

Effect of orally administered combination of *Caulerpa racemosa* and *Eleutherine americana* (Aubl) Merr extracts on phagocytic activity of macrophage

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Abstract

Background and purpose: Polysaccharide sulfate is a major active phytochemical constituent of *Caulerpa racemosa*, whereas the *Eleutherine americana* (Aubl) Merr has antioxidant properties. The aim of this research was to investigate the combined effect of polysaccharide sulfate that was isolated from *C. racemosa* and *E. americana* on the macrophage activity.

Experimental approach: The phenolic contents and antioxidant activities of *E. americana* extracts in water and various ethanol concentrations were studied using the Folin-Ciocalteu and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) methods, respectively. Polysaccharide sulfate was isolated from *C. racemosa* by precipitation method. To assess the macrophage activity, mice were treated orally for 14 days with either a combination of polysaccharide sulfate and *E. americana* 96% ethanol extract at a specific ratio or with each extract alone. Macrophages were isolated and the phagocytic activity was measured by assessing the ability of the macrophages to phagocytose latex particles and nitric oxide (NO) levels were assessed using a colorimetric assay.

Findings / Results: The *E. americana* crude extract in water exhibited the highest yield (13.04%), compared with the extract in 96% ethanol, which had the highest phenolic content (6.37 ± 0.16 mg/g gallic acid equivalent) and the strongest antioxidant activity (IC_{50} , 22.63 ± 1.09 μ g/mL). The combination of extracts, when both extracts were administered at 65:65 mg/kg BW, resulted in the highest increases in phagocytosis activity ($62.73 \pm 5.77\%$) and NO levels (16.43 ± 1.37 μ mol/L).

Conclusion and implications: The results of this study confirmed the non-specific immunostimulant properties of the combination of polysaccharide sulfate and *E. americana* and justified their use in traditional medicine. The observed increase in macrophage activity appeared to be correlated with the increased ability of mice to fight infection.

Keywords: Antioxidant; *Caulerpa racemosa*; *Eleutherine americana* (Aubl) Merr; Phagocytic; Phenolic content; Polysaccharide sulfate.

INTRODUCTION

Infectious diseases remain an important public health issue worldwide. Antibiotics are often specific for the types of bacteria they can treat and, in general, cannot be easily interchanged between infection types. When antibiotics are used correctly, they are generally safe, with few side effects (1,2).

However, the inappropriate use of antibiotics to treat infectious diseases has resulted in a major increase in multidrug resistance (MDR) among

bacteria. Microorganisms that have been reported to have developed MDR include *Escherichia coli* (3), *Klebsiella pneumoniae* (4), *Staphylococcus aureus* (5), *Streptococcus pneumoniae* (6), *Salmonella enterica* serovar Typhi (7), *Shigella species* (8), and *Neisseria gonorrhoea* (9), which are all considered to be significant agents of human infection.

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The use of antibiotics can also endanger the natural microbial flora in the body, which represent essential components of the body's self-defense (immune) system (10). This situation has resulted in the search for alternative substances that can be used to treat infections, with minimal negative side effects, including those with immunostimulant properties.

Immunostimulants are natural or synthetic products, associated with a variety of chemical characteristics and mechanisms of action, which is capable of activating various components of the human immune system to protect against infections caused by bacteria and viruses. These products can also be used to counter the effects of various diseases that pathologically reduce immune system capabilities (11). Two natural sources of substances with immunostimulant activities that are abundant in South Sulawesi are the seaweed *Caulerpa racemosa* (*C. racemosa*) and the bulb of *Eleutherine americana* (Aubl) Merr (*E. Americana*).

In South Sulawesi, *C. racemosa* is known as *lawi-lawi* and is intensively cultivated in some areas, including the Takalar District (12). The primary chemical component in *C. racemosa*, polysaccharide sulfates (13,14), has been shown to display antiviral activity towards measles (15), herpes simplex virus (16), human immunodeficiency virus (HIV) (17), dengue virus, and respiratory syncytial virus (18). The polysaccharide sulfates from the edible green alga *C. racemosa* var *peltata* has been shown to have immunostimulatory effects by increasing the levels of tumor necrosis factor (TNF)- α , interleukin-1 β (IL-1 β), nitric oxide (NO), and reactive oxygen species (ROS), in a dose-dependent manner, in macrophage cells (19,20).

