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RESEARCH ARTICLE

Evaluation of the Bioactive Properties of the Combined Ethanolic Extracts of *Cordyceps militaris* and *Suaeda maritima*

Urarux Romruen[1*], Eakaphun Bangyeekhun [2]

[1,2]Department of Microbiology, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand

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***Corresponding Author:**
urarux12@gmail.com

ABSTRACT

This research work aims to evaluate the bioactive properties of combined ethanolic extracts of *Cordyceps militaris* (CME) and *Suaeda maritima* (SME) and identify their potential health benefits. Seven different ratios of these extracts were prepared and analyzed for their antioxidant and antidiabetic activities. Extracts no. 6 (20:80, CME: SME) and 7 (pure SME) exhibited the highest 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity, with IC50 values of 377.66 ±13.97 and 305.28 ±28.70 µg/mL, respectively. This particular extract, number 7, also had the best ability to reactive with ABTS radicals, with an IC50 value of 172.15 ±15.48 µg/mL in the ABTS detection test. In the FRAP assay, extracts no. 6 and 7 displayed the highest antioxidant activity, with IC50 values of 6.30 ±0.80 and 6.48 ±0.22 µg/mL, respectively. Notably, extract no. 1 (pure CME) had the strongest α-amylase inhibitory effect, with an IC50 value of 281.35 ±31.71 µg/mL. Extract no. 6 had a much stronger α-amylase inhibitory effect than extract no. 7. Furthermore, LC-QTOF-MS/MS analysis identified four compounds with potential biological activities in extract no. 6. These findings highlight the potential health benefits of the combined ethanolic extracts of CME and SME and the presence of bioactive compounds in the extracts.

INTRODUCTION

Medicinal plants are increasingly preferred over pharmaceuticals due to their lower side effects, safety profile, and cost-effectiveness. According to the WHO (2001), they are easily accessible and act as the primary source of healthcare, particularly in the development of antibacterial and antioxidant medications. In recent years, the exploration of natural sources for bioactive compounds with potential therapeutic applications has gained significance in the field of pharmacology (Li et al., 2017; Mustafa et al., 2017). Fungi and plants, known for their rich biochemical diversity, have emerged as promising reservoirs for the discovery of novel drugs (Tiwari and Bae, 2022). The goal of this study is to look at the bioactive properties of ethanolic extracts made from the combined biomass of the parasitic fungus *Cordyceps militaris* and the halophytic plant *Suaeda maritima*, with a focus on how they might work together to have more powerful effects.

C. militaris, commonly known as the golden cordycepd mushroom, has a long history in East Asian medicine and has recently gained considerable attention for its diverse bioactive components. A nucleoside

analogue called cordycepin has been shown to fight tumours, reduce inflammation, and change the immune system (Xu et al., 2019). Polysaccharides from *C. militaris* have been shown to be antioxidants and change the immune system (Wang et al., 2019). These compounds contribute to the pharmacological potential of *C. militaris* (Phull et al., 2022; Abdullah et al., 2023). Recent research has shown that *Cordyceps* increases the energy of cells and fights bacteria, fungi, viruses, inflammation, diabetes, free radicals, tumours, sexual dysfunction, HIV, and the immune system.

S. maritima is a plant that grows in mangrove forests, saltwater swamps, and beaches. It has been recognized for its pharmacological significance (Mohamed et al., 2022), and its young leaves are high in protein, carbs, fibre, calcium, and β-carotene, making them useful for cooking. Its adaptation to high-salinity environments further adds to its appeal as a source of unique bioactive compounds, and it has a wide range of biological activities, including hepatoprotective effects, antibacterial activity, antioxidant capabilities, and antidiabetic potential (Sudjaroen, 2015; Mohamed et al., 2022). The chemical compounds found in *S. maritima* include n-tetradecanyl dihydrocaffeate, n-nonanyl-n-octadec-9-enoate, and n-hexadecanyl dihydrocaffeate. A closer look at the plant under a microscope shows that it is made up of septate hyphae and lateral, "tear-shaped" microconidia that may be many, scarred, or missing (Mohamed et al., 2022). *S. maritima*, a mangrove-associated plant, has been found to contain diverse chemical ingredients and has been evaluated for its antimicrobial properties, making it a valuable subject for further study.

