

The effect of the combination of *Eucheuma Cottonii* plus zinc on higher FoxP3Treg expression and IFN- γ levels compared to the control group

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ABSTRACT

Background

Allergic disease cases were still increasing. Corticosteroids and antihistamines are still standard therapies to relieve and prevent the recurrence of allergies. However, the side effects of long-term treatment drugs are a serious problem. *Eucheuma Cottonii* has the potential as a strong antioxidant and a high-fiber Carrageenans agent. These properties make it a potential immunomodulator to control allergies. Zinc has been proven and recommended for several diseases such as diarrhea, malnutrition, and others.

Objective

This study aimed to prove the effect of the combination of *Eucheuma cottonii* with zinc on blood IFN- γ levels, intestinal Foxp3Treg expression, and prevention of asthma symptoms in young asthma-induced mice.

Methods

Post-test-only control group design experimental study, in 5 groups of young female BLAB/C mice with asthma models: healthy group (K1), negative control with sterile aquabidest (K2), positive control with corticosteroids (K3), treatment with 20mg *Eucheuma Cottonii* Extract (ECE) + 0.08mg Zinc (K4), and 40 mg ECE+0.08mg Zinc, orally for 14 days.

Result

The K4 group had the highest levels of intestinal FoxP3Treg expression compared to other groups, $p: 0.008$. IFN- γ K4 levels were higher than K3 (corticosteroids), $p: 0.004$, but not significantly different from the other three groups, $p > 0.05$. IFN- γ K4 levels were still higher than the healthy group and K5, but $p > 0.05$. In group K2, the macroscopic appearance of the lungs had several pale, thicker circles in some areas and was accompanied by more severe asthma symptoms than the other groups.

Conclusion

The effect of the combination formulation of ECE 20mg + zinc supports the presence of higher intestinal FoxP3Treg expression and maintains blood IFN- γ levels above the levels of healthy groups and corticosteroid drugs.

Keywords

E. *Cottonii* Extract; Zinc; Foxp3Treg Expression; IFN- γ ; Asthma mice

INTRODUCTION

Climate change in the form of the greenhouse effect causes global warming of temperatures on the earth's surface. Warming and air pollution increase Asthma triggers, especially airborne allergens. Climate change including frequent rain is associated with increased cases of allergies and the severity of allergic asthma attacks in vulnerable populations. ¹ The theory of "the hygiene hypothesis" states that the decrease in the prevalence of infection has increased the prevalence of allergic diseases in the world. ² The Indonesian Ministry of Health noted that one of the non-communicable diseases "asthma", is the most common allergic disease in society, until the end of 2020, the number of asthma sufferers in Indonesia was 4.5 percent of the total population of Indonesia or more than 12 million. Asthma attacks disrupt daily activities and even pose a dangerous risk of respiratory failure. ³

Allergy drugs, such as corticosteroids, are available fairly cheap but have less good long-term side effects, including susceptibility to infection and impaired bone growth. While anti-leukotriene drugs are better, they are quite expensive, as is immunotherapy. Considering

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this, research is still needed on easier and more natural allergy treatments as immunomodulators to prevent asthma in the long term. Traditional medicine utilizes biological riches that are easily obtained in Indonesian sea waters, one of which is *Eucheuma Cottonii*, which needs to be used and continued to be developed for treatment.

Eucheuma cottonii contains flavonoid antioxidants that reduce inflammation and antioxidant properties.⁴ Seaweed including high-fiber carrageenan properties has the potential to be a prebiotic that supports normal intestinal microbiota through short-chain fatty acid (SCFA) products that stimulate secretory IgA (sIgA) intestinal regulatory antibodies secreted into the lumen of the intestinal mucosa and respiratory organs.^{5,6} Secretory IgA Together with FoxP3 + Treg tends to control excessive inflammatory effects due to exposure to inhaled or food allergens so that it can prevent asthma allergies.^{7,8,9}

Research has shown that newborn zinc is associated with allergies before the age of 6 months.¹⁰ Low levels of newborn zinc are associated with symptoms and signs of asthma and allergic rhinitis allergies at the age of 3 years.¹¹ Indonesia is one of the countries at risk of zinc deficiency.¹² The combination formulation of EEC plus zinc is expected to have a mutually reinforcing immunoregulation effect. The research aims to prove the effectiveness of the *Eucheuma cottonii* extract formulation with zinc as an immunomodulator in increasing IFN- γ and FoxP3+Treg and preventing or reducing asthma symptoms in young asthma model mice. The research met the research ethics requirements approved by the Medical/Health Research Bioethics Commission, Faculty of Medicine, Sultan Agung Islamic University, Semarang.