The bulb of *E. americana*, Dayak union, is traditionally used in the Jeneponto District to treat measles that is known locally as *campak*. Preliminary research indicated that treatment with *E. americana* extract, at 0.08 mg/30 g BW, was able to reduce the germinal diameter of lymph nodes and increase serum immunoglobulin G (IgG) levels in mice (21). The IC₅₀ for the antioxidant activity of an *E. americana* extract in 70% ethanol was 31.97

$\mu\text{g/mL}$ (strong activity), as assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (22).

The aim of the present study was to develop an herbal product, by combining the polysaccharide sulfates derived from *C. racemosa* with the antioxidants properties from *E. americana*, to achieve a synergistic effect and increase the immune power, as determined by evaluating the effects of treatment on the phagocytotic capacity of macrophages and the level of NO. This study also determined the effects of various polarity solvents on the phenolic contents and antioxidant capacities of *E. americana* and examined the correlations between these two characteristics.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, amphotericin B, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and phosphate-buffered saline (PBS) pH 7.4 were purchased from Gibco® (USA). Ethanol, methanol, Giemsa, barium chloride (BaCl₂), Folin-Ciocalteu, 1,1-diphenyl-2-picryl hydrazyl (DPPH), cetylpyridinium chloride, sodium acetate, cysteine, ethylenediaminetetraacetic acid (EDTA), crude papain, sodium chloride, sodium carbonate, potassium sulfate, and latex beads were obtained from Sigma-Aldrich (USA).

Sample collection and preparation

The green alga, *C. racemosa*, was collected from the coast of Takalar, Indonesia, on the Flores Sea, Indonesia. The alga was cleaned under running water, cut into small pieces, and oven-dried at 60 °C. The dried *C. racemosa* was extracted *via* an enzymatic technique, using papain, as described by Rodrigues *et al.* with some modifications (23). The samples were first ground and then rehydrated in 100 mM sodium acetate (pH 5.0), containing cysteine and EDTA (5 mM), to which a solution of crude papain (30 mg/mL) was added. The mixture was heated to 60 °C for 6 h, to extract the polysaccharide, and then the mixture was strained and centrifuged (5,000 rpm, 4 °C, 30 min). The supernatant was added to 16 mL

of 10% cetylpyridinium chloride to precipitate the polysaccharide sulfate (25 °C, 24 h). The precipitate was cleaned with 0.05% cetylpyridinium chloride, dissolved in a 100:15 mixture of 2 M sodium chloride and ethanol, and precipitated through the addition of ethanol (4 °C, 24 h). After precipitation, the polysaccharide sulfates were centrifuged, washed twice with 80% ethanol, and washed with 96% ethanol. Finally, the polysaccharide sulfates were oven-dried (60 °C, 4 h).

Fresh bulbs of *E. americana* were collected from a local market in Gowa, Indonesia. The bulbs were air-dried, after the hand-picked removal of unwanted particles, chopped into small pieces, and ground into a coarse powder using a blender (Miyako, Indonesia). The dried bulbs were extracted in various polarity solvents, including water and 30, 50, 70, and 96% ethanol, using a sonicator for 72 h. Then, the samples were filtered and concentrated using rotary evaporation (Büchi Rotavapor, Germany).

