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Original Article

Effect of *Cordyceps militaris* extract on T-lymphocyte, Th2, and Th17 cytokines *in vitro* and *in vivo*

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Abstract

Cordyceps militaris has been used in traditional medicine due to its immune-boosting functions, especially in elderly and cancer patients. The study aims to assess the impact of *C. militaris* extract on the immune response in LPS-induced splenocytes and male Sprague Dawley rats. The study found that CME contains various bioactive compounds such as adenosine, cordycepin (3' deoxyadenosine), and phenolic compounds. *In vitro* studies showed that CME has the potential to enhance $CD3^+$ T cell and $CD45RA^+$ B cell populations in LPS-induced splenocytes with a cell viability of over 80%. Moreover, we found that CME can enhance the immune response in rats by increasing $CD3^+$ T cell proliferation and up-regulating IL-4, IL-6, and IL-17a expression, without affecting the rat's body weight. The findings suggest that CME could be a promising immune enhancer for patients with weakened immune systems. However, further animal experiments with varying doses are essential to determine the optimal dosage for the successful development of CME as a therapeutic agent.

Keywords: *Cordyceps militaris*, Immunostimulatory effects, Pro-inflammatory cytokines, Cordycepin

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Introduction

The immune system is a complex network that communicates between lymphoid organs, cells, and humoral factors to protect the body against a variety of harmful pathogens, including viruses, bacteria, fungi, parasites, toxins, other foreign substances, as well as abnormal cells (Boshtam et al., 2017). The invasion of pathogens triggers inflammation within the host body, a response that is regulated by various mediators and

regulators such as cytokines, growth factors, eicosanoids, complements, and peptides to contain and eliminate pathogens or damaged cells. Cytokines are important proteins that act as mediators and help in communication for an immune response in the host body. They play a critical role in activating cells, promoting division, inducing apoptosis, and directing cell movement to the site of injury or infection (Parkin and Cohen, 2001; Turner et al., 2014; Yahfoufi et al., 2018). To maintain a healthy immune system, natural agents and bioactive compounds derived from natural products are used to stimulate adaptive and innate immunity processes (Wang et al., 2018). Type 1 T helper cells, also known as Th1 cells, are specialized immune cells that have a crucial function in defending the body against intracellular pathogens such as certain bacteria and viruses. Th1 cells produce specific cytokines, including IFN-γ, TNF, and IL-2, which aid in the immune response against these pathogens. Conversely, Th2 cells stimulate the humoral immune response, induce antibody production, and promote B cell proliferation (Parkin and Cohen, 2001; Shin et al., 2018).

Cordyceps militaris (Scarlet caterpillar club) belongs to the *Ascomycota* (family Clavicipitaceae) which has been used in Asian ethnomedicine for centuries as a tonic food and herbal medicine (Liu et al., 2014). This fungus contains various bioactive compounds, including nucleosides, amino acids, vitamins, phenolic compounds, and polysaccharides, that exhibit diverse biological activities and pharmaceutical functions, including antioxidative, antihyperglycemic, antitumor, anti-angiogenic, anti-inflammatory, and immunomodulatory effects (Das et al., 2010; Jedrejko et al., 2022; Kang et al., 2014; Liu et al., 2014; Li et al., 2019; Lou et al., 2019).

Water and ethanolic extracts derived from *C. militaris* were found to possess immunomodulatory properties. These extracts have demonstrated the potential to enhance an effective immune response in healthy men by upregulating IL-2 expression and IFN-γ secretion by macrophages via IL-18 (Kang et al., 2015; Kim et al., 2008). Polysaccharides extracted from *C. miliaris* exhibit immunoregulatory properties in animal studies. They effectively stimulate immune responses by activating the MAPKs and NF-κB pathways, resulting in elevated levels of TNF-α, nitric oxide (NO), and reactive oxygen species (ROS). Additionally, these polysaccharides are associated with improved macrophage function and enhanced spleen lymphocyte activity (Lee and Hong, 2011; Wang et al., 2012). Lipopolysaccharide (LPS), a major component of endotoxin derived from the cell walls of gram-negative bacteria, is widely used in both in vitro and in vivo studies to evaluate endotoxic shock and acute systemic inflammation (Choi et al., 2014; Jo et al., 2010). This study aims to assess the impact of *C. militaris* extract on the immune cells including $CD45RA^+$ B cells and $CD3^+$ T cells, and the expression levels of Th1 (IFN-γ), Th2 (IL-4 and IL-6), and Th17 (IL-17a) cytokines in LPS-induced splenocytes, and animal experiments.

