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In Vitro Anthelmintic Evaluation of *Acanthophora spicifera* Macroalgal Extract Against *Haemonchus contortus* in Sheep

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ABSTRACT

This study investigated the chemical composition and anthelmintic activity of *Acanthophora spicifera* against *Haemonchus contortus* from sheep *in vitro*. Fresh macroalgae were collected from Sepanjang Beach, Gunungkidul, Indonesia. Proximate analysis, fiber fractions, mineral composition, and color profile analysis were conducted to determine chemical profile of the macroalga. It was extracted by maceration with 96% ethanol (1:5) and ultrasonication. Secondary metabolites analyzed from the extract included total flavonoids, phenols, tannins, and saponins. The anthelmintic activity was tested *in vitro* through *H. contortus* adult worm motility and egg hatch inhibition tests. Extract treatments included P0: 0.9% physiological NaCl (negative control); P1: 0.5 mg/mL macroalgal extract; P2: 1 mg/mL macroalgal extract; P3: 1.5 mg/mL macroalgal extract; and Palb: 0.5 mg/mL albendazole (positive control). Motility test results showed that the use of macroalgae at levels of 0.5, 1, and 1.5 mg/mL significantly reduced *H. contortus* motility ($p<0.05$). However, effective LD_{50} was reached in 1 and 1.5 mg/mL within the first 10 hours, and LD_{100} after 21 hours ($p<0.05$). None of the extract levels matched the efficacy of albendazole ($p<0.05$). The egg hatch inhibition test revealed that the use of macroalgae at levels of 0.5, 1, and 1.5 mg/mL significantly inhibited *H. contortus* egg hatching by more than 85.73% after 24 hours ($p<0.01$), demonstrating comparable efficacy to albendazole. Based on these findings, *A. spicifera* extract contains various secondary metabolites with anthelmintic activity, inhibiting both *H. contortus* adult worm motility and egg hatching. This suggests that *A. spicifera* has potential for further development as a bioanthelmintic for ruminant parasite control, although further *in vivo* studies are needed.

Keywords: Acanthophora spicifera; anthelmintic; Haemonchus contortus; macroalgae; sheep

INTRODUCTION

Gastrointestinal parasites in ruminants continue to be a neglected concern, often emerging unexpectedly in grazing pastures and spreading quickly. They affect domestic and wild ruminants of all ages and are widespread throughout tropical and subtropical areas (Besier *et al*., 2016; Niciura *et al*., 2022; Palevich *et al*., 2022). *Haemonchus contortus*, one of the most significant parasites inhabiting the abomasum, decreases nutrient bioavailability, lowers nitrogen intake, and reduces the digestibility of organic matter and energy due to its high prevalence (Sakti *et al*., 2024d). Particularly, up to 50 µL blood per adult worm can be consumed daily by this parasite. Severe anemia, hypoproteinemia, and even death can be caused by infection with this parasite. Up to 100 mL of blood per day may be lost by infected animal (Adduci *et al*., 2022).

Rotational grazing, comprehensive biosecurity, phytochemical utilization from various plants, and the use of anthelmintics serve as key strategies to control and reduce infections of *H. contortus* and other parasites in animals (Adiwimarta *et al*., 2010; Ali *et al*., 2021; Niciura *et al*., 2022; Sakti *et al*., 2018, 2020). Consequently, the widespread use and heavy dependence of anthelmintics has caused *H. contortus* to develop resistance to theese drugs (Baihaqi *et al*., 2024). This has resulted in insufficient dosage and the emergence of multidrug-resistant parasites, which presented a considerable challenge to the global ruminant production sector (Babják *et al*., 2023; Barbosa *et al*., 2023). Outside of resistance,

anthelmintic application provokes area of interest regarding ecological disturbance around ranches and drug residues in animal products. These concerns have determined the exploring for new anthelmintic compounds that are more effective, safer, environmentally friendly, and sustainable. Marine biodiversity has arisen as an encouraging source for these new anthelmintic candidates (Baihaqi *et al*., 2023; Panhwer *et al*., 2023; Sakti *et al*., 2024c; Vonthron-Sénécheau, 2016).

