PD shake. In case of 10 mg/mL concentration, their activity to inhibit lipid peroxidation of strain AG-1 extracts ranged are decreased as from: 67.00 ± 1.15–79.67 ± ± 1.20% (FD) and 62.00 ± 0.58–77.67 ± 1.45% (OD) while the ranges of strain PSJ-1 extracts was: 64.67 ± ± 1.45–72.83 ± 1.01% (FD) and 58.67 ± 0.67–70.33 ± ± 0.33% (OD), respectively. Here, the mean values of the two strains AG-1 and PSJ-1 of *C. militaris* mycelial extracts were not significantly ($P < 0.05$) different and showed the highest activity. For 2 mg/mL concentration extracts, the two strains AG-1 and PSJ-1 of *C. militaris* mycelial extract showed significantly ($P < < 0.05$) highest activity in comparison with BHT. In the Fig. 4, the significantly strongest lipid peroxidation inhibition was identified in an extract of strain AG-1 grown on the formula PD shake in both cases of freeze drying and oven drying at almost concentrations with the lowest values of IC_{50}: 1.91 mg/mL (FD), 1.88 mg/mL (OD) (Fig. 4). However, the results presented the methanolic extract of *C. militaris* revealed the lowest EC_{50} value for lipid peroxidation inhibition (1.05 mg/ml), respectively (Filipa, 2013). The water extracts from natural *Ophiocordyceps. sinensis* and cultured *Cordyceps* mycelial inhibited the microsomal lipid peroxidation as induced by Fe (III)–ADP/NADPH system. All *Cordyceps* showed similar potency, the overall inhibition activities were lower than the other anti-oxidation assays; the inhibition was below 20% in all concentrations that we tested. The extrapolated IC_{50} from the tested *Cordyceps* could reach 5 mg/ml, respectively (Li, 2001). From *C. militaris* waster medium (CMWM), while inhibition of lipid peroxidation of the CMWM extracts at 15.0 mg/mL reached nearly 50%, at a concentration of 4.0 mg/mL, respectively (Wang, 2015).

IC_{50} values in antioxidant activity. As shown in Fig. 3, the IC_{50} values of the tested samples were calculated for comparison. With regard to the mycelial extract of two strains AG-1 and PSJ-1 showed positive antioxidant activity by methanol extract of hydroxyl radical scavenging assay, scavenging activity of $ABTS^+$ radical cation; inhibition of lipid peroxidation, formulas containing liquids medium and MYPS media of submerged Culture method by of two strains AG-1 and PSJ-1 had higher values of the freeze dry (–40°C) were more effective than oven dry (40°C). The effectiveness of the nine mycelial extracts for both the strains AG-1 and PSJ-1as evidenced by lower IC_{50} values a descending order IC_{50} of ABTS + radical cation > hydroxyl radical scavenging assay > inhibition of lipid peroxidation in Fig. 4. Their activity to radical scavenging, scavenging activity, and inhibition lipid peroxidation of both strain AG-1 and PSJ-1 extracts ranged and the overall results were decreased in the order of PVC static > MYPS static > PD static > PVC static + shake > MYPS static + + shake > PD static + shake > PVC shake > MYPS shake > PD shake. In addition, the submerged culture method of PVC static media was more effective than all the formulas. For the methanol extract with freeze dry of liquids static activity of mycelial strains AG-1 and PSJ-1extracts the IC_{50} values were: AG-1: 4.12 mg/mL and PSJ-1: 3.12 mg/mL of hydroxyl radical, AG-1: 6.68 mg/mL and PSJ-1: 5.07 mg/ml of $ABTS^+$ radical cation, and AG-1: 2.64 mg/mL and PSJ-1:2.10 mg/mL of inhibition of lipid peroxidation, respectively. It is obvious that the scavenging activity of $ABTS^+$ radical cation activity of PVC static products was greatly enhanced as mycelial grew. In case of oven dry of PVC static activity of mycelial strains AG-1 and PSJ-1extracts the IC_{50} values were: AG-1: 3.57 mg/mL and PSJ-1: 3.01 mg/mL of hydroxyl radical, AG-1: 4.57 mg/mL and PSJ-1: 4.03 mg/ml of $ABTS^+$ radical cation, and AG-1: 2.14 mg/mL and PSJ-1: 1.83 mg/mL of inhibition of lipid peroxidation, respectively (Fig. 3). However, with regard to inhibition of lipid peroxidation, the oven dried saples showed the highest activity with the lowest IC_{50} values, and the freeze dried (–40°C) products showed higher activity than the oven dry (40°C) showed the lowest activity (IC_{50} values: AG-1: 1.91 mg/mL and PSJ-1: 1.67 mg/mL), and there was no significant difference between the ethanol extracts of freeze dry (–40°C) and oven dry (40°C).