Testing polysaccharide sulfates

The polysaccharide sulfate contents in the *C. racemosa* extracts were analyzed for sulfate groups (-SO₃H) using a BaCl₂-gelatin method (24). In a typical procedure, the gelation solution (0.3%) was prepared in hot water (60-70 °C) and incubated at 4 °C overnight. Then 2.0 g of BaCl₂ was dissolved in the gelatin solution and incubated for 2 h, at 25 °C. Approximately 0.5 mL of polysaccharide sulfate solution (10.0 mg/mL) was combined with 3.8 mL of 0.5 M HCl and 1.0 mL of BaCl₂-gelatin reagent, and the mixture was incubated for 15 min. The released Ba-sulfate concentration was measured at λ 360 nm by ultraviolet-visible (UV-Vis) spectrophotometry (Agilent 8453, USA), using potassium sulfate to generate the standard curve. This experiment was performed in triplicate.

Determination of phenolic content

The phenolic contents of the *E. americana* extracts were determined using the Folin-Ciocalteu method (25). A 0.1 mL volume of 50% Folin-Ciocalteu was added to 0.1 mL of each extract (10 mg/10 mL). A 0.1 mL volume of 7.5% sodium carbonate was added to the

mixture, and the mixture was filtered and water was added to bring the total volume of 10 mL. The mixture was incubated at room temperature for 5 min. The absorption of the mixture was recorded using a spectrophotometer (Agilent 8453, USA) at λ 730 nm. A standard curve was produced using several concentrations of gallic acid. The phenolic contents were expressed as mg/g gallic acid equivalent (mg/g GAE). This experiment was performed in triplicate.

Determination of antioxidant activity

The DPPH free radical scavenging activity was evaluated using the method proposed by Liaudanskas *et al.* (25). Ten mg of each *E. americana* extract was dissolved in 10 mL of ethanol (1,000 µg/mL). Stock solutions of each extract at volumes of 2.5, 5.0, 10.0, 20.0, and 40.0 µL were added to the wells of a microplate, 75 µL of 240 µM DPPH was added to each well using a multichannel micropipette, and the volume of each well was brought to 200 µL using ethanol. The microplate was incubated at 37 °C for 30 min, and absorbance was recorded at 515 nm using a microplate reader (Biotek ELx808, USA). The antioxidant activity of the *E. americana* extracts is given as the half-maximal inhibitory concentration (IC₅₀).

Animals

All animal experiments were conducted with local ethical approval from the Faculty of Medicine, Hasanuddin University (Ethics No. 169/H4.8.4.5.31/PP36-KOMETIK/2017). Male 8-10-week-old Balb/c mice (20-30 g) were housed in a 12/12-h light/dark cycle unit, with free access to food and water.

Preparation of sheep red blood cell suspension antigen

Sheep red blood cells were used as antigenic materials. Sheep red blood cell suspensions, 20% in PBS pH 7.4, were used to challenge the animals throughout the study.

Experimental design

The mice were randomly divided into 6 groups, 3 each. Group I, received polysaccharide sulfate 130 mg/kg BW; group II, received *E. americana* 130 mg/kg BW; groups III-V, received the combination of

polysaccharide sulfate and *E. americana* (30 and 100; 100 and 30; 65 and 65 mg/kg BW); and group VI, vehicle (0.5% sodium carboxymethylcellulose). All animals were treated orally with extracts or vehicle control daily for 14 days. On day 8, all groups were challenged with 0.1 mL of 20% sheep red blood cell suspension, intraperitoneally (i.p). On day 15, the mice were sacrificed by cervical dislocation under anesthetic conditions.

Isolation and preparation of macrophages

Resident peritoneal macrophages were collected by washing out the mouse peritoneal cavities with cold PBS. The macrophage cells (1.5×10^5 cell/mL), attached to glass coverslips (d = 13 mm), were placed into 24-well tissue culture plates and cultured in RPMI medium, supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.5% amphotericin B. Cultures were incubated a 37 °C cell culture incubator, with 5% CO₂, for 24 h.