Material and Methods

Chemicals and reagents

The following reagents were used in the experiments: adenosine, concanavalin A, and cordycepin (3'-deoxyadenosine) were purchased from Sigma (Sigma-Aldrich Pte. Ltd, Singapore). FITC anti-rat CD3 antibody (dilution 0.5 mg/mL), APC anti-rat CD335 (dilution 0.2 mg/mL), PE/Cy7 anti-rat CD45RA (dilution 0.2 mg/mL), and propidium iodide (dilution 0.5 mg/mL) were purchased from BioLegend (BioLegend, Inc., CA). Super-X Plex™ Flow cytometry assay multi-plex panel (Lot #1392407) was ordered from Antigenix America Inc. (USA).

Preparation of *C. militaris* extract

C. militaris was cultured in 100 mL potato dextrose broth with 0.25 g/L of ferrous sulfate, 20 g/L of glucose, and 20 g/L yeast extract in 475 mL glass jars (dimensions 80 mm × 120 mm) at 25 °C for 28 d under static liquid fermentation. The mycelia were harvested and dried at 65°C until constant weight. A 5 g sample of the dried mycelium was pulverized into a powder and extracted with distilled deionized water for 1 h using a magnetically stirred mixer, twice. The extraction was centrifuged for 5 minutes at 5,000 x *g* and then filtered through Whatman paper No. 1 (diameter 47 mm). Total phenolic content from *C. militaris* extract was measured using the Folin-Ciocalteu method, following the procedure outlined by Aryal et al. (2019). Results are expressed as mg GAE/g dry extract, with data recorded in triplicates. To minimize interference from each sample, blank control was also performed, and data was recorded in triplicates for each sample.

Measurement of bioactive compound profiles using an HPLC assay

The bioactive compounds obtained from *C. militaris*

were analyzed using reversed-phase High-performance liquid chromatography with a C18 column (particle size of 5 µm; dimensions 250 mm x 4.6 mm). Elution was performed by employing a gradual increase in methanol concentration within a linear gradient combined with distilled water. The gradient ranged from 0% to 40% methanol and 100% to 60% distilled water in 25 min with a flow rate of 1.0 mL/min. The detection of adenosine and 3' deoxyadenosine (cordycepin), the biomarkers of interest, was done using an injection volume of 20 µL and a wavelength of 260 nm.

Animal ethics statement

Male Sprague Dawley (SD) rats (6 weeks old, 250 g body weight) were obtained from M-CLEA BIORESOURCE Co., Ltd. (Thailand). All animal experiments were conducted following the guidelines of the Institutional Animal Care and Use Committee of Khon Kaen University, approved under animal protocol number IACUC-KKU-76/64.

In vitro

Isolation of primary splenocytes from male Sprague Dawley rats

The spleens were aseptically removed from sacrificed SD rats and subsequently pulverized using cell strainers with a 40 µm mesh. The cell suspension was treated with red blood cell lysis buffer (pH 7.3) to remove lysed cells. Following multiple washes with PBS buffer (pH 7.4), the cell pellets were resuspended in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 2.05 mM L-glutamine, and 1% (v/v) penicillin/streptomycin (100 U/mL), and then adjusted to the final concentration of 1×10^7 cells/mL.