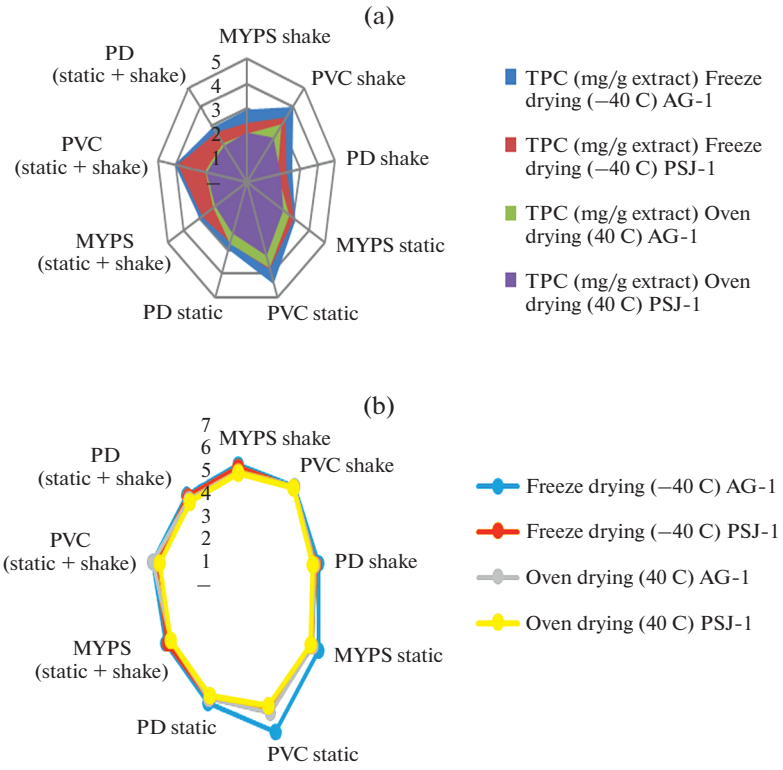

Fig. 4. Total phenolic (a) and flavonoids (b) contents in *Cordyceps militaris* submerged culture methanol extracts.

Total phenolic and flavonoids contents

Total phenolic contents. Phenolic are secondary metabolites commonly found in plants, mushrooms, and fungi and have been reported to exert multiple biological effects (Dimitrios, 2006; Kim, 2008). Phenolic compounds which were known as antioxidant components defend our body against free radicals. The most antioxidant properties found in mushrooms are mainly in the form of phenolic acids and flavonoids (Ferreira, 2009). The recovery of phenolic content in different samples is affected by the polarization of the extracting and the solubility of the compound in the solvent used for extraction (Alothman, 2009; Sulaiman, 2011). Therefore, it is hard to select an appropriate solvent for the extraction of phenolic contents from all samples.

