Radical Scavenging Activities of *Asterina pectinifera* Fermented with *Cordyceps militaris* Mycelia

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Abstract *Asterina pectinifera* was fermented with *Cordyceps militaris* mycelia for improvement of anti-oxidant activities. DPPH, alkyl, hydroxyl, and superoxide radical scavenging activities were evaluated using electron spin resonance. Anti-oxidant activities were also determined based on the ferric reducing anti-oxidant power assays and the ABTS radical scavenging activity. The lipid peroxidation inhibition activity was confirmed using ferric thiocyanate and thiobarbituric acid assays. The free radical scavenging activity and anti-oxidative effects of *A. pectinifera* fermented with *C. militaris* mycelia (FACM) extracts were higher than for *A. pectinifera* and *C. militaris* mycelia extracts alone. FACM extracts contained different biochemical ingredients due to fermentation of *A. pectinifera* and provide a beneficial anti-oxidant activity. FACM extracts are a promising source of beneficial anti-oxidants for use in food industries.

Keywords: *Asterina pectinifera*, *Cordyceps militaris* mycelia, anti-oxidant activity

Introduction

Marine organisms have been widely used as human food stuffs for many years. Recently, much research has focused on biochemical components and biological activities of oceanic organisms. Therefore, isolation of natural anti-oxidants from marine organisms and study of diverse anti-oxidative (1), anti-coagulation (2), anti-tumor (3), anti-viral (4), anti-infection, anti-thrombotic (5,6), anti-inflammatory (7,8), and anti-HIV (9,10) activities has flourished. Starfish have long been used as a traditional medicine in Korea for treatment of stomach symptoms (11). Recently, research has focused on structural elucidation and the pharmacological importance of starfish-derived polysaccharides (12,13). However, starfish play a negative role in ecosystems and fisheries. Starfish outbreaks have caused damage to coral reefs. Starfish have also caused adverse effects for reef-feeding fish and benthic organisms (14,15).

Starfish are major invasive organisms of Phylum Echinodermata. Increased starfish populations are a threat to commercially important bivalve populations (16,17). In addition, most starfish, which are predators of shellfish, cause damage to aquatic farms in Korea. Up to 50 shellfish can be killed by a starfish every 6 days. Thus, Korean farmers have devoted much effort to combating starfish. However,

there is no suitable method for use of starfish waste, except composting (18). Therefore, conversion of starfish waste into a bioactive resource using fermentation was attempted in this study. Anti-oxidant activities of *A. pectinifera* fermented using *C. militaris* mycelia (FACM) are first reported herein. FACM showed increased anti-oxidant activities, compared with unfermented *A. pectinifera* and *C. militaris*. The anti-oxidant activity of FACM indicates that separation and identification of anti-oxidative components are desirable for further investigation.

Materials and Methods

Materials The *A. pectinifera* was collected from the Tongyeong marine waterfront area of Tongyeong, Korea in August of 2015. The fungal species *C. militaris* was obtained from Dr. Jae-Hyun Jeong of the Korea National University of Transportation in Jeungpyeong, Chungbuk, Korea. 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), AAPH, DPPH, and (4-pyridyl-1-oxide)-*N*-tert-butyl nitron (4-POBN) were obtained from Sigma-Aldrich. (St. Louis, MO, USA). All other reagents were of analytical grade.

Extract preparation The *A. pectinifera* was inoculated with *C. militaris* mycelia and the culture maintained in an incubator at 25°C for 20 days. After fermentation of *A. pectinifera*, those were dried using freezing drier. The dried *C. militaris*, fermented and unfermented *A. pectinifera* was boiled with 10 times of distilled water for 2 h separately. Then the extracts were filtered with Whatman No. 41 paper, evaporated and lyophilized. All the extracts were vacuum dried and stored at -20°C until used. The extraction yields from FACM are shown in Table 1.