Cell viability assay

A cell viability assay was conducted using propidium iodide dye (dilution 0.5 mg/mL) to assess the cytotoxic effect of CME. Approximately 5×10^5 cells/well of splenocytes were seeded into a 96-well plate with varying concentrations of CME (0.01, 0.1, 1.0, and 10.0 mg/mL) and incubated at 37°C with 5% CO_2 under humidified conditions for 72 h. After the incubation period, the CME-treated splenocytes were stained with propidium iodide dye and measured using flow cytometry (Attune NxT Acoustic Focusing Cytometer, Invitrogen, Thermo Fisher Scientific).

Effect of CME on lymphocyte proliferation

To assess the proliferation of T and B cells, Flow cytometry was performed using specific monoclonal anti-rat associated with fluorescence dye following anti-rat CD3-FITC and anti-rat CD45RA-PE/Cy7. Approximately 5×10^5 cells/well of splenocytes were seeded in 96-well plates with varying concentrations of CME (0.02, 0.2, and 2.0 mg/mL) in the presence or absence of LPS (2.5 µg/mL). The cells were incubated at 37°C in a humidified environment with 5% CO_2 for 72 h. After 72 h of incubation, splenocytes treated with CME were harvested and stained using specific monoclonal anti-rat antibodies to measure $CD3^+$ T cell and $CD45RA^+$ B cell proliferation.

In vivo

Animals and experiment design

Ten SD rats were employed to assess the impact of CME on the immune response. Prior to the experiment, the rats were allowed to acclimate for at least 7 d under standard conditions, including a 12 h light-dark cycle, humidity maintained between 30–60%, and a constant temperature of 23 ± 2°C. We randomly divided the rats into two groups (n = 5/group): (i) orally administered distilled water (control group) and (ii) orally administered 50 mg/kg body weight of CME (Eq. 1) for 14 consecutive days. During the experiment, the rats had ad libitum access to water and food. After the treatment, the rats were euthanized, and their spleens and blood were collected for measuring the $CD3^+$ T and $CD45RA^+$ B cell proliferation.

Animal equivalent dose (AED) mg/kg = (Human dose mg/kg) x K_m ratio Eq. (1)

The K_m ratio values are calculated by dividing the human K_m factor (37) by the animal K_m factor (6) or vice versa. Therefore, the K_m ratio value for a rat is 6.2 (Nair and Jacob, 2016).

Cytokines profiling assay

The Antigenix America Super-X Plex™ multiplex assay was applied to assess levels of IL-4, IL-6, IL-17a, and IFN-γ production following the manufacturer's protocol. Initially, a 96-well flat bottom plate was coated with antibody beads designed for capturing multiplex panels. Subsequently, 30 µL of assay buffer and 50 µL of each rat serum were added individually to their corresponding wells. Following cytokine capture, a series of washing steps were performed, and the captured cytokines were detected using specific biotinylated detection antibodies. Finally, the streptavidin-PE conjugate reagent was added and resuspended in a reading buffer. The

resulting data were analyzed using a flow cytometer.