Observation of macrophage phagocytosis activity

Phagocytosis activity was observed using latex beads suspended in PBS. A 200 µL volume of suspended latex beads (d = 2.0 µm) was placed in each well and incubated for 4 h, in 5% CO₂, at 37 °C. The medium was removed from the well, leaving the macrophage cells adhered to the coverslips, which were washed 3 times with PBS and dried at room temperature. The coverslip was then immersed in cold methanol, stained with 20% Giemsa, and washed with distilled running water. The number of macrophages engaged in the phagocytosis of latex beads was counted under a microscope, using 20× magnification. Phagocytosis activity was provided as the percentage of macrophage cells observed to be phagocytosing latex beads.

Calculation of NO concentrations

The NO level in the culture supernatant was measured after 24 h incubation. Briefly, 100 µL of supernatant was incubated with 2% (w/v) sulfanilamide in 10% (v/v) o-phosphoric acid, for 15 min. Then, 50 µL of 0.2% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride was added, and the mixture was incubated for

an additional 15 min at room temperature. The absorbance of the sulfanilamide-N-(1-naphthyl)-ethylenediamine dihydrochloride complex was recorded at 570 nm using a microplate reader (Biotek ELx808, USA). The quantification of nitrite in the sample was standardized against 0-100 µmol/L of NaNO₂ (26).

Statistical analysis

The results are expressed as the mean ± standard deviation (SD) from 3 independent experiments which were analyzed by one way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered as statistically significant.

RESULTS

Extract yields

The extraction of the dried *E. americana* bulb was performed using the maceration technique at room temperature. Maceration allows the extraction of all components, without heat, which can damage chemical components. The solvents used in this study, based on polarity, were water and 30, 50, 70, and 96% ethanol, which allowed the bioactive compounds to be extracted according to polarity. The yields for the dried extracts in each solvent are presented in Table 1. The results showed a pattern for percent yield, as follows: water > 30% ethanol > 50% ethanol > 70% ethanol > 96% ethanol. These results indicated that the *E. americana* bulb primarily contains polar compounds.

Approximately 51.90 kg of wet *C. racemosa* was collected for this study. After cleaning and oven drying, the final dry powder mass was 520.00 g (yield = 1.00%). Estimation of the polysaccharide sulfates contents showed that 1 mg of *C. racemosa* contains 171.2 µg polysaccharide sulfates.

Table 1. The yields of *Eleutherine americana* extracts prepared using different extraction solvents.

Extraction solvent	Dried bulb (g)	Dried extract (g)	Yield (%)
Water	50	6.52	13.04
Ethanol (30%)	50	5.24	10.48
Ethanol (50%)	50	4.45	8.90
Ethanol (70%)	50	4.13	8.26
Ethanol (96%)	50	2.41	4.82

Table 2. Total phenolic concentrations and IC₅₀ values of the *Eleutherine americana* bulb extracts prepared using different extraction solvents.

Treatment	Total phenolic concentration (mg/g GAE)	IC ₅₀ (µg/mL)
Water	1.00 ± 0.13	195.20 ± 11.42
30% Ethanol	0.96 ± 0.06	70.95 ± 1.89
50% Ethanol	3.42 ± 0.37	40.55 ± 19.03
70% Ethanol	4.72 ± 0.05	38.99 ± 2.67
96 % Ethanol	6.37 ± 0.16	22.63 ± 1.09
Vitamin C	-	1.00 ± 0.13

GAE, gallic acid equivalent.

Total polyphenol content

The total phenolic content was determined in *E. americana* ethanolic extracts, with the highest phenolic content observed in the extract prepared with 96% ethanol (6.37 ± 0.16 mg/g GAE), followed by 70% ethanol (4.72 ± 0.05 mg/g GAE), and 50% ethanol (3.42 ± 0.37 mg/g GAE), whereas water and 30% ethanol were the lowest levels of 1.00 ± 0.13 and 0.96 ± 0.06 mg/g GAE, respectively (Table 2). The total polyphenol content of the extracts decreased with the increasing polarity of the solvent, indicating that *E. americana* bulbs primarily

contain semi-polar and/or non-polar compounds.

Antioxidant activity

The DPPH assay was used to determine the antioxidant activity of the different *E. americana* extracts. All of the extracts showed concentration-dependent increases in the IC₅₀ values (Fig. 1). Antioxidant activity depends on the total polyphenolic compound concentration.