Diverse applications, such as food, feed, cosmetics, and fertilizers, have been developed from macroalgae. Macroalgae also serve as a valuable source of minerals due to the considerable biomass of certain species (Charles *et al*., 2020; Winarni *et al*., 2022). Between them, red macroalgae are regarded as a potential feed additive for ruminants due to their high contents of protein, carbohydrates, digestible fibers, and secondary metabolites (Narvaez-Izquiedo *et al*., 2024). In Indonesia, the red macroalga *Acanthophora spicifera* is rarely utilized for food or animal feed, even though it contains high levels of protein and functional bioactive compounds. Recent findings have primarily highlighted its antioxidant, antimicrobial, anticancer, antihemolytic properties, and protective effects against gastrointestinal mucosal injury (Akbar & Hasan, 2024; Guillén *et al*., 2024; Júnior *et al*., 2021; Kumar *et al*., 2023; Salamat *et al*., 2022; Sivakumar *et al*., 2024). The direct application of *A. spicifera* as animal feed may be limited by its high level of mineral, which has the potential to disrupt rumen fermentation activity (Hidayah *et al*., 2023). Hence, extraction processes are required to obtain only the beneficial bioactive compounds. This macroalga contains a wide range of compounds, including flavonoids, polyphenols, tannins, steroids, saponins, alkaloids, phospholipids, glycosides, terpenes, acetogenins, and various fatty acids (Akbar & Hasan, 2024; Guillén *et al*., 2024; Kumar *et al*., 2023; Narvaez-Izquiedo *et al*., 2024; Samrit *et al*., 2024), many of which possess potential anthelmintic properties. However, its efficacy against *H. contortus* is not well-documented. Consequently, evaluating the potential of macroalgal extracts as eco-friendly anthelmintic agents is essential, especially for sheep commonly raised in the region where the macroalga was collected. This study aimed to evaluate the bioanthelmintic activity of the red macroalga *Acanthophora spicifera*, collected from the southern coast of Yogyakarta Province, Indonesia, in inhibiting the motility and egg hatching of *H. contortus* in sheep.

MATERIALS AND METHODS

Ethical Clearance Approval

Ethical clearance approval No. 012/KE.02/SK/01/2024 was issued by the Animal Care and Use Ethics Committee of the National Research and Innovation Agency (BRIN), Jakarta, Indonesia. It was subsequently used as the guideline for implementing all procedures in this study.

Sample Collection and Preparation Procedures

Fresh biomass of the red macroalga *A. spicifera* was collected from its natural habitat at Sepanjang Beach, located south of the Special Region of Yogyakarta, at coordinates 8°08'14.9" S and 110°34'07.9" E, as depicted in Figure 1. The collection was carried out during low tide in the morning at the beginning of February 2024. The preparation of biomass followed the Sakti *et al*. (2024d) procedure, with minor modifications in the washing duration. The fresh samples of *A. spicifera* (Figure 2) were transported to the laboratory, which is less than an hour, at room temperature, and then washed to remove contaminants and rinsed with running tap water and drained (Ramin *et al*., 2019). Furthermore, the biomass was transferred to stainless steel dishes and then frozen at -20 °C overnight. The frozen samples were freeze-dried at -50 °C for 30 h, with the process repeated once to achieve a fully freeze-dried state, and then ground to a size of 80 mesh.

Proximate Analysis and Dietary Fiber Profiling

The proximate analysis of the -50 °C samples was conducted following the Latimer & Horwitz (2010) methods, including moisture content, crude protein (CP), crude fiber (CF), ether extract (EE), and ash. Nitrogen-free extract (NFE) was calculated based

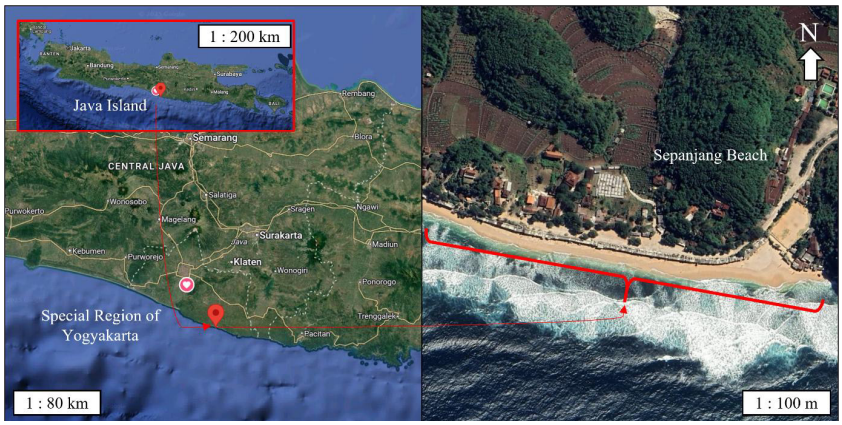


Figure 1. Map of the sampling site for *Acanthophora spicifera* collection at Sepanjang Beach, Gunungkidul, Yogyakarta, Indonesia