Statistical analysis

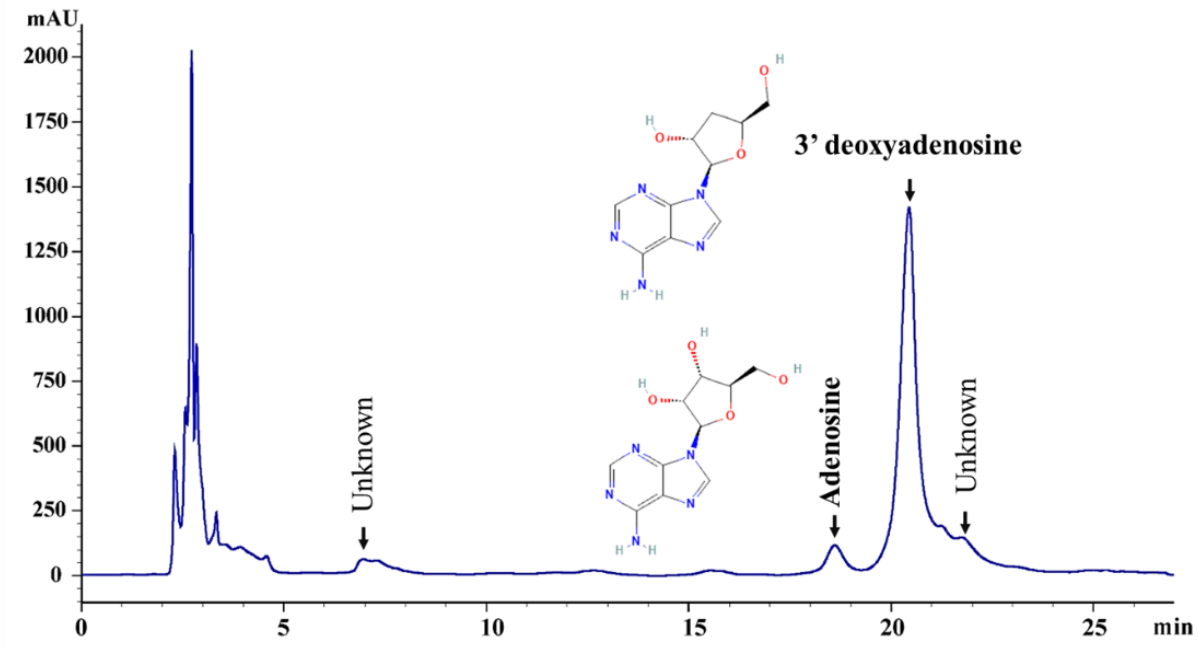
One-way analysis of variance (ANOVA) coupled with Dunnett's multiple range test and Two-independent t-tests were employed to establish the statistical significance of value between the sample groups. Differences were considered statistically significant at a *p*-value of < 0.05. All values were expressed as the mean ± standard deviation.

Results

The bioactive compound profiles derived from *C. militaris* using an HPLC assay

The RP-HPLC chromatogram displayed noticeable peaks of adenosine and 3' deoxyadenosine (cordycepin) at a retention time of 18.6 and 20.4 min, respectively (Figure 1A). Our analysis contained the quantification of cordycepin and phenolic contents, which may have a significant impact on immune response. The results indicated that CME contained 9.26 ± 3.07 mg/g dry weight of cordycepin and 1.74 ± 0.04 mg GAE/g dry weight of total phenolic content (Figure 1B).

(A)



(B)

Bioactive compounds	Amount
Cordycepin (mg/g dry weight)	9.26 ± 3.07
Total phenolic content (mg GAE/g)	1.74 ± 0.04

Figure-1. (A) RP-HPLC chromatogram of bioactive compound profiles obtained from *C. militaris* extraction. The adenosine and 3' deoxyadenosine (cordycepin) were eluted at retention times of 18.6 and 20.4 min, respectively (n=3). (B) Total phenolic content was measured using the Folin-Ciocalteu assay and expressed as mg/gram of gallic acid equivalent (GAE).

Cell viability

After 72 h incubation, the cytotoxic effects of CME were evaluated by applying propidium iodide staining. The results indicated a dose-dependent impact on the spleen lymphocyte viability (Figure 2). At higher concentrations, the % of cell viability decreased significantly below 15.5%. However, a concentration that could maintain viability above 80% was considered for subsequent experiments.