In result Fig. 4 showed that the case of freeze drying, strain AG-1 extracts grown on different submerged culture method formulas varied in the following order: 2.61 ± 0.24 to 4.44 ± 0.31 mg GAE/g extract. The highest value was achieved at formulas containing PVC static (4.44 ± 0.31 mg/g extract), followed by formulas MYPS static (3.15 ± 0.18 mg/g extract), PD static (2.92 ± ± 0.18 mg/g extract), PVC static + shake (4.01 ± ± 0.26 mg/g extract); MYPS static+shake (3.04 ± ± 0.18 mg/g extract), PD static + shake (2.88 ± 0.10 mg/g extract) and PVC shake (4.01 ± 0.27 mg/g extract); MYPS shake (2.92 ± 0.12 mg/g extract), PD shake (2.61 ± 0.24 mg/g extract). Formulas PVC static, MYPS static, PD static, PVC static+shake, MYPS static + shake, PD static + shake, PVC shake, MYPS shake, PD shake gave the lowest values of strain PSJ-1 in the following order: (2.25 ± 0.31 to 3.82 ± 0.21 mg/g extract). On the contrary, in the case of oven drying, TPC of strain AG-1 mycelial extract ranged in the following order: 1.75 ± 0.07–3.74 ± 0.18 mg/g extract. Formulas containing PVC static also gave the highest TPC of strain AG-1 (3.74 ± 0.18 mg/g extract). Formulas containing PVC static, MYPS static, PD static, PVC static + shake, MYPS static + shake, PD static + + shake, PVC shake, MYPS shake, PD shake were significantly different with formulas PD static in termss of TPC of strain AG-1. The TPC of strain mycelial PSJ-1 were low than those of strain AG-1, which ranged was: 1.94 ± 0.21 to 3.23 ± 0.04 mg/g extract in case of (OD) and 2.25 ± 0.31 to 3.82 ± 0.21 mg GAE)/g dry extract (FD), respectively (Fig. 4).

The results of our study were similar to some previous reports. The difference in different ten submerged culture method formulas of *C. militaris* powder (CM) and cordycepin-enriched *C. militaris* powder (CCM) and fermented CCM showed the difference in TPC (CM: 2.68, CCM:3.07, CCM + Lh: 3.19, CCM + La: 3.82, CCM + Sc:3.72, CCM + Bs:4.03, CCM + Mp: 3.54, CCM + Ao:3.25, CCM + Ak:3.53, CCM + Ro: 2.56 mg GAE/g extract, respectively) (Ahn, 2013; Wang, 2015) indicated that TPC of *C. militaris* waster medium was 3.91 mg GAE/g extract (100% distilled water). However, in other research, the TPC of *C. mil-*

itaris water and ethanol extract were 3.9 mg GAE/g extract. (Lee, 2013). The results in this study were lower than those of some reports. TPC of solvent extracts of fermented wheat and the unfermented steamed wheat, samples were freeze dried, including water (acidified with 1% HCl, pH = 3), 70% ethanol and 70% acetone extracts of *C. militaris* were: unfermented wheat (70% ethanol: 36.94 mg/g; 70% acetone: 54.16 mg/g; water: 29.37 mg/g) and fermented (70% ethanol: 41.63 mg/g; 70% acetone: 66.37 mg/g; water: 32.79 mg/g) extract, respectively) (Zhang, 2012; Xiao, 2014) also indicated that TPC of unfermented and *C. militaris*-fermented wheat of methanolic (80%) and ethanolic (80%) extracts of unfermented chickpea and *C. militaris*-fermented chickpea (CFC), samples were extracted with 40 folds of methanol (80%) or ethanol (80%) in water was: UFC (methanol (80%): 7.08 mg/g; ethanol (80%): 7.36 mg/g; water: 6.07 mg/g extract, respectively) (Xiao, 2014). Reports were demonstrated that the antioxidant potential is positively correlated with the total phenolic compounds (Kaur, 2002). On the contrary, the TPC in our study were much higher to compare with the results of some previous researchers. The TPC in methanolic extract of aqueous extracts from natural and cultured *Ophiocordyceps sinensis* was: 17.07 mg GAE/g extract, while the values of three natural materials were almost equivalent. Varying from 12.02 to 12.14 mg GAE/g extract, respectively (Junqiao, 2015). TPC of extracts from the two strains AG-1and PSJ-1of *Cordyceps militaris* mycelial extracts in this study depended on different nine submerged culture method formulas, two strains AG-1 and PSJ-1of *C. militaris* but also on drying methods. In comparison to a freeze drying method, oven drying method of strain AG-1 possessed the higher TPC. Strain PSJ-1 grown on different nine submerged culture method formulas also achieved the significantly higher values of TPC in the case of freeze drying to compare with oven drying method. Strain PSJ-1 grown on other submerged culture method formulas had no significant difference between two drying methods (Fig. 4).