The antioxidant capacities of the *E. americana* extracts were expressed as the IC₅₀ values of the extracts. During the DPPH assay, the 96% ethanol extract had the highest antioxidant, and the remaining extracts showed the following pattern: 70% ethanol > 50% ethanol > 30% ethanol > water (Table 2). The antioxidant activities of the extracts decreased as the polarity of the solvent increased, indicating that semi-polar and/or non-polar compounds play roles in antioxidant activity.

Correlation

Furthermore, the total phenolic contents and the antioxidant activities of the extracts (IC₅₀) were found to be highly correlated (correlation = - 0.730), with higher polyphenol levels correlating with higher antioxidant activity.

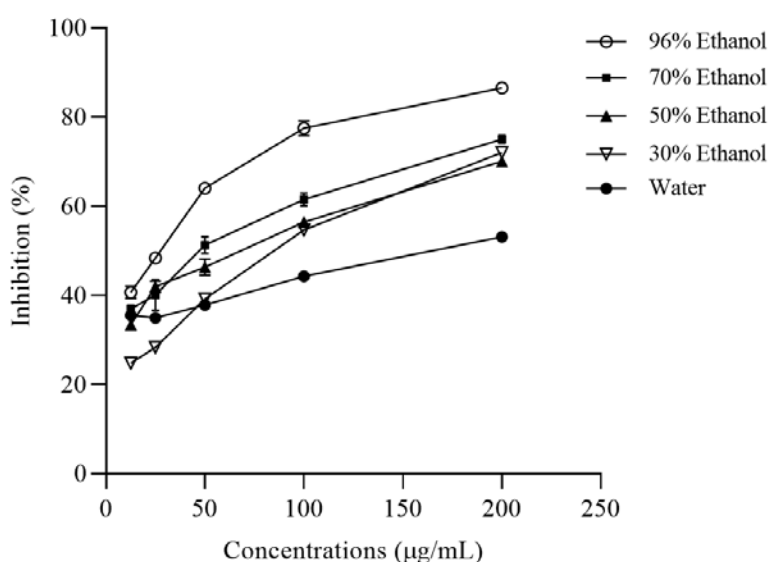


Fig. 1. The DPPH radical scavenging activity of *Eleutherine americana* in various polarity solvents. The absorbance of the DPPH solution alone at 517 nm was 0.821 ± 0.011 (experimental control). DPPH, 2,2 -diphenyl-1-picryl-hydrazyl-hydrate.

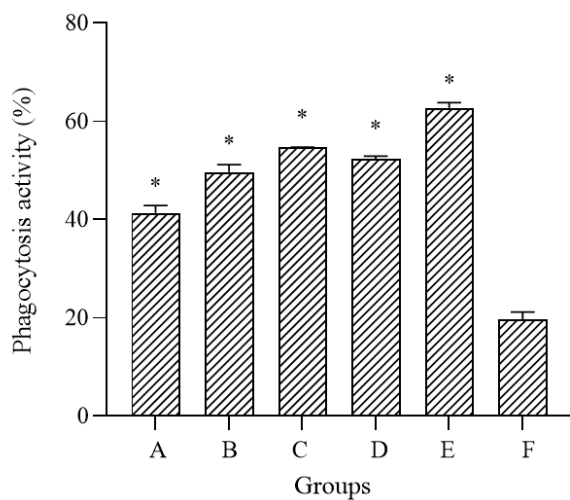


Fig. 2. The effects of polysaccharide sulfates and *E. americana*, either alone or in combination, on the phagocytosis activity of peritoneal macrophages. After incubation at 37 °C for 4 h, the numbers of macrophages engaged in the phagocytosis of latex beads were counted under a microscope, using 20× magnification. **P* < 0.05 represents significant differences compared to the control group. *E. Americana*; *Eleutherine americana*; BW, body weight; A, polysaccharide sulfate 130 mg/kg BW; B, *E. americana* 130 mg/kg BW; C, Polysaccharide sulfate and *E. americana* 30:100 mg/kg BW; D, polysaccharide sulfate and *E. americana* 100:30 mg/kg BW; E, polysaccharide sulfate and *E. americana* 65:65 mg/kg BW; F, negative control.