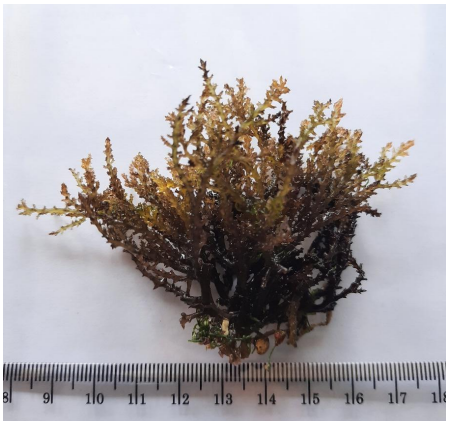


Figure 2. Red macroalgae *Acanthophora spicifera*

on proximate composition. Moisture content was determined stepwise from fresh to dried samples and by heating at 105 °C. Crude protein was analyzed using the Kjeldahl method, where nitrogen content was measured and converted into CP. Ether extract was determined using the Soxhlet method by extracting samples with petroleum ether for 16 hours, followed by solvent evaporation. Crude fiber was quantified by treating samples with sulfuric and nitric acids to isolate fiber residues. Ash content was measured by incinerating samples in a furnace at 550 °C for 5 h. NFE was calculated as 100% minus the sum of crude fiber, crude protein, ether extract, and ash. Fiber fractions, including neutral detergent fiber (NDF, cell wall components) and acid detergent fiber (ADF, lignocellulose), were analyzed using the Goering & Van Soest (1970) method.

Mineral Composition and Colorimetry

Mineral content, including macro minerals (Ca, Mg, S, K, P, Cl, Na), micro minerals (Al, Si, Ti, Sn, Cs, Pd, Sb, I, Ba, Te, Mo, Rh, Tc, Ru), and heavy metals (Hg, Cd, Pb, As), was analyzed using X-Ray Fluorescence (XRF) and presented as elements and oxides. Color profile analysis was performed on fresh and freeze-dried (-50 °C) samples using a chromameter, with color coordinates measured as L^* (lightness), a^* (green to red), and b^* (blue to yellow).

Ultrasound-Assisted Maceration for Macroalgae Extraction

Extraction followed the method of Hodhodi *et al*. (2022) with modifications based on Cikoš *et al*. (2022), using maceration with a solvent followed by ultrasonic exposure. Ethanol (96%) was used as the solvent, as water and ethanol are considered safer for food, feed, and pharmaceutical applications (Cikoš *et al*., 2022; Garcia-Vaquero *et al*., 2020). Freeze-dried (-50 °C) macroalgae were macerated at a solvent-to-sample ratio of 5–20 mL/g (5–20:1) according to Cikoš *et al*. (2022), preferably 5:1 for 24 h with occasional stirring. The macerate was subjected to ultrasonication at 20–25 kHz, with an ultrasonic pulse of 2 s, an interval of 3 s, and a total exposure of 10 min. The ultrasonicated extract was filtered using filter paper, and the filtrate was collected, while the residue was retained and then re-macerated with the same solvent ratio. This process was repeated three times. Furthermore, to obtain a thick extract, the combined filtrates were concentrated using a vacuum evaporator, which was subsequently freeze-dried at -50 °C for approximately 30 h. It produced a dry macroalgae extract.

$AlCl_3$-Based Flavonoid Analysis

The total flavonoid content was determined using the aluminium chloride ($AlCl_3$) colorimetric method (Chang *et al*., 2002). The procedure was conducted as follows: dried macroalgae extract (3 mg) was dissolved in 3 mL of methanol to obtain a sample concentration of 1,000 ppm. Subsequently, from this solution, 10 µL was transferred into a microplate well, while quercetin standard solutions at various concentrations were prepared similarly and added to separate wells. To each well, 60 µL of methanol, 10 µL of 1 M potassium acetate (KCH_3COO), 10 µL of 10% $AlCl_3$ solution, and 120 µL of distilled water were then added sequentially. The mixture was incubated at room temperature for 30 min to allow the formation of the flavonoid-$AlCl_3$ complex, after which the absorbance was measured 415 nm using a microplate reader. The standard solutions at different concentrations were used to construct a calibration curve. The total flavonoid content was calculated based on the quercetin calibration curve and expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g extract).