Total flavonoid contents. Total flavonoid contents was important for human health because of their high pharmacological activities as radical scavengers (Cook, 1996). Some mechanisms have been suggested for how flavonoids may help prevent hormone-dependent steroid cancer, but randomized clinical trials are still in progress. More research is needed to clarify the nature of the impact and interactions between these bioactive constituents and other dietary components, and to determsine how efficient and practical it would be to reduce cancer risk and other human illness. Therefore, an overview of the recent achievements in the flavonoid study is mandatory, with special reference to the study progress on dietary appearance and health aspects of flavonoids (Yao, 2004). Expected these properties, flavonoids can be used as ingredients in the production of cosmetics and pharmaceutical products (Chuarienthong, 2010; Malinowska, 2013).

In the Fig. 4 showed that the case of freeze drying, strain AG-1 extracts grown on different submerged culture method formulas varied in the following order: 5.06 ± 0.13 to 6.87 ± 0.16 mg QE/g extract. The highest value was achieved from formulas containing PVC static (6.87 ± 0.16 mg/g extract), followed by formulas MYPS static (5.90 ± 0.11 mg/g extract), PD static (5.55 ± 0.29 mg/g extract), PVC shake (5.62 ± 0.10 mg/g extract); MYPS shake (5.23 ± 0.29 mg/g extract), PD shake (5.20 ± 0.30 mg/g extract), and PVC static + + shake (5.51 ± 0.08 mg/g extract); MYPS static + + shake (5.25 ± 0.12 mg/g extract), PD static + shake (5.06 ± 0.13 mg/g extract). Formulas PVC static, MYPS static, PD static, PVC static + shake, MYPS static + shake, PD static + shake, PVC shake, MYPS shake, PD shake gave the lowest values of strain PSJ-1 in the following order: (4.97 ± 0.04 to 5.80 ± 0.10 mg/g extract). On the contrary, in the case of oven drying, TFC of strain AG-1 mycelial extract ranged in the following order: 4.93 ± 0.28–6.01 ± 0.22 mg/g extract. Formulas containing PVC static also gave the highest TFC of strain AG-1 (6.01 ± 0.22 mg/g extract). Formulas containing PVC static, MYPS static, PD static, PVC static + shake, MYPS static + shake, PD static + + shake, PVC shake, MYPS shake, PD shake were significantly different with formulas PD static in termss of TFC of strain AG-1. The TFC of strain mycelial PSJ-1 were lower than those of strain AG-1, which ranged was: 4.70 ± 0.07 to 5.70 ± 0.21 mg/g extract in the case of oven drying, respectively (Fig. 4). Total flavonoid contents in our study were significantly different and were depended on two strains AG-1 and PSJ-1, on oven drying method different nine submerged culture formulas, two strains AG-1 and PSJ-1of *C. militaris* used (Wang, 2015) indicated that TPC of *C. militaris* medium was 3.91 mg GAE/g extract (100% distilled water). The total flavonoid contents (TFC) of different nine submerged culture formulas, two strains AG-1 and PSJ-1 of *C. militaris* in our study was lower than that of TFP indicated by (Zhang, 2012) (unfermented wheat (70% ethanol: 65.23 mg/g; 70% acetone: 29.56 mg/g; water: 5.82 mg/g) and fermented (70% ethanol: 72.65 mg/g; 70% acetone: 32.27 mg/g; water: 13.29 mg/g) extract, respectively), but much higher than *C. militaris* powder and Cordycepin-enriched *C. militaris* powder (CCM) and fermented CCM hot water extract showed the difference in TPC (CM: 0.44, CCM: 0.55, CCM + Lh: 0.83, CCM + La: 0.59, CCM + Sc: 0.60, CCM + Bs: 0.68, CCM + Mp: 0.91, CCM+Ao: 0.82, CCM + Ak: 0.92, CCM + Ro: 0.73 mg GAE/g extract, respectively) (Ahn, 2013). And higher than that indicated that TFC of *C. militaris* waster medium was 4.26 mg of QE/g DW (100% distilled water) (Wang, 2015). However, indicated that TFC of unfermented and *C. militaris* fermented wheat on methanolic (80%) and ethanolic (80%) extracts of unfermented chickpea (UFC) and *C. militaris*

fermented chickpea, samples were extracted with 40 folds of methanol (80%) or ethanol (80%) in water was: UFC (methanol 80%): 7.08 mg/g; ethanol (80%): 7.36 mg/g; water: 6.07 mg/g extract, respectively) (Xiao, 2014).