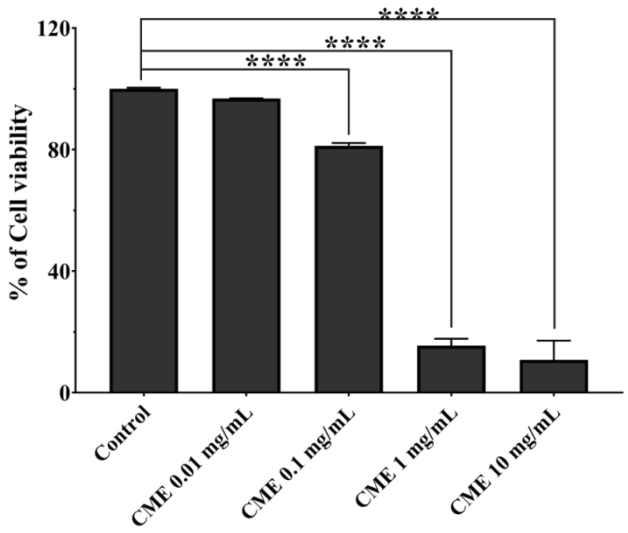


Figure-2. Effect of CME on the viability of spleen lymphocytes. The cells were treated with indicated concentrations of CME or without CME. The % of cell viability was assessed after 72 h by Flow cytometer using propidium iodide staining and expressed as the mean ± standard deviation of three independent experiments (*n*=3). **, *p* < 0.0001, statistically significant differences versus the control group.**

Effect of CME on lymphocyte proliferation

To investigate the impact of CME on immune response, rat splenocytes were incubated with CME in the presence or absence of LPS. The proliferation of T and B cells was assessed using specific antibodies targeting $CD3^+$ T cells (FITC) and $CD45RA^+$ B cells (PE/Cy7). The findings demonstrated that CME significantly stimulated $CD3^+$ T cell and $CD45RA^+$ B cell proliferation in LPS-induced splenocytes (Figure 3).

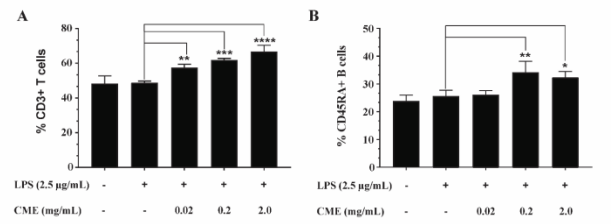


Figure-3. Effect of CME on LPS-induced spleen lymphocyte proliferation. Spleen lymphocyte was incubated with indicated concentrations of CME (0.02, 0.2, and 2.0 mg/mL) in the absence and presence of LPS (2.5 µg/mL) for 72 h. The proliferation of (A) % of $CD3^+$ T cells and (B) % of $CD45RA^+$ B cells are presented as the mean ± standard deviation of three independent experiments (*n*=3). *, *p* < 0.0332, and **, *p* < 0.0021, *, *p* < 0.0002, and ****, *p* < 0.0001 statistically significant differences against the LPS-treated control.**

Effect of CME on lymphocyte proliferation in healthy male Sprague Dawley rats

We carried out a study on healthy male SD rats to assess the impact of CME on the immune response using two vital types of immune cells: PBMCs and splenocytes. Within PBMC cells, CME possesses a potent ability to stimulate $CD3^+$ T cells as shown in Figure 4C. Conversely, there was no noticeable impact on $CD3^+$ T cells and $CD45RA^+$ B cells in splenocytes (Figure 4A and Figure 4B, respectively).

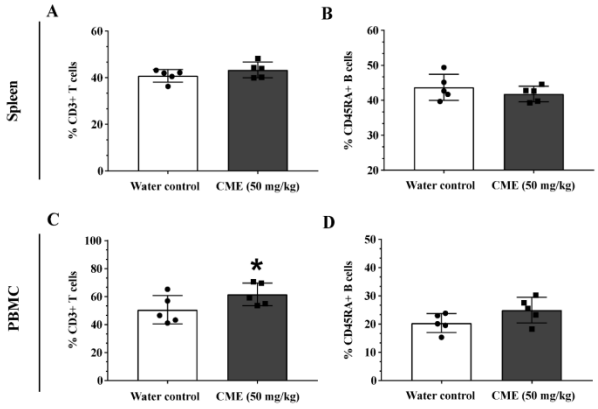


Figure-4. Effects of CME on spleen lymphocyte and PBMCs proliferation after 14 days of administered intragastrically CME (50 mg/kg body weight) and distilled water once daily. The proliferation of %$CD3^+$ T cells and %$CD45RA^+$ B cells were evaluated in spleen lymphocytes (A and B) and PBMCs (C and D). Data are expressed as the mean ± standard deviation (*n*=5). *, *p* < 0.0332, statistically significant differences versus a control group.