Individual studies have extensively explored the medicinal properties of *C. militaris* and *S. maritima* separately. However, more research has yet to explore the potential synergistic effects when their extracts are combined. This study aims to fill in that gap by looking at the bioactivity of the ethanolic extracts of the two plants together. The goal is to find any interactions that might improve the effectiveness of therapy. It is well recognized that interactions between two crude extracts, each containing various bioactive compounds, can result in synergistic, antagonistic, and additive effects. For instance, the combination of green tea and oak fruit extracts demonstrated a wide range of interaction effects on antioxidant activity (Ranjbar Nedamani et al., 2015). Similarly, an aqueous extract of *Ganoderma lucidum* mixed with an ethanolic extract of *Chlorella vulgaris* has shown that they work well together to fight inflammation and free radicals (Abu-Serie et al., 2018).

We looked into the biological activities, mainly the antioxidant and α-amylase-inhibiting activities, of a mix of extracts from *C. militaris* and S*uaeda Maritima* in this study. The inhibition of α-amylase was used as a marker for evaluating the antidiabetic activity of the extracts. Additionally, the bioactive compounds present in the extract were evaluated through the liquid chromatography-quadrupole time of flight mass spectrometry (LC-QTOF-MS/MS). The findings from this study are expected to contribute valuable insights into the synergistic effects of *C. militaris* and *S. maritima* extracts, thereby opening a new way for the development of novel therapeutic formulations.

MATERIALS AND METHODS

Ethanol extraction

The fruiting bodies of *C. militaris* and *S. maritima* (seablite) leaves were collected from Nakhon Pathom and Samut Songkhram, Thailand, respectively. The samples were dried at 60°C and subsequently ground into a fine powder by using a mortar and pestle. The dry sample powder (50 g) was extracted with 250 mL of absolute ethanol in 15 cycles using Soxhlet equipment. The resulting extract was then filtered through Whatman filter paper no. 1, and the solvent was evaporated using a rotary evaporator. The dried extracts were re-suspended in dimethyl sulfoxide. The ethanolic extract from *C. militaris* (CME) was then combined with the ethanolic extract of *S. maritima* (SME) in seven different ratios: 100:0, 80:20, 60:40, 50:50, 40:60, 20:80, and 0:100.

Measurement of polysaccharide content

The procedure involved mixing 0.5 mL of 3 mg/mL extract with 0.5 mL of 5% (w/v) phenol solution using a vortex mixer and then adding 2.5 mL of 98% sulfuric acid. The mixture was then incubated at 30°C for 30 minutes before being measured at 490 nm with a spectrophotometer. D-glucose was used as a standard.

Determination of total phenol compounds

To measure the total phenolic content, 12.5 µL of extract (100 mg/mL), 50 µL of d/water, and 12.5 µL of Folin-Ciocalteu's phenol reagent were added to a 96-well plate, mixed well, and incubated at 30°C in the dark for 6 minutes. Subsequently, 125 µL of Na_2CO_3 (7% w/v) and 100 µL of water were added and thoroughly mixed. The reaction was then incubated at 45°C in the dark for 45 minutes. Absorbance was measured at 765 nm using a microplate reader, and gallic acid (20–200 mg/mL) was used as a standard.

Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging assay

In a 96-well plate, the extract was diluted twice. Then, 134 µL of 150 µM 2,2-diphenyl-1-Picrylhydrazyl (DPPH) was mixed with 66 µL of the diluted extract. The reaction was then incubated at 30°C in the dark for 45 minutes, and the absorbance was measured at 517 nm by using a microplate reader. Trolox was used as a standard. The percentage of DPPH radical scavenging activity was calculated by using the following equation:

$$\text{DPPH radical scavenging activity } (\%) = \frac{\text{Abs DPPH} - \text{Abs sample}}{\text{Abs DPPH}} \times 100$$

AbsDPPH is the absorbance of the DPPH solution mixed with DMSO. Abssample is the absorbance of the DPPH solution mixed with the extract.

Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging assay

The ABTS radical scavenging activity assay was performed by incubating a mixture of 7 mM ABTS solution and 2.45 mM potassium persulfate at 30°C in the dark for 16 hours. A total of 2.8 mL of the mixed solution was diluted with 65 mL of 0.1 M sodium acetate buffer (pH 4.5) to prepare the ABTS working solution. The extract was then serially twofold diluted in a 96-well plate, and 10 µL of the diluted extract was mixed with 200 µL of the ABTS working solution. After incubation at 30°C in the dark for 7 minutes, the absorbance of the reaction was measured at 734 nm by using a microplate reader. Trolox was used as a standard, and the percentage of ABTS radical scavenging activity was calculated with the following equation:

$$\text{ABTS radical scavenging activity } (\%) = \frac{\text{AbsABTS-Abs sample}}{\text{Abs ABTS}} \times 100$$