Measurement of radical scavenging activities using ESR

DPPH radical scavenging activity: DPPH radical scavenging activities were assessed using the method described by Nanjo *et al.* (19). A reaction mixture containing 60 µL samples at different concentrations was added to 60 µL of 60 µM DPPH in a methanol solution. After vigorous mixing for 10 sec, the solution was transferred to a 100 µL Teflon capillary tube and the DPPH scavenging activity was measured using an ESR spectrometer (JES-FA100; JEOL Ltd., Tokyo, Japan). After 2 min, the spin adduct was measured using the ESR spectrometer. Experiments were conducted under conditions of a central field of 3,745 G, a modulation frequency of 100 kHz, a modulation amplitude of 2 G, a microwave power of 5 mW, and a temperature of 298 K.

Hydroxyl radical scavenging activities: Hydroxyl radical scavenging activities were assessed using an ESR spectrometer. Hydroxyl radicals were generated in an iron-catalyzed Haber-Weiss reaction (Fenton-driven Haber-Weiss reaction), and hydroxyl radicals rapidly reacted with nitron spin-trap DMPO. The resultant DMPO-OH adduct was detected using the ESR spectrometer. A reaction mixture containing 0.2 mL of each sample at different concentrations, 0.2 mL of 0.3 M DMPO, 0.2 mL of 10 mM FeSO₄, and 0.2 mL of 10 mM H₂O₂ containing a pH 7.2 phosphate buffer solution was transferred to a 100 µL Teflon capillary tube. After 2.5 min, the ESR spectrum was recorded using a JES-FA ESR spectrometer. Experiments were conducted under conditions of a central field of 3,475 G, a modulation frequency of 100 kHz, a modulation amplitude of 2 G, a microwave power of 1 mW, and a temperature of 298 K.

Alkyl radical scavenging activities: Alkyl radicals for assay of alkyl radical scavenging activities were generated using AAPH. A reaction mixture containing PBS (pH 7.4), 0.1 mL of 10 mM AAPH, 0.1 mL of 10 mM 4-POBN, and 0.1 mL of tested samples at different concentrations was incubated at 37°C in a water bath for 30 min. Samples were then transferred to 100 µL Teflon capillary tubes. The spin adduct was observed using an ESR spectrometer. Experiments were conducted under conditions of a central field of 3,475 G, a modulation frequency of 100 kHz, a modulation amplitude of 2 G, a microwave power of 1 mW, and a temperature of 298 K.

Superoxide radical scavenging activities: Superoxide radicals for assay of superoxide radical scavenging activities were generated via UV irradiation of a riboflavin/EDTA solution. A reaction mixture containing 0.1 mL of 0.8 mM riboflavin, 0.1 mL of 1.6 mM EDTA, 0.1 mL of 800 mM DMPO, and 0.1 mL of different sample concentrations

was exposed under a UV lamp at 365 nm for 1 min. The mixture was then transferred to a 100 µL quartz capillary tube and measured using an ESR spectrometer. Experiments were conducted under conditions of a central field of 3,475 G, a modulation frequency of 100 kHz, a modulation amplitude of 2 G, a microwave power of 10 mW, and a temperature of 298 K.

Ferric reducing antioxidant power (FRAP) assays: The FRAP assay has been used for assessment of anti-oxidant activities of compounds due to simplicity and accuracy. A reaction mixture containing a 3 mL aliquot of FRAP reagent, a 0.3 M acetate buffer, and 40 mM HCl containing 10 mM TPTZ and 20 mM ferric chloride (10:1:1, v/v/v) was mixed with 1 mL of sample. Absorbance values were compared with those obtained value from 0-10 mM standard curves of FeSO₄. Anti-oxidant activities were expressed as mM FeSO₄ equivalents in mg of extract (mM FeSO₄ equivalents/mg of extract).