METHODS AND MATERIALS

This research was conducted at the Indonesian SCCR (Stem Cell and Cancer Research) laboratory in Gunungpati, Semarang City. Preparation of *Eucheuma Cottonii* Extract in the Chemistry Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang. It involved an experimental study on mice with a post-test-only control group design. Female BLAB/C mice induced asthma by peritoneal injection and inhalation of ovalbumin for 35 days, treatment started on day 21 for 14 days. Mice were divided into 5 groups, one healthy control (K1), and 4 intervention groups. The negative

control group received sterile Aquabidest (K2), the positive control group received methylprednisolone 0.15mg/day (K3), the first treatment group (K4) received *Eucheuma Cottonii* extract at a dose of 20mg+ zinc 0.08mg/20g body weight, and the second group treatment (K5) with a dose of 40mg *Eucheuma Cottonii* Extract + zinc 0.08mg/20g BW.

Procedure for induction of asthma model mice

Female BLAB/c mice with an Asthma model were obtained as follows: Mice were sensitized on days 0 and 7 by intraperitoneal injection of a mixture of 20 μ L ovalbumin + 1mg aluminum hydroxide in 500 μ L Normal saline. Mice were provoked intranasally (inhalation) every day from day 14 to day 35, inhalation in groups in a chamber (containing 7 mice), the dose of inhaled ovalbumin for one chamber was 1% with an inhalation rate of 0.25ml/minute for 30 minutes/day. Ovalbumin solution was around 10mg/ml, diluted with water for injection.¹³

Asthma model mice are considered valid if at least score 1: itching, scratching the nose and head, score 2: fur erect, restless or less active, respiratory frequency, score 3: shortness of breath, cyanosis around the mouth or tail, score 4: none activity after being pushed or tremors or seizures, score 5: death.¹⁴

Ingredients and procedures for preparing *Eucheuma Cottonii* Extract

Eucheuma cottonii was collected from Karimunjawa Beach in Jepara. *E. cottonii* was washed with running water and cut into small pieces. They were dried in an oven at 40°C until the water content in the plant was less than 10%, and the air-dried *E. cottonii* was ground into powder. 430 g of *E. cottonii* powder was macerated with 2 L of 96% ethanol at 40 °C for 10 days, the supernatant was collected and filtered through Whatman No. 1 filter paper in a Buchner funnel for 8 days. The filtrate was concentrated by evaporation at 40 °C using a rotary evaporator for 6 days. The extract was dehydrated under reduced pressure, stored at 0 to 4 °C, and used in the experiment. Obtained *Eucheuma Cottonii* Extract 5.7% from 430g of *E. Cottonii* dry powder, around 24.6g. Next, an analysis of antioxidant content, total phenols, and flavonoids was carried out.

RESULTS

Observation for 35 days from the induction of intraperitoneal ovalbumin sensitization on days 1, 7,

followed by provocation induction with ovalbumin inhalation for 3 weeks from day 14 to day 35. Until the 20th day before treatment, the validation mice model of asthma in the induction group (K2, K3, K4, and K5) all showed a score of 1: nose itching, scratching the nose repeatedly as well as frequently scratching the head, and restlessness, unable to rest during the provocation process of albumin inhalation. On the 14th day or the 1st day of ovalbumin inhalation provocation, mice from K2 scored 4: less active, did not move even when pushed, had rapid breathing frequency, cyanotic tail, finally died after the inhalation.