AbsABTS is the absorbance of the ABTS solution mixed with DMSO. Absample is the absorbance of the ABTS solution mixed with the extract.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP test needed a 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution, which was made by mixing 10 mM TPTZ with 40 mM HCl, 20 mM FeCl3, and 300 mM acetate buffer (pH 3.6). Subsequently, a twofold diluted extract (25 µL) was added to a 96-well plate, followed by 175 µL of TPTZ solution. The mixture was then incubated at 37°C in the dark for 30 minutes. After incubation, absorbance was measured at 593 nm by using a microplate reader. Trolox was used as a standard. The percentage of Ferric-Reducing Antioxidant Power (FRAP) was calculated with the following equation:

$$\text{FRAP value } (\%) = \frac{\text{Abs sample} - \text{Abs TPTZ}}{\text{Abs sample}} \times 100$$

Where Abssample is the absorbance of TPTZ solution mixed with the extract. AbsTPTZ is the absorbance of the TPTZ solution mixed with DMSO.

α-Amylase inhibition assay

The α-amylase inhibition assay was conducted using a modified method described by Kusano et al. (2011). For this purpose, 60 µL of extract dissolved in 0.1 M sodium acetate buffer (pH 6.5) was added to a 96-well plate, followed by 120 µL of 5 mg/mL starch solution (pH 7.0) and incubated at 37°C for 3 minutes. Subsequently, 20 µL of α-amylase (50 µg/mL) was added to the mixture and incubated at 37°C for 15 minutes. To terminate the reaction, 240 µL of 0.1 M HCl and 300 µL of 1 mM iodine were added to the reaction mixture, and absorbance was then measured at 650 nm by using a microplate reader, with a carbohydrate used as a standard. The percentage of enzyme inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{1-(\text{Abs2}-\text{Abs1})}{\text{Abs4}-\text{Abs3}} \times 100$$

Where Abs_1 is the absorbance of the reaction with the extract, enzyme, and starch. Abs_2 is the absorbance of the reaction with the extract, buffer, and starch. Abs_3 is the absorbance of the reaction with DMSO, enzymes, and starch. Abs4 is the absorbance of the reaction with DMSO, buffer, and starch.

LC-QTOF-MS/MS analysis of extract composition

The composition of the extract was analyzed using an Agilent 1290 Infinity II LC system for LC-QTOF-MS/MS analysis. The mobile phases A and B consisted of 0.1% (v/v) formic acid in water and acetonitrile, respectively. An Agilent Poroshell EC-C18 column (2.1 ´ 150 mm, 2.7 µm) was employed at 35°C. The gradient profile was initially set at 0%–5% B (0–1 minute), 5%–17% B (10–13 minutes), and 17%–100% B (20–25 minutes), and then decreased to 5% (27–33 minutes). A total of 1 µL of the extract was injected at a flow rate of 0.3 mL/minute. Mass detection was performed over the range of 50–1100 m/z using Masshunter Qualitative Analysis version B.08.00. Nitrogen gas atomization was performed at a speed of 11 L/minute at 300°C under 45 psi, with a sheath gas velocity of 12 L/minute at 250°C.

Statistical analysis

The data from three independent experiments were analyzed through one-way ANOVA and Tukey's test using SPSS 18.0.

RESULTS AND DISCUSSION

Polysaccharide and phenolic contents

The study analyzed the polysaccharide and phenolic content of the combined ethanolic extracts of *C. Militaris* (CME) and *S. Maritima* (SME). Extract no. 1 (CME: SME; 100:0) had the highest polysaccharide concentration of 912.74 ±15.68 µg/mL, while extracts no. 5 and 6 (CME: SME; 40:60 and 20:80) had the lowest polysaccharide concentration of 297.31 ± 23.31 µg/mL, as shown in table 1. There was a decrease in the polysaccharide concentration in the extract mixture as the ratio of *C. Militaris* decreased, except for extract No. 7, where CME: SME was 0:100. The polysaccharide content in the fruiting body of *C. Militaris* is higher than that in the stroma, sclerotium, and mycelium, as noted by (Nie et al., 2017). Huang et al. (2006) reported a polysaccharide content of 140.1 mg/g in the ethanolic extract derived from the fruiting body of C. militaris. Polysaccharide is the most essential component of C. militaris, with a wide range of biological actions such as immunological activity, antioxidant activity, anticancer activity, and anti-inflammatory activity, among others (Zhang et al., 2019). The difference in the polysaccharide and phenolic contents in the extracts may be due to different extraction methods, plant and mushroom strains, and cultivation conditions.