CONCLUSION

In this study showed that single factor design and identify optimal culture conditions which improved biomass mycelial production by *C. militaris* two strains AG-1 and PSJ-1. Optimal PVC media contained: 30 g/l glucose; 10 g/L corn powder; 0.25 g/L $FeSO_4 \cdot \cdot 7H_2O$; 0.5 g/L vitamin B1, 0.6 g/L K_2HPO_4; 0.7 g/L $MgSO_4 \cdot 7H_2O$; 1 g/L KH_2PO_4; 6 g/L peptone. Using these static culture conditions a maximum biomass production of maycelial was PSJ-1: 18.20 ± 1.84 g/L; AG-1: 21.85 ± 1.00 g/L for 250 mL working volume in the 500 mL glass bottle. The total content of freeze drying and methanol extracts reached AG-1: 27.79 ± ± 0.59 mg/g; PSJ-1: 24.56 ± 0.64 mg/g and AG-1: 9.73 ± 0.13; PSJ-1: 8.84 ± 0.27 mg/g. Furthermore, the study also revealed that the antioxidant activity of the extracts varied depending on the extracted solvent. In conclusion, the antioxidant activity of the *C. militaris* mycelial had resulted in higher values of TPC, TFC as well as high efficiency of DPPH radical scavenging ability, ABTS. Whereas that, submerged culture formulas PVC reduced TPC and TFC had directly linked to a decreased antioxidant activity of two strains mycelial extracts. These results suggested that PVC static and MYPS static can be used to replace some parts or all PVC in substrate formulas for two strains cultivation which also improved antioxidant component and activities of two strains mycelial. Finally freeze drying methodof the *C. militaris* mycelial extracts had higher contents of total polyphenols, making it the most effective antioxidant fraction in the methanol extracts. Further investigation on the *in vivo* antioxidant ability of the *C. militaris* mycelial two strains was underway in our laboratory and submerged culture method with *C. militaris* was applied as a tool to develop mycelial as health food ingredient possessing multifunctional properties including antioxidant activity, which might be used as an alternative supplement to synthetic antioxidants since it is environmentally friendly and safe for consumption.

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ВЛИЯНИЕ СОСТАВА ЖИДКИХ ПИТАТЕЛЬНЫХ СРЕД НА АНТИОКСИДАНТНЫЕ СВОЙСТВА ЭКСТРАКТОВ МИЦЕЛИЯ *CORDYCEPS MILITARIS* (ШТАММЫ AG-1 И PSJ-1)

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Были исследованы антиоксидантные свойства экстракта мицелия двух штаммов *Cordyceps militaris* (AG-1 и PSJ-1) при глубинном культивировании. Экстракт мицелия был получен сублимационной сушкой (FD) и сушкой в печи (OD), а экстрагирован абсолютным алкоголем, горячей водой и метанолом. Содержание антиоксидантов определялось в метаноловых экстрактах; антиоксидантный потенциал определялся по изменению содержания DPPH, ионов двухвалентного железа, гидроксильного радикала, ABTS-катиона и перекисному окислению липидов. Результаты поверялись значениями IC_{50} с установлением минимальной ингибирующей концентрации. Обилие соединений с антиоксидантным действием подтвердило данные, полученные из экстрактов. Культивирование мицелия с более высоким содержанием статических сред привело к более высоким значениям общего содержания фенола и флавоноидов, а также к высокой активности DPPH и $ABTS^{+}$. Метаноловые экстракты штаммов AG-1 и PSJ-1 показали высокую антиоксидантную активность и влияние на перекисное окисление липидов. Были оценены антиоксидантные свойства и содержание антиоксидантов, состав экстрактов глубинных культур штаммов AG-1 и PSJ-1 и продемонстрирован значительный фармакологический потенциал этих штаммов.

Ключевые слова: антиоксидантные свойства, культуральные жидкости, лиофилизация, экстракты, *Cordyceps militaris*