Validation results before treatment on day 19, one mouse in the induction group (K3) had a clinical score of 4: less active, did not move with encouragement, shortness of breath, and cyanosis of the tail, after the nebulizer the mouse got worse so termination was carried out as validation of the induction group. The macroscopic image shows that the lung tissue has pale, thickened bumps. The condition of the group of healthy mice (K1) was quite good, the mice were sleeping and calm. Validation of the healthy control group (K1) was carried out at the end, the macroscopic appearance of the lungs was brighter in color and there were no pale bumps. See Figure 1.

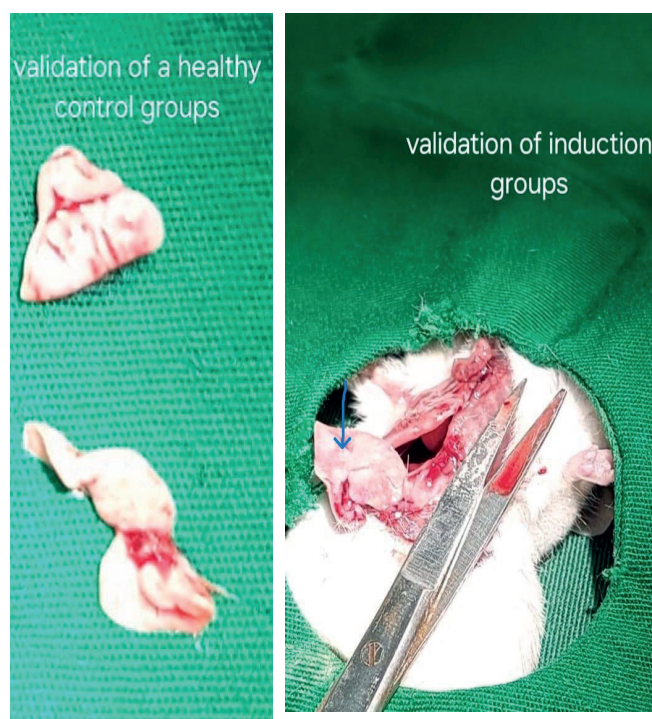
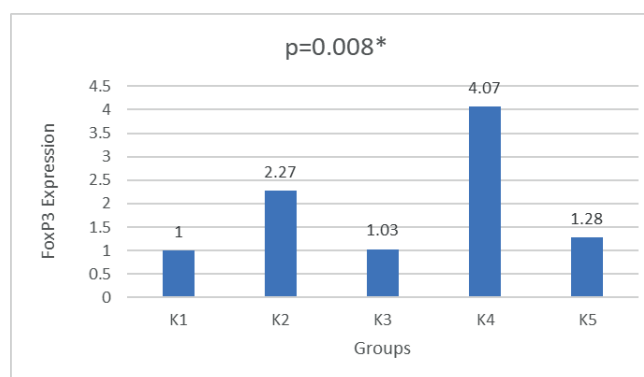


Figure 1. Macroscopic lung tissue of the healthy control group and the induction group.

Blue arrows show several thickened pale circular shapes. The animal model for asthma induction using this research protocol was sufficient both clinically and macroscopically for the lung organs to show signs of asthma.

Administration of a combination of *Eucheuma Cottonii* Extract with zinc for 14 days starting from day 21 to day 35. Analysis of *E. Cottonii* Extract levels from an Extract weight of 24.6g: antioxidant IC₅₀ around 483.55 ppm (weak), flavonoids 1.7% (0.42g or 16,660ppm), total Phenol 2.06%(0.5g or 20,623ppm). This research has not analyzed the oligosaccharide content.

If you see at Figure 2, there is a significant difference in FoxP3 expression between groups, $p = 0.008$. The expression of K4 (intervention group 20mg *E. Cottonii* Extract + 0.08mg Zinc) was higher than the other groups, however, the addition of a double dose of *E. Cottonii* Extract was proven to reduce FoxP3 expression in K5 even though the figure was still above the healthy control and positive control groups.



*One-way ANOVA

Figure 2. FoxP3 expression between groups

Figure 1 and Table 1 illustrate the differences in FoxP3 expression between the groups.