Table 1: Polysaccharide and phenolic content of the combined ethanolic extracts between *C. militaris* (CME) and *S. maritima* (SME)

Extract	CME: SME	Concentration (µg/mL)	
		Polysaccharide	Phenolic
1	100:0	912.74 ± 15.68^{d}	111.27 ± 2.96^{b}
2	80:20	350.55 ± 4.80^{b}	88.01 ± 4.23^{a}
3	60:40	385.87 ± 13.04^{b}	114.44 ± 2.08^{b}
4	50:50	353.03 ± 17.17^{b}	94.62 ± 4.93^{a}
5	40:60	297.31 ± 23.31^{a}	90.79 ± 4.82^{a}
6	20:80	297.31 ± 26.02^{a}	145.49 ± 3.59^{c}
7	0:100	475.92 ± 23.71^{c}	192.72 ± 4.48^{d}

Values are the means of triplicate experiments; *SD* and letters indicate statistical grouping ($p < 0.05$).

Table 1 illustrates that the highest phenolic content (192.72 ± 4.48 µg/mL) and the lowest phenolic content (88.01 ± 4.23 µg/mL) were observed in extract no. 7 (CME: SME; 0:100) and extract no. 2 (CME: SME; 80:20), respectively. The phenolic content of extract no. 7 corresponds to a gallic acid equivalent of 1.93 mg/g of the extract or 0.19% dry weight of extract. Patra et al. (2011) observed that the phenolic content was notably higher in leaf extracts of *S. maritima* compared to stem extracts in different

solvents. However, Sudjaroen (2015) found that the extract had less phenolic content, with only 6.93 mg of gallic acid equivalent/g. This was different from what Patra et al. (2011) found. The ethanolic leaf extract was found to have strong antioxidant characteristics and contain 0.77% dry weight of extract for phenol content.

Antioxidant activities

Extracts no. 6 and 7 demonstrated significant antioxidant activity in the study. In the DPPH assay, they exhibited the highest DPPH radical scavenging activity, with IC50 values of 377.66 ±13.97 and 305.28 ±28.70 µg/mL, respectively, as shown in Table 2. Furthermore, extract No. 7 showed the highest ABTS radical scavenging activity, with an IC50 value of 172.15 ± 15.48 µg/mL. In the FRAP assay, extracts no. 6 and 7 displayed the highest antioxidant activity, with IC50 values of 6.30 ±0.80 and 6.48 ±0.22 µg/mL, respectively. Although there are various reports on the antioxidant activities of *C. Militaris* and *S. Maritima* separately (Eiamthaworn et al., 2022; Mohamed et al., 2022; Sudjaroen, 2015), the antioxidant activities of combined extracts of *C. Militaris* and *S. Maritima* have not been previously reported. In this study, the combined extracts of *C. Militaris* and *S. Maritima* showed promising antioxidant activities, indicating a positive correlation between phenolic compounds and antioxidant activity (Roy and Dutta, 2021; Truyen and Patacsil, 2017). The study also highlighted the need for continued research on bioactive compounds with antioxidant capacity from medicinal plants.

Table 2: Antioxidant activities of the combined ethanolic extracts between *C. militaris* (CME) and *S. maritima* (SME)

Extract	Ratio (CME: SME)	IC_{50} (µg/mL) DPPH Assay	ABTS Assay	FRAP Assay
1	100:0	1,342.04 ± 94.48^{e}	910.65 ± 62.23^{f}	36.29 ± 6.86^{f}
2	80:20	1,074.07 ± 51.74^{d}	493.34 ± 21.87^{c}	10.76 ± 0.15^{e}
3	60:40	622.55 ± 23.69^{c}	772.04 ± 37.38^{e}	8.47 ± 0.44^{de}
4	50:50	563.56 ± 22.68^{c}	501.66 ± 31.94^{cd}	8.23 ± 0.65^{cd}
5	40:60	529.14 ± 50.19^{c}	512.75 ± 18.37^{cd}	7.73± 0.26b^{cd}
6	20:80	377.66 ± 13.97^{b}	584.89 ± 21.60^{d}	6.30 ± 0.80^{b}
7	0:100	305.28 ± 28.70^{b}	172.15 ± 15.48^{b}	6.48 ± 0.22^{bc}
Trolox		12.51 ± 0.48^{a}	5.26 ± 0.25^{a}	3.96 ± 0.09^{a}

Values are the means of triplicate experiments; *SD* and letters indicate statistical grouping ($p < 0.05$).