Cytokine profiling assay

We confirmed the impact of CME on the immune response in male SD rats by assessing cytokine production. The mean fluorescence intensity (MFI) results indicated a significant elevation in IL-4, IL-6, and IL-17a production in rats due to CME treatment. Although there was an induction in IFN-γ levels, no statistically significant difference was observed compared to the control group (Figure 5A-D).

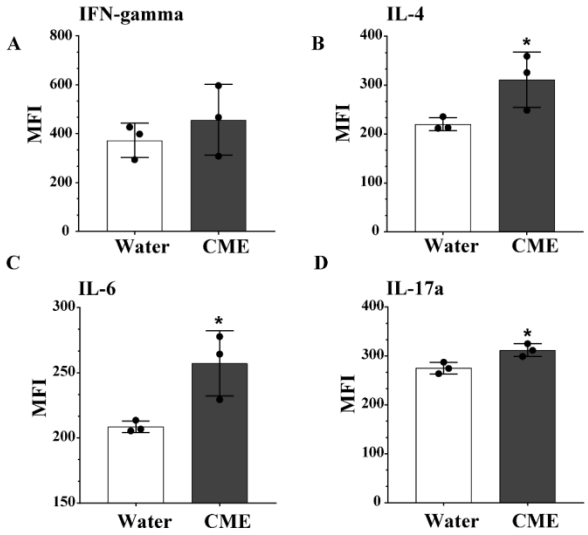


Figure-5. Effect of CME on cytokines production. The levels of cytokine were measured using a Super-X Plex™ Flow cytometry assay kit. Levels of (A) interferon-gamma; (B) interleukine-4; (C) interleukine-6; (D) interleukine-17a are represented as the mean ± standard deviation of the mean fluorescence intensity (MFI) of PE (*n*=3). *, $p < 0.05$, statistically significant differences against control.

Discussion

Cordyceps militaris has potent therapeutic benefits to enhance the immune system's function in both elderly people and cancer patients (Friedman, 2016; Lee and Hong, 2011). Various bioactive substances extracted from *C. militaris* have been investigated for their immunomodulatory effects and pharmaceutical activities in humans and animals. Previous studies have reported that bioactive compounds from mushrooms can stimulate B cells, T cells, and natural killer cells to enhance the host immune system (Wang et al., 2012). Herein, bioactive compounds, including adenosine, cordycepin, and phenolic compounds were found in CME (Figure 1). Cordycepin is a nucleoside analog derived from adenosine that can be found primarily in the mycelia, fruiting bodies, and fermented broth of *Cordyceps* species. This compound has been extensively researched for its potential therapeutic properties, including antioxidant, anticancer effects, and anti-inflammatory (Lee et al., 2020). The attribution of phenolic compounds and flavonoids is associated with lymphocyte proliferation in the spleen and affects the production of cytokines and pro-inflammatory gene expression, contributing to inflammation (Lin and Tang, 2007; Yahfouf et al., 2018).