Table 1. Mean FoxP3 expression between groups

	MEAN	SD
K1	1,00	0,00
K2	2,27	1,69
K3	1,03	0,54
K4	4,07	2,05
K5	1,28	1,06

K1 : Normal K2: negative control K3: positive control
K4 : treatment with *E. Cottonii* dose 20mg + zinc 0.08mg
K5: treatment with *E. Cottonii* dose 40mg + zinc 0.08mg

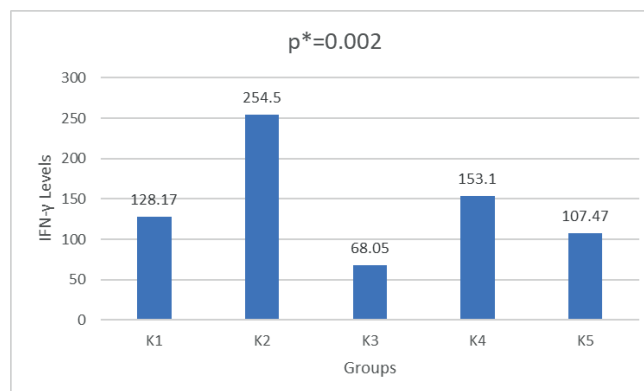
The results of Table 1 prove that the administration of 20 mg of E.Cottoni extract and zinc (Group 4) had significantly higher FoxP3 expression than the other groups, $p < 0.05$, except the negative control group. FoxP3Treg expression in group 5 was not significantly different compared to all control groups when increasing the dose of E. Cottoni Extract twofold, $p > 0.05$. FoxP3Treg expression in group 4 was significantly higher than the other three groups ($p < 0.05$) except for the negative control.

Table 2 Post Hoc test of FoxP3 expression

Between Groups	p-value	95% Confidence Interval	
		Lower Bound	Upper Bound
K1 vs K2	0.140	-0.6111	0.0929
K1 vs K3	0.734	-0.2939	0.4102
K1 vs K4	0.003	-0.9150	-0.2110
K1 vs K5	0.972	-0.3580	0.3461
K2 vs K1	0.140	-0.0929	0.6111
K2 vs K3	0.075	-0.0348	0.6692
K2 vs K4	0.087	-0.6560	0.0481
K2 vs K5	0.149	-0.0989	0.6052
K3 vs K1	0.734	-0.4102	0.2939
K3 vs K2	0.075	-0.6692	0.0348
K3 vs K4	0.001	-0.9732	-0.2691
K3 vs K5	0.708	-0.4161	0.2880
K4 vs K1	0.003	0.2110	0.9150
K4 vs K2	0.087	-0.0481	0.6560
K4 vs K3	0.001	0.2691	0.9732
K4 vs K5	0.004	0.2051	0.9091
K5 vs K1	0.972	-0.3461	0.3580
K5 vs K2	0.149	-0.6052	0.0989
K5 vs K3	0.708	-0.2880	0.4161
K5 vs K4	0.004	-0.9091	-0.2051

vs :versus

Figure 3 shows that there is a difference in mean IFN- γ levels between groups, $p: 0.002$. The highest was in the negative control (K2). The highest levels were in the two K4 treatment groups, the lowest in the group given oral methylprednisolone (K3).



*One-way ANOVA

Figure 3. IFN- γ levels between groups

Post Hoc test results from Table 3, it was found that IFN- γ levels were significantly higher in the ECE 20mg+zinc 0.08mg (K4) treatment group than in the methylprednisolone (K3) treatment group, $p: 0.004$. IFN- γ levels were not significantly different between the E. Cottonii extract (ECE) 20 mg+zinc (K4) dose group and the 40mg ECE+zinc dose group (K5), $p: 0.214$. The IFN- γ level in the negative control group (K2) was the highest, but statistically, it was not significantly different from the ECE treatment group with a dose of 20mg+zinc (K4), $p: > 0.05$. The K2 group was significantly higher than all control and treatment groups K5, $p < 0.05$.