Macrophage phagocytosis activity

The phagocytic capacity of peritoneal macrophages is expressed as the means ± SD of the percentage of phagocytosing macrophages (Fig. 2). There were significant differences in the phagocytosis activity between all the treated groups and control. The use of either 130 mg/kg BW of polysaccharide sulfate or *E. americana* increase the phagocytosis activity to 41.33 ± 1.53 and 49.67 ± 1.53%; their combination at 30:100 and 100:30 mg/kg BW were 54.73 ± 0.06 and 52.33 ± 0.58%; and the highest activity, 62.73 ± 1.05%, was found at a combination of 65:65 mg/kg BW. This result indicated a synergistic effect between polysaccharide sulfate and *E. americana* on the immune system.

Nitric oxide concentration

In the present study, polysaccharide sulfate and *E. americana*, both alone and in combination, were evaluated for their abilities to inhibit NO production in peritoneal

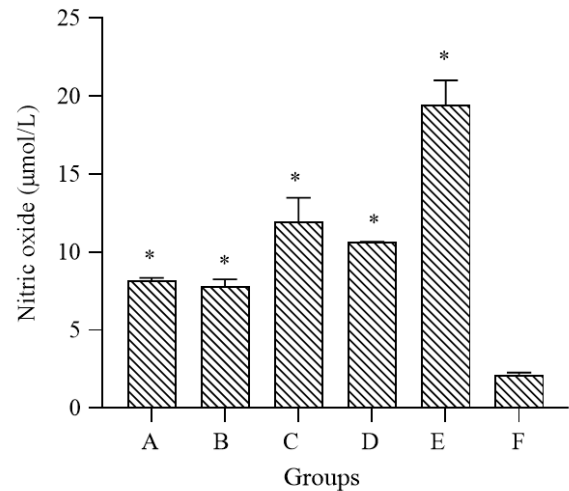


Fig. 3. The inhibitory effects of polysaccharide sulfates and *E. americana*, either alone or in combination, on nitric oxide production by peritoneal macrophages after incubation at 37 °C for 4 h. **P* < 0.05 represents significant differences compared to the control group. *E. Americana*; *Eleutherine americana*; BW, body weight; A, polysaccharide sulfate 130 mg/kg BW; B, *E. americana* 130 mg/kg BW; C, Polysaccharide sulfate and *E. americana* 30:100 mg/kg BW; D, polysaccharide sulfate and *E. americana* 100:30 mg/kg BW; E, polysaccharide sulfate and *E. americana* 65:65 mg/kg BW; F, negative control.

macrophages. Nitrite accumulation in cells increased following sheep red blood cell suspension treatments. The lowest level of NO was observed for the negative control (2.24 ± 0.38 µmol/L). Polysaccharide sulfate alone, *E. americana* alone, and their combination at 30:100; 100:30, and 65:65 mg/kg BW increased the NO accumulation in peritoneal macrophages compared with the negative control group (*P* < 0.05). A combination of 65:65 mg/kg BW indicated the highest ability to increase NO levels to 19.48 ± 1.53 µmol/L (Fig. 3).

DISCUSSION

This article discusses the synergistic effects of polysaccharides that isolated from *C. racemosa* and *E. americana* on the phagocytic activity of macrophage, which is characterized by an increased ability of macrophages to eliminate pathogens. The combination of polysaccharide sulfate and *E. americana* is based on the fact that *C. racemosa* containing

polysaccharides have already been reported as immunostimulants (19), and *E. americana* contains high polyphenol content and high antioxidant power (22) whose potential in boosting immune system has been proven. The combination of these two ingredients is expected to have a synergistic effect so that it can reduce the dose given compared to giving alone. This study also reports on how to obtain polysaccharide sulfates and the best solvents used to extract *E. americana*, which is characterized by the highest content of polyphenols and antioxidants.