Spleen is an important organ for the immune system where thymocytes differentiate into lymphocytes, which have a crucial role in immunological defense (Shin et al., 2018; Zhu et al., 2016). The isolated lymphocytes from the spleen were used as the preliminary indexes to assess the cytotoxicity. The regulation of different immune cell types involves various markers, such as the Cluster of Differentiation 3 (CD3), which activates CD45RA (B-cell specific marker) and stimulates both T helper cells and cytotoxic T cells (Nayak et al., 2009; Porciello et al., 2022). $CD3^+$ T cells can differentiate into diverse subtypes, including cytotoxic T cells, T helper cells (Th1, Th2), memory T cells, and regulatory T cells, each contributing uniquely to immune responses. Our finding demonstrated that CME obtained from *C. militaris* exhibited a dose-dependent cytotoxicity in splenocytes (Figure 2). It was also observed that CME significantly induces $CD3^+$ T cell and $CD45RA^+$ B cell proliferation in splenocytes. Furthermore, the animal experiments demonstrated that CME had no effect on the rat's body weight, indicating its safety for consumption (Figure S1). However, we found that CME had potentially induced the proliferation of $CD3^+$ T cells in PBMCs only (Figure 4).

Herein, we assessed the effects of CME on the production of Th1, Th2, and Th17 cytokines, which are secreted in extracellular due to the host immune response. Our findings indicate that CME effectively stimulates the immune response by increasing IL-4, IL-6, and IL-17a production in healthy SD rats (Figure 5). These cytokines play crucial roles in the immune response. IL-4, a cytokine produced by various immune cells including basophils, mast cells, and T cells, promotes the differentiation of naïve T cells into Th2 cells. This cytokine plays a crucial role in driving the activation and development of Th2 responses while suppressing Th1 differentiation (Luzina et al., 2012) to maintain immune balance. IL-6 is another cytokine that promotes inflammation and helps regulate the differentiation of immune cells,

specifically B cells that produce antibodies. It also plays a vital role in hematopoiesis, which is the process of blood cell formation (Song and Kellum, 2005). The production of IL-6 can be enhanced due to the immune response process when lipopolysaccharides are secreted from bacteria (Riaz et al., 2015). Lastly, IL-17a is a pro-inflammatory cytokine that links T cell activation to neutrophil mobilization and activation (Zenobia and Hajishengallis, 2015).

Conclusion

This research has found that *Cordyceps militaris* extract can potentially enhance the immune system in rats by increasing $CD3^+$ T cell proliferation and up-regulating IL-4, IL-6, and IL-17a production. CME has shown promise as an immune enhancer for patients with weakened immune systems. However, for the successful development of CME as a therapeutic agent, animal experiments with varying doses need to be conducted to determine the optimal dosage. Additionally, studying changes in spleen and thymus indexes could provide valuable insights into the underlying mechanisms of CME's immune-enhancing effects.

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Contribution of Authors

Mongmonsin U: Conceptualization, research methodology development & validation, software & formal analysis, writing of original draft of manuscript.
Rungsa P: Research methodology development, writing and editing of manuscript.
Teajaroen W: Research methodology development and literature review.
Somdee T: Supervision of study, literature review and editing of manuscript.
Daduang J: Literature review, editing of manuscript and resource provision for study.
Daduang S: Conceptualization, supervision & resource provision for study, writing, reviewing & editing of manuscript, project administration and funding acquisition.

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Supporting information

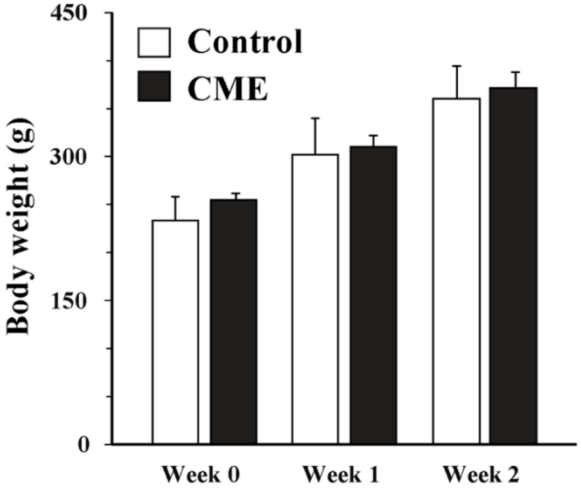

Figure supplementary-1. Body weight changed in healthy rats after 14 days of administered intragastrically CME (50 mg/kg body weight) and distilled water once daily.