Table 3. Post Hoc tests for IFN- γ levels

Groups	p-value	95% Confidence Interval	
		Lower Bound	Upper Bound
K1 vs K2	0.014	-0.5220	-0.0662
K1 vs K3	0.036	0.0171	0.4728
K1 vs K4	0.325	-0.3382	0.1175
K1 vs K5	0.787	-0.1980	0.2577
K2 vs K1	0.014	0.0662	0.5220
K2 vs K3	0.000	0.3112	0.7669
K2 vs K4	0.108	-0.0441	0.4116
K2 vs K5	0.008	0.0961	0.5518
K3 vs K1	0.036	-0.4728	-0.0171
K3 vs K2	0.000	-0.7669	-0.3112

Groups	p-value	95% Confidence Interval	
		Lower Bound	Upper Bound
K3 vs K4	0.004	-0.5832	-0.1274
K3 vs K5	0.063	-0.4430	0.0127
K4 vs K1	0.325	-0.1175	0.3382
K4 vs K2	0.108	-0.4116	0.0441
K4 vs K3	0.004	.1274	0.5832
K4 vs K5	0.214	-0.0877	0.3680
K5 vs K1	0.787	-0.2577	0.1980
K5 vs K2	0.008	-0.5518	-0.0961
K5 vs K3	0.063	-0.0127	0.4430
K5 vs K4	0.214	-0.3680	0.0877

Vs: versus

Severe signs of asthma in mice from this research were marked with a score of 3 to 5, the mice experienced shortness of breath, and cyanosis around the mouth or tail, continued to have no activity after pushing and had convulsions and then died.

K1 mice lived healthily until the 35th day. K2 mice died on the 14th day or after inhalation provocation on day 1, then 1 mouse was killed on the 19th or 6th day after inhalation provocation for validation because the condition worsened, they were inactive, the tail was cyanotic, and shortness of breath score 3-4.

One K3 died on the 17th day or the 4th day after inhalation provocation, score 5. One K5 died on the 17th day or the 4th day after inhalation provocation, score 5. In the K4 treatment group, none of the mice died. Mice died or worsened during the asthma induction period before being given treatment

The results of macroscopic photos of the lung organs are as follows in the image below

Clinical symptoms of allergic asthma during observation days 21 to 35. When provoked by inhalation of ovalbumin, all mice in the induction group were found to have symptoms of scratching their nose and head, but were still active. Starting from the second week of inhalation provocation, the condition of the K2 group compared to the other groups, all mice were more restless, such as shortness of breath, 2-3 mice were less active and their tails were slightly cyanotic. Groups K4 and K5 during the second and third weeks of ovalbumin inhalation provocation were calmer, with only 1-2 mice scratching their noses and heads, still active, and sometimes huddled together