α-Amylase inhibition assay

The highest α-amylase inhibition activity with an IC50 value of 281.35 ±31.71 µg/mL was observed in Extract No. 1, and the lowest α-amylase inhibition activity was shown in Extract No. 7 (pure SME). Although there was no synergistic effect between the two extracts, extract No. 6 showed more significant inhibition activity than extract No. 7, as shown in Table 3. α-Amylase plays a vital role in carbohydrate degradation by hydrolyzing glycosidic bonds and releasing simple sugars. The inhibition of this enzyme has a positive effect on people with hyperglycemia or diabetes mellitus (Ogunyemi et al., 2022; Wang and Wang, 2019). This result suggested that the addition of 20% (v/v) CME can significantly increase the inhibition of α-amylase. Interestingly, the IC50 value of extract no. 6 in α-amylase inhibition was not significantly different from that of acarbose.

Table 3: α-Amylase inhibition of the combined ethanolic extracts between *C. Militaris* (CME) and *S. Maritima* (SME)

Extract	(CME: SME)	α-Amylase Inhibition IC_{50}; (µg/mL)
1	100:0	281.35 ± 31.71^{a}
6	20:80	383.10 ± 11.09^{b}
7	0:100	459.48 ± 24.52^{c}
Acarbose		376.93 ± 34.31^{b}

Values are the means of triplicate experiments; *SD* and letters indicate statistical grouping ($p < 0.05$).

LC-QTOF-MS/MS analysis of extract composition

Extract no. 6 underwent analysis using LC-QTOF-MS/MS, where compounds were scrutinized with a mass error tolerance of ±5 ppm and scores exceeding 80, comparing MS/MS spectra from a chosen database. Among the 70 compounds, four exhibited potential biological activities, which are detailed in Table 4. The application of LC-MS/MS-QTOF allows for the screening and detection of bioactive compounds with high accuracy and has the potential to identify novel, unknown bioactive compounds. The use of LC-MS/MS-QTOF enables accurate screening and detection of bioactive compounds, providing a reliable method for identifying both known and novel bioactive compounds with high accuracy.

Table 4: The bioactive compounds in the extract no. 6 which were analyzed by LC-QTOF-MS/MS

No.	Compound Name	Molecular Formula	Mass (DB)	Score (DB)	Diff. (DB, ppm)
1.	Choline	$C_5H_{15}NO_2$	121.1103	87.85	-1.2
2.	5-Formylsalicylic Acid	$C_8H_6O_4$	166.0266	87.44	0.42
3.	Nonanedioic Acid	$C_9H_{16}O_4$	188.1049	85.59	2.82
4.	Palmitoleoyl Ethanolamide	$C_{18}H_{35}NO_2$	297.2668	85.25	-0.8

Choline, an essential nutrient for various metabolic processes and neurotransmitter synthesis, is found in many mushrooms. Its deficiency may result in neurological disorders (Agarwal and Fulgoni III, 2021; Zeisel and Da Costa, 2009). Extract No. 6 contains several compounds with potential biological activities, including 5-Formylsalicylic acid (Compound 2), which displays anti-inflammatory, pain-relieving, and fever-reducing activities (Lu et al., 2010). Charnock et al. (2004) and Spaggiari et al. (2023) say that azelaic acid (Compound 3) in the extract can help with stress and skin diseases because it kills bacteria. Palmitoleoyl ethanolamide found in the extract exhibits anti-inflammatory properties (Beggiato et al., 2019; Petrosino and Di Marzo, 2017). The results suggest that extract No. 6 contains compounds with antibacterial and anti-inflammatory activities and can be suggested for health benefits.

CONCLUSION

In conclusion, the study assessed the bioactive characteristics of combined ethanolic extracts of *C. militaris* and *S. maritima*. Extract No. 6 (20:80 CME: SME) was shown to have effective antioxidant and antidiabetic properties, as well as antibacterial and anti-inflammatory components. The LC-QTOF-MS/MS analysis of extract no. 6 revealed four compounds with potential biological activity. The study found that adding 20% (v/v) CME can dramatically inhibit α-amylase. Extract no. 6 has considerably stronger α-amylase inhibitory activity than extract no. 7 (pure SME). Thus, extract No. 6 is recommended due to its high antioxidant and antidiabetic properties. Further research can be done in the future to isolate and identify the bioactive components in extract No. 6, as well as examine their mechanism of action.

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