Folin–Ciocalteu-Based Phenolic Analysis

The total phenolic content was determined using a colorimetric approach based on the method of Baek *et al*. (2021). Briefly, 15 mg of the sample was dissolved in 3 mL of 80% methanol to prepare a stock solution with a concentration of 5,000 ppm. From this solution, 10 µL was transferred into a microplate well, along with gallic acid standard solutions prepared at various concentrations. Subsequently, 130 µL of distilled water and 10 µL Folin–Ciocalteu reagent were added, and the mixture was vortexed and incubated for 6 min. Thereafter, 100 µL of 7% Na_2CO_3 solution was added, vortexed, and incubated for 90 min. The absorbance was measured at 750 nm using a microplate reader. A calibration curve was constructed from the gallic acid standards, and the total phenolic content was expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

Phloroglucinol-Reactive Phenolics Analysis

The phloroglucinol-reactive phenolics (PRPs) content was determined following the methods of Montero *et al*. (2014) and Stengel & Connan (2015), with modifications described by Chouh *et al*. (2022) and Vissers *et al*. (2017), using a 2,4-dimethoxybenzaldehyde (DMBA) assay with phloroglucinol as the reference standard. A 2% (w/v) DMBA stock solution was prepared by dissolving 2 g of DMBA in 100 mL of 99% glacial acetic acid. A 6% (v/v) HCl stock solution was prepared by mixing 16 mL of 37% HCl in 84 mL of 99% glacial acetic acid. The working reagent solution was freshly prepared prior to analysis by combining the DMBA and HCl stock solution in a 1:1 ratio. For the assay, 50 µL of a 5,000 ppm macroalgae extract (5 mg/mL in methanol) was mixed with 250 µL of the freshly prepared reagent solution and incubated in the dark at room temperature for 1 h. A phloroglucinol standard curve (0–0.25 mg/mL) was constructed using methanol as the solvent. Absorbance of both samples and standards was recorded at 515 nm using a microplate reader. The PRPs content was expressed as milligrams of phloroglucinol equivalent per gram of extract (mg PGE/g extract).

Saponin Content Analysis

The total saponin content was analyzed according to El Shafay *et al*. (2022), with modifications to the solution ratio and absorbance measurement using a microplate reader. A 5,000 ppm stock solution of macroalgae extract was prepared using methanol as the solvent. Diosgenin was used as the saponin standard and dissolved in methanol. A total of 100 µL of macroalgae extract was mixed with 100 µL of 8% vanillin solution (in ethanol) in an ice bath. Then, 1 mL of 72% sulfuric acid (prepared by diluting 72 mL of concentrated sulfuric acid in 100 mL of distilled water) was added, followed by incubation at 60 °C for 10 min in a water bath. The mixture was then cooled in an ice bath. A total of 250 µL of the sample and standard solutions were transferred to a microplate, and absorbance was measured at a wavelength of 544 nm. Saponin content was expressed as milligrams of saponin equivalent per gram of extract (mg SE/g extract).

Adult Worm Motility Test

The motility assay of adult *H. contortus* followed the method of Sakti *et al*. (2018), employing a factorial completely randomized design (CRD) with two factors: extract concentration and observation time. *H. contortus* specimens were collected from the abomasum of Thin-Tailed sheep (<12 months old) slaughtered at an abattoir in Yogyakarta. Age determination was based on the absence of permanent incisor eruption, ensuring that only young sheep were used to minimize the risk of acquiring anthelmintic-resistant worms commonly found in older livestock (1–5 years) with a history of synthetic anthelmintic administration.

The selection criteria for *H. contortus* included: (a) female worms, identified by their characteristic intertwined red and white bands; (b) adult stage, with a body length of approximately 20 mm and a diameter of 2 mm; and (c) healthy and intact morphology, exhibiting active movement upon stimulation and normal swimming behavior in 0.9% sodium chloride. In this study, we specifically used adult female *H. contortus* because they are responsible for egg production. Assessing the efficacy of the extract on female worms provides a more direct indicator of its potential to reduce egg output. A total of 125 selected worms were manually transferred to petri dishes containing 0.9% sodium chloride for cleaning. The bioassay was conducted using three concentrations of macroalgae extract prepared in 0.9% sodium chloride, following the method of Medeiros *et al*. (2020). Albendazole was used as the positive control, whereas 0.9% sodium chloride served as the negative control. The experimental groups were designated as follows: 5 mL of 0.9% sodium chloride (negative control, P0); 5 mL of 0.5 mg/mL extract (P1); 5 mL of 1 mg/mL extract (P2); 5 mL of 1.5 mg/mL extract (P3); and 5 mL of 0.5 mg/mL albendazole (positive control, Palb).