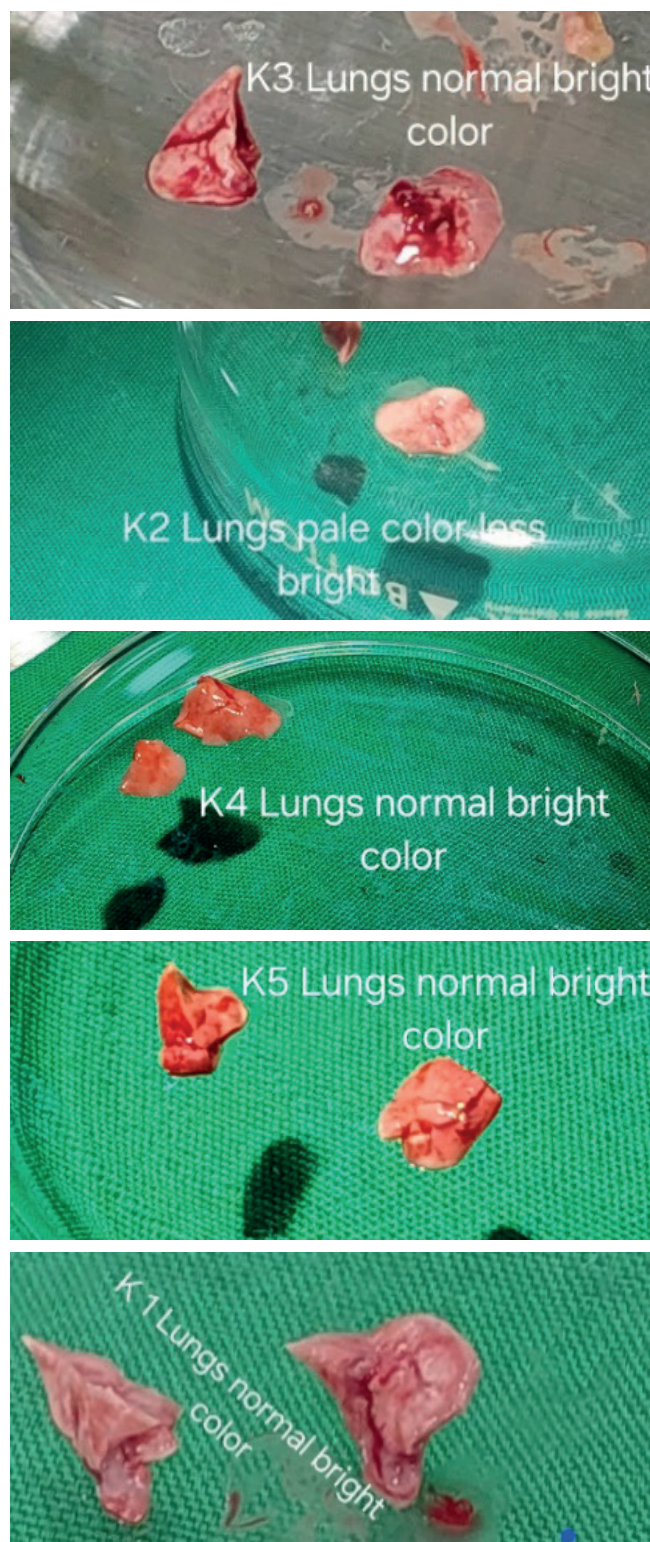


Figure 4. Macroscopic examination of the lung organs after treatment was complete. The lungs of group K2 (negative control) were paler and less fresh than the other groups.

trying to sleep. The condition of K3 during ovalbumin inhalation provocation, similar to K4/K5 was still active, sometimes, 1-2 animals were restless as if short of breath, but no one tried to huddle together to sleep

DISCUSSION

Extracts of *E. Cottonii* seaweed showed low antioxidant activity, with $LC_{50} > 150$ $\mu\text{g/mL}$. The antioxidant activity of *E. Cottonii* seaweed extracts from fresh samples was more potent than extracts from dried samples.⁴ Similar to the ECE results from this research, the antioxidant IC_{50} was around 483.55 ppm (weak). Limitations of this research have not assessed the potential of carrageenan. According to other studies, the best quality of carrageenan was obtained from seaweed when it undergoes a seawater bleaching process by immersion in seawater.⁵ The properties of carrageenan are important as a high-fiber food, including oligosaccharide components necessary for the development of a healthy gut microbiome, preventing allergic and autoimmune diseases.⁶⁻¹⁰

IFN- γ strengthening is needed in cases of allergic diseases including asthma, to control the allergic disease cytokine IL-4. IL-4 is often responsible for activating IgE product antibody-type B cells, mast cells, eosinophils, and basophils that release active substances that trigger type 1 hypersensitivity (atopic allergy).^{8,9,10} This research proves the effect of EEC + zinc in maintaining immune tolerance through the intestinal mucosa, as evidenced by the high expression of FoxP3Treg in the group administered 20mg EEC + 0.08mg Zinc compared to the control group. In addition to IFN- γ amplification, the role of Treg is very important in controlling Th2 activity. Th2 dominance increases the risk of asthma symptoms.^{11,12} The general observation of the less fresh appearance of the lung organs (some pale round areas) and the appearance of severe symptoms of asthma in this study were more prominent in the negative control group (K2) than the other four groups.