ABTS radical scavenging activities: The total anti-oxidant activity of samples was estimated using an ABTS⁺ radical cation decolorization assay (20). A reaction mixture containing 7 mM ABTS and 2.45 mM potassium persulfate (K₂S₂O₈) was prepared. The assay mixture was kept in the dark at room temperature overnight for completion of radical generation, then diluted with distilled water until the absorbance value was 0.70±0.02 at 734 nm. ABTS radical scavenging activities of samples were determined using 0.9 mL of ABTS reagent mixed with 0.1 mL of extract for absorbance measurements at 734 nm after 6 min of reaction at room temperature using ethanol as a control. Anti-oxidant activities of FACM extracts were expressed as a Trolox equivalent anti-oxidant capacity (TEAC) as mM Trolox equivalents/mg of extract.

Lipid peroxidation inhibition activities

Ferric thiocyanate (FTC) method: The lipid oxidation inhibition activity of samples was measured following the ferric thiocyanate (FTC) method described by Kikuzaki and Nakatani (21). A reaction mixture containing 4 mg of sample in 99.5% ethanol, 2.51% linoleic acid in 4.1 mL of 99.5% ethanol, 8 mL of a 0.05 M phosphate buffer at pH 7.0, and 3.9 mL of distilled water was mixed in a screwcap container. The reaction mixture was kept in the dark at 40°C. Then, 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate were added to 0.1 mL of this solution. After 3 min, 0.1 mL of 3.5% hydrochloric acid containing 0.02 M ferrous chloride was added to the reaction mixture. The absorbance of the resulting red solution was measured at 500 nm. Vitamin C and α-tocopherol were used as positive controls, while a mixture without a sample was used as a negative control.

Thiobarbituric acid (TBA) assay: A TBA assay was used for assessment of lipid peroxidation following the method described by Ottolenghi (22). A reaction mixture containing 2 mL of 20% trichloroacetic acid (TCA), 2 mL of a 0.67% TBA solution, and 1 mL of a sample solution prepared following the FTC method were mixed. This mixture was kept in a boiling water bath for 10 min, followed by centrifugation at 1,900xg for 20 min. The absorbance of the supernatant was measured

at 532 nm. Vitamin C and α -tocopherol were used as positive controls, while a mixture without a sample was used as a negative control.

Statistical analysis Statistical analyses were performed using Tukey's test and a one-way analysis of variance (ANOVA), followed by post-hoc Duncan's multiple range tests. Graph Pad Prism software version 5.00 (GraphPad Software Inc., La Jolla, CA, USA) was used. Results were expressed as a mean $\pm$ standard deviation (SD) at a significance level of $p < 0.05$.

Results and Discussion

Fermentation and extraction Twenty days after *A. pectinifera* inoculation with *C. militaris* mycelia, the fermentation product was confirmed via visual observation. *C. militaris* mycelia grew well on both interior and exterior surfaces of the *A. pectinifera* fermentation product (Fig. 1). Extraction yields of *A. pectinifera*, *C. militaris* mycelia, and FACM were 13.94, 2.15, and 21.35%, respectively (Table 1). FACM extraction yields of *A. pectinifera* and *C. militaris* mycelia were significantly ($p < 0.05$) increased, compared with *A. pectinifera* and *C. militaris* mycelia, probably related to decomposition of *C. militaris* due to mycelial fermentation.

Radical scavenging activities The DPPH assay is widely used for evaluation of free radical scavenging activities of biomolecules in the fields of food and biomedical research (23). DPPH radical scavenging

activities of FACM, unfermented *A. pectinifera*, and *C. militaris* mycelial extracts were determined. The FACM extracts (IC_{50} , $10.06 \pm 1.25 \mu\text{g/mL}$) showed higher DPPH radical scavenging activities than *A. pectinifera* (IC_{50} , $313.40 \pm 4.26 \mu\text{g/mL}$) and *C. militaris* mycelia (IC_{50} , $318.00 \pm 1.96 \mu\text{g/mL}$) (Table 1). After fermentation, a 30 times increase in DPPH radical scavenging activities was observed. The alkyl radical spin adduct of the 4-POBN/free radical was generated from AAPH, and FACM extracts (IC_{50} , $71.38 \pm 3.74 \mu\text{g/mL}$) showed higher alkyl radical scavenging activities than *A. pectinifera* (IC_{50} , $416.70 \pm 11.2 \mu\text{g/mL}$) and *C. militaris* mycelia (IC_{50} , $372.00 \pm 2.92 \mu\text{g/mL}$) (Table 1). Thus, FACM extracts showed increased alkyl radical scavenging activities.