Each treatment was conducted in five replicates, with five adult female worms allocated to each petri dish (n= 5). The number of live and dead worms was recorded at 0 min, 15 min, 30 min, and hourly until all worms were dead. Complete mortality was defined as the absence of movement for at least 10 s upon probing with an inoculation loop. Morphological responses to treatments were documented descriptively using an optical binocular microscope and a Scanning Electron Microscope (SEM).

Egg Hatch Inhibition Test

The *H. contortus* eggs used for the *in vitro* egg hatch inhibition test of *H. contortus* (Sakti *et al*., 2024d) were obtained from thin-tailed sheep naturally infected with *H. contortus* at an infection level exceeding 5,000 epg (eggs per gram of feces). The infected animals were slaughtered, and their abomasum was collected following the same procedure as in the adult *H. contortus* motility test. The egg hatch inhibition test followed a completely randomized design (CRD) with a one-way pattern, consisting of three different concentration levels of the test extract in a total volume of 3 mL solution, which was pipetted into test tubes. Albendazole was employed as the positive control, whereas 0.9% sodium chloride served as the negative control. Each treatment was conducted in three replicates with the following groups: 3 mL of 0.9% sodium chloride (P0); 3 mL of extract at 0.5 mg/mL (P1); 3 mL of extract at 1 mg/mL (P2); 3 mL of extract at 1.5 mg/mL (P3); 3 mL of albendazole at 0.5 mg/mL (Palb).

From the abomasum, a total of 45 female *H. contortus* worms were collected and randomly placed into 15 test tubes representing five treatment groups, each tube containing three worms. Eggs were released from the reproductive tract into the test solution by rotating a blunt glass spatula on the worm until homogeneous. The number of eggs was counted under a binocular microscope by sampling the solution at 0 and 24 hours after incubation at room temperature, this follows the method described by the World Association for the Advancement of Veterinary Parasitology (WAAVP) to detect anthelmintic resistance in gastrointestinal nematodes, including *H. contortus*, as referenced by Sakti *et al*. (2018).

Egg counts were conducted by first shaking the test tube, then withdrawing a 0.3 mL sample and placing it on a specialized double-object glass slide. Subsequently, 0.3 mL of saturated sugar solution was added, mixed with an inoculation needle, and left to stand for 5 minutes. The prepared slides were examined under a binocular microscope connected to a computer (Optilab™) at a total magnification of 100× (10× objective lens and 10× ocular lens). All eggs visible within the field of view were counted using a manual counter. The number of unhatched eggs at 24 h was compared with the initial count at 0 hours to determine the percentage of inhibition.

Morphological Analysis of Parasites Using SEM

Samples from each treatment group were mounted onto carbon tape and left to air-dry for several minutes until fully dried. The samples were then coated with

gold (Au) using an MC1000 ion sputter coater (Hitachi Corp.) at 10 mA for 60 s before being observed under a scanning electron microscope (SEM) (5 kV, SE detector, high vacuum mode) (Suryani *et al*., 2021). The magnifications used were 1,000× and 3,500× for the anterior and posterior regions, 200× for the genital region, and 15,000× for the cuticle.

Statistical Data Analysis

Statistical data were analyzed using analysis of variance (ANOVA), followed by Duncan's Multiple Range Test at a probability level of 0.05. Statistical analysis was performed using the CoStat statistical software version 6.0 (Cohort, 2022).

RESULTS

Nutrient Composition

The chemical composition of *A. spicifera* is presented on a 100% dry matter basis (Table 1). Freshly collected *A. spicifera*, a red macroalga, exhibits a high moisture content, exceeding 93%, resulting in a dry matter content of less than 7%. Freeze-drying at -50 °C effectively reduced the moisture content to below 16%, yielding a dried preparation with approximately 85% dry matter. In this study, the NDF and ADF contents of *A. spicifera* were less than 44% and 35%, respectively.