Although this research ECE formulation has weak antioxidant power, the potential of ECE plus zinc is likely stronger as an anti-inflammatory that can prevent symptoms or alleviate allergic asthma. This result follows previous studies that ECE suppresses proinflammatory cytokines, and hepatoprotectors, and reduces histamine which is the source of allergic symptoms.^{15,16} Our previous study confirmed the relationship between

low zinc levels in newborns and allergic infants.¹⁷ The addition of zinc to this research formula can strengthen regulatory cytokines so that FoxP3Treg expression in the terminal intestine increases sharply compared to the control at a dose of 20mg, however, increasing the dose of extract is less beneficial because it reduces Treg expression in the range of values similar to normal mice. In general, it can be said that *E. cottonii* extract plus zinc has the effect of maintaining the tolerance of the immune system through the presence of increased intestinal Treg. In line with our previous study, low levels of zinc in newborns are associated with the appearance of allergy symptoms and signs at the age of 3 years.¹⁸ Indonesia is a high-risk area for zinc intake, which is related to the dietary habits of its people.¹⁹ So this research added zinc in *E. Cottonii* extract.

However, it is important to consider the pharmacodynamic and pharmacokinetic effects of interactions between herbal ingredients and conventional drugs, particularly zinc. The group of asthma model mice that were not given intervention also still had higher expression of the regulatory factor (FoxP3) and the Th2-controlling cytokine (IFN- γ) than the corticosteroid intervention group and the highest dose of EEC intervention group.

Modern integrative medicine often combines herbs with conventional drugs, leading to herb-drug interactions. These interactions can impact clinical response, side effects, and interfere with other drug absorption. Herbs can interfere with liver and intestinal enzymes, which impact metabolism. EEC is an extract containing several compounds in addition to antioxidants and carbohydrate fiber, which may affect the absorption of food and zinc itself.²⁰ The highest dose of EEC may interfere with the absorption of Zinc or vice versa; this may be due to the interaction effect between herbs and drugs.

Other research has shown that differences in extraction stages and the types of solvents used, such as ethanol, n-hexane, ethyl acetate, butanol, and H₂O, provide different results based on the analysis of Brine shrimp bioassay, phytotoxicity, and inhibition of enzyme activity.²¹ Extraction method, solvent properties, and bioactivity influence the active compound content. Therefore, this research extraction method still requires further study.

Other results from this study have not yet proven its effect on increasing levels of short-chain fatty acids in mouse feces. Research will continue to examine *E. cottonii* as a high-fiber herbal ingredient. The drying

and dissolution methods used must be different, as one reference is the results of previous research that *E. cottonii* contains high-carbohydrate fiber in the form of dry carrageenan powder.²² High-fiber foods increase intestinal SCFAs, which play a role in microbiota balance, and sIgA, which supports immunotolerance and prevents allergic diseases.^{7,10} Previous studies have shown that the dried powder form of the fruit is safe for consumption. It showed no signs of toxicity, and microscopic examinations of the internal organs of test animals remained normal.²³

This study uses zinc levels of mice above the daily dose, if according to the formula of the conversion factor to an adult human 70 kg weight of about 31mg / day. The recommended adult zinc intake is 11mg/day.²⁴ The basis of the zinc dose uses the results of previous research on 200g healthy Wistar strain rats administered 0.15 mg zinc supplements which produced the best effect for strengthening IFN- γ cytokines while still maintaining levels of the anti-inflammatory cytokine TGF- β which is still above the value of the levels of the group given daily or twice daily doses.²⁵ Following the condition of allergic diseases that require regulating the balance of immune responses to normal, to prevent Th2 dominance by strengthening Treg.¹¹ The combination of probiotics and zinc has been shown to improve intestinal villus length and mucosal epithelial thickness in animal models of malnutrition. Zinc may

be a good choice to add to herbal supplements such as *E. cottonii*.²⁶ Previous studies have proven the effect of *E. Cottonii* as an anti-inflammatory and a good source of oligosaccharides in reducing allergic inflammation, eosinophil infiltration, and increasing the presence of Treg in food allergies.^{27,28} *E. Cottonii* has the potential as a food source of prebiotics, it has been known the benefits of prebiotics in modulating normal immune responses in the digestive tract to prevent allergies.²⁹

CONCLUSION

The combination formulation of *Eucheuma Cottonii* Extract plus Zinc could prove to be a hope as a phytopharmaceutical to alleviate allergy symptoms by strengthening Treg expression, but it must be at a measured dose that is safe for the balance of normal immune responses.

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CONFLICT OF INTEREST

There are no conflicts of interest

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