Hydroxyl radicals generated in the $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ system can be trapped using a DMPO forming spin adduct, which can be detected using an ESR spectrometer. FACM extracts (IC_{50} , $863.12 \pm 20.35 \mu\text{g/mL}$) showed higher hydroxyl radical scavenging activities than *A. pectinifera* (IC_{50} , $3,468.90 \pm 24.84 \mu\text{g/mL}$). However, FACM extracts showed lower activities than *C. militaris* mycelia (IC_{50} , $515.00 \pm 12.46 \mu\text{g/mL}$) (Table 1). Superoxide radical scavenging activities of samples are shown in Table 1. FACM extracts (IC_{50} , $12.40 \pm 1.20 \mu\text{g/mL}$) showed higher superoxide radical scavenging activities than *C. militaris* mycelia (IC_{50} , $866.00 \pm 2.54 \mu\text{g/mL}$). However, IC_{50} values for the superoxide radical scavenging activities of unfermented *A. pectinifera* extracts were not calculated. FACM extracts affected radical scavenging activities and the scavenging activity of FACM extracts was higher than for unfermented *A. pectinifera* (Table 1). Thus, biomolecules of *A. pectinifera* were probably converted via fermentation to produce enhanced biological activities.

Fig. 1. Observation of the exterior (A) and interior (B) of *A. pectinifera* fermented with *C. militaris* mycelia (FACM).

Table 1. DPPH, alkyl, hydroxyl, and superoxide radical scavenging activities of *A. pectinifera*, *C. militaris*, and FACM extracts based on ESR measurement

Materials	Yield (%, w/w)	DPPH radical (IC_{50} , $\mu\text{g/mL}$)	Alkyl radical (IC_{50} , $\mu\text{g/mL}$)	Hydroxyl radical (IC_{50} , $\mu\text{g/mL}$)	Superoxide radical (IC_{50} , $\mu\text{g/mL}$)
<i>A. pectinifera</i>	13.94 ¹⁾	313.40 ± 4.26^b	416.70 ± 11.20^b	3468.90 ± 24.84^c	ND ²⁾
<i>C. militaris</i>	2.15	318.00 ± 1.96^b	372.00 ± 2.92^b	515.00 ± 12.46^a	866.00 ± 2.54^b
FACM	21.35	10.06 ± 1.25^a	71.38 ± 3.74^a	863.12 ± 20.35^b	12.40 ± 1.20^a

¹⁾Values represent means $\pm$ SD ($n=3$).

²⁾Not detected.

^{a-c}Values of radical scavenging activity marked by different letter are significantly different at $p < 0.05$.

Table 2. Anti-oxidant activities of *A. pectinifera*, *C. militaris*, and FACM extracts

Materials	TEAC ¹⁾ (mM Trolox equiv./ mg extract)	FRAP (mM FeSO ₄ equiv./ mg of extract)
<i>A. pectinifera</i>	0.182±0.007 ^b	0.054±0.002 ^a
<i>C. militaris</i>	0.116±0.002 ^a	0.053±0.003 ^a
FACM	0.530±0.002 ^c	0.875±0.004 ^b

¹⁾TEAC: Trolox equivalent anti-oxidant capacity, FRAP: Ferric reducing anti-oxidant power.

^{a-c}Values of anti-oxidant activity marked by different letter are significantly different at $p < 0.05$.