The primary phenolic compounds in *E. americana* extract belong to the flavonoid group, which are powerful antioxidants with immunomodulatory effects (27,28). Phenolic compounds are characterized by the presence of one or more hydroxyl (R-OH) groups bound to benzene. The OH group is polar and easily dissolves in polar solvents. However, increasing the number of carbon chain complex compounds can reduce polarity (29,30). *E. americana* bulbs are primarily composed of polar compounds, as demonstrated by the higher yield observed for the water-based extract compared with ethanol-based extracts (Table 1), which is similar to the results reported by Agustin *et al.*, who found the highest yield with water. Most polyphenol compounds are polar to semi-polar; therefore, the highest polyphenol contents and antioxidant activities are likely to be observed for extracts containing semi-polar solvents, such as ethanol. The addition of water to ethanol can increase the polarity (31).

The combination of polysaccharide sulfate and *E. americana* has not previously been examined. Polysaccharide sulfates not only provide structural support for the cell membrane but also have bioactive therapeutic effects on the immune system (32). Hao *et al.* found that treatment with *C. racemosa* extracted in water boosted the immune system by increasing NO, ROS, TNF- α , and IL-6 levels in RAW264.7 macrophage cells, in a dose-dependent manner (19). The compounds reported from *E. americana* were eleutherin, eleutherinol, isoeleutherin, dihydroeleutherinol, and hongconin inhibit RAW 264.7 lipopolysaccharide-activated mouse

macrophage cell (33). *C. racemosa* polysaccharides have been reported to increase the percentages of CD3⁺, CD4⁺, natural killer (NK), and CD4⁺/CD8⁺ cells in female Kunming mice (34).

The high levels of phenolic contents and the strong antioxidant potential of *E. americana* bulbs led us to examine the effect of *E. americana* extracts on NO production. Both polysaccharide sulfates and *E. americana*, alone and in combination, increased the NO production of peritoneal macrophages. This effect indicates that the presence of the extract may be responsible for this activity. NO is a multifunctional signaling molecule and at high concentrations is involved in immune responses by cytokine-activated macrophages (35). In addition, NO has been shown to have positive effects on the cardiovascular system and act as a potent neurotransmitter. However, NO can also act as a proinflammatory mediator that induces inflammation during abnormal situations (36,37).

Immune cells are particularly sensitive to oxidative stress because of the high polyunsaturated fatty acid contents in their plasma membranes, which results in the increased production of ROS; therefore, the immune system generally has higher antioxidant concentrations (38). We predicted that the antioxidant effect of *E. americana* could supply an endogenous antioxidant, protecting immune cells, such as macrophages.

A synergistic effect was demonstrated between polysaccharide sulfates and *E. americana*, in the present study, with the optimal combination observed at both extracts were administered at 65:65 mg/kg BW. This combination may represent an ideal natural immunomodulator that deserves further study for development as a potential therapeutic agent.

CONCLUSION

In conclusion, to optimize the phenolic content and antioxidant capacity of *E. americana* extracts, 96% ethanol should be used. Treatment with a combination of polysaccharide sulfates derived from *C. racemosa* and 96% ethanol extract of

E. americana, at both extracts were administered at 65:65 mg/kg BW, was able to improve macrophage function in mice. The present study provided a novel perspective, examining the nutraceutical use of medicinal plants as adjuvant therapies with immunostimulant effects. Further studies focusing on how polysaccharide sulfates and *E. americana* modulate the immune system are warranted.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this study

AUTHORS' CONTRIBUTION

E. Pakki, R. Tayeb, I.A. Ridwan, and L. Muslimin conceived and designed the experiments. E. Pakki, R. Tayeb, and U. Usmar performed the experiments and analyzed the data. E. Pakki, R. Tayeb, and L. Muslimin wrote the manuscript.

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