Fig. 2. Anti-oxidant activity of *A. pectinifera*, *C. militaris*, and FACM extracts in a linoleic acid auto-oxidation system based on ferric thiocyanate (FTC) measurement. Data are represented as mean±SD ($n=3$). * $p < 0.05$ versus control group.

ABTS radical scavenging activities The ABTS radical scavenging activities of samples was determined following the decolorization assay method, which is applicable for assessment of both lipophilic and hydrophilic anti-oxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma anti-oxidants (24). Generation of ABTS radical cation formation was neutralized by extracts based on measurement using spectrophotometric methods (25,26). The neutralizing ability of extracts was attributed to anti-oxidant activities. The ABTS method is valid for analysis of both water-soluble and lipid-soluble anti-oxidants (24). The TEAC value of the FACM extract of 0.530 ± 0.002 mM Trolox equiv./mg was higher than for the *A. pectinifera* extract with a value of 0.182 ± 0.007 mM Trolox equiv./mg of extract and the *C. militaris* extract with a value of 0.116 ± 0.002 mM Trolox equiv./mg of extract (Table 2), indicating that the FACM extract exhibited greater anti-oxidant activities.

FRAP assay The antioxidant capacities of extracts are shown in Table 2. The results showed that the FACM extract (0.875 ± 0.004 mM

Fig. 3. Anti-oxidant activity of *A. pectinifera*, *C. militaris*, and FACM extracts in a linoleic acid auto-oxidation system based on thiobarbituric acid (TBA) measurement. Data are represented as mean±SD ($n=3$). * $p < 0.05$ versus control group.

FeSO_4 equiv./mg extract) had higher FRAP antioxidant activity followed by the extract of *A. pectinifera* (0.054 ± 0.002 mM FeSO_4 equiv./mg extract) and *C. militaris* (0.053 ± 0.003 mM FeSO_4 equiv./mg extract). The FRAP assay, now commonly used for screening of anti-oxidant activities of molecules in different fields of study, is characterized by reduction of Fe^{3+} to Fe^{2+} due to available reducing species, followed by a color change from yellow to blue observed using a spectro-photometer (27,28).

Lipid peroxidation inhibition assay Lipid peroxidation inhibitory effects of samples were assessed based on an ability to prevent peroxidation formation of 10 mg/mL linoleic acid using an FTC assay. Sample effects were compared with the commercial anti-oxidants vitamin C and α -tocopherol at 1.0 mg/mL as a positive control. The FTC assay was used for measurement of peroxide levels during the initial stage of linoleic acid oxidation (29). A control group of linoleic acid auto-oxidation without a sample was attended by a rapid increase in the peroxide value after day 2 of testing, reaching a maximum level on day 4, followed by a decline on day 6 (Fig. 2). With addition of different samples, FACM and α -tocopherol had a significant ($p < 0.05$) inhibitory activity against lipid peroxidation, compared with controls. Absorbance values for linoleic acid peroxidation of FACM were higher than for *A. pectinifera* and *C. militaris* and significantly ($p < 0.05$) extended the induction period of linoleic acid auto-oxidation. Moreover anti-oxidant activities of FACM extracts were similar to α -tocopherol activities. The TBA assay was used for analysis of later stages of lipid oxidation in which peroxide decomposes to form carbonyl compounds (29). Controls exhibited gradually increasing peroxide values from day 2, reaching a maximum level on day 8, followed by a decline on day 10 (Fig. 3). Until day 8, FACM showed

stronger inhibition of lipid oxidation, compared to controls and other extracts. Anti-oxidant activities of FACM can be inhibited for extension of the period of lipid oxidation.

C. militaris mycelial fermentation successfully enhanced inhibition of lipid peroxidation and anti-oxidant activities of *A. pectinifera*. Evaluation of the fermentation effect of *A. pectinifera* is reported herein for the first time. The bioactive potential of FACM can probably be attributed to biochemical conversion occurring during fermentation. Therefore, FACM is a potential source of natural anti-oxidants that is deserving of further study.

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Disclosure The authors declare no conflict of interest.

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