Minerals and Color Profile Analysis

Potassium, chlorine, and calcium were identified as the most abundant macrominerals in the red macroalga (Table 2). Among the microminerals, titanium, iodine, and tin were dominant, detected at ppm levels. Notably, no heavy metals were detected in the biomass of *A. spicifera*.

Color profile analysis (Table 3) revealed a highly significant difference ($p<0.01$) among the three forms of *A. spicifera* preparations. The drying process significantly altered the color profile values. All color variables increased following both freeze-drying at -50 °C and oven drying at 40 °C, with the exception of *a** value, which decreased due to oven drying at 40 °C.

Yield and Secondary Metabolite Composition of *A. spicifera*

The yield data, expressed on a 100% dry matter basis, along with the secondary metabolites content (Table 4), were analyzed from *A. spicifera* samples subjected to freeze-drying at -50 °C. Each secondary metabolite is presented as an equivalent to its respective standard compound.

Adult Worm Motility Test

The *in vitro* motility test of *H. contortus* exposed to *A. spicifera* macroalga extract at different incubation times indicated no significant reduction in motility during the initial 9 h of treatment (Table 5). However,

Table 1. The chemical composition of *Acanthophora spicifera*

Components	Values (%)
Dry matter (DM)	6.77
Ash	35.51
Crude protein (CP)	24.63
Ether extract (EE)	1.66
Crude fiber (CF)	5.17
NFE	33.04
NDF	43.14
ADF	34.68

Note: The data are presented on a 100% dry matter basis; NFE: nitrogen free extract; NDF: neutral detergent fiber; ADF: acid detergent fiber.

Table 2. Macromineral, micromineral, and heavy metal composition of *Acanthophora spicifera* subjected to freeze-drying at -50 °C, presented in elemental and oxide forms

Mineral (element; compound)	Value
Macromineral (%)	
Calcium (Ca; CaO)	8.25
Magnesium (Mg; MgO)	0.51
Sulfur (S; SO_3)	3.61
Potassium (K; K_2O)	18.77
Phosphorus (P; P_2O_5)	0.19
Chlorine (Cl; Cl)	12.35
Sodium (Na; Na_2O)	-
Aluminium (Al; Al_2O_3)	0.20
Silicon (Si; SiO_2)	0.94
Micromineral (ppm)	
Titanium (Ti; TiO_2)	375.00
Iron (Fe; Fe_2O_3)	-
Tin (Sn; Sn_2O_3)	97.60
Cesium (Cs; Cs_2O)	12.20
Palladium (Pd; PdO)	2.20
Zinc (Zn; ZnO)	-
Antimony (Sb; Sb_2O_3)	26.70
Iodine (I; I)	210.90
Barium (Ba; BaO)	28.40
Tellurium (Te; TeO_2)	72.30
Manganese (Mn; MnO)	-
Scandium (Sc; Sc_2O_3)	-
Indium (In; In_2O_3)	-
Copper (Cu; CuO)	-
Molybdenum (Mo; MoO_3)	3.60
Selenium (Se; SeO_2)	-
Rhodium (Rh; Rh)	0.50
Technetium (Tc; Tc)	0.20
Flour (F; F)	-
Ruthenium (Ru; RuO_2)	0.40
Vanadium (V; V_2O_5)	-
Cobalt (Co; Co_3O_4)	-
Heavy metal (ppm)	
Mercury (Hg; HgO)	-
Cadmium (Cd; CdO)	-
Lead (Pb; PbO)	-
Arsenic (As; As_2O_3)	-

Note: Analysis was conducted using X-Ray Fluorescence (XRF).

Table 3. Color profile analysis of fresh *Acanthophora spicifera*, freeze-dried at -50 °C, and oven-dried at 40 °C

Color variables	Drying methods		
	Fresh	Freeze-dried at -50 °C	Oven-dried at 40 °C
Degree of lightness (*L**)	13.69 ± 0.13^{c}	33.73 ± 0.08^{a}	33.42 ± 0.01^{b}
Green to red degree (*a**)	2.66 ± 0.03^{b}	2.86 ± 0.02^{a}	1.01 ± 0.02^{c}
Blue to yellow degree (*b**)	5.18 ± 0.12^{c}	7.16 ± 0.01^{a}	6.51 ± 0.01^{b}
Chroma value (C)	5.82 ± 0.12^{c}	7.71 ± 0.02^{a}	6.59 ± 0.01^{b}
Hue value (h°)	62.83 ± 0.37^{c}	68.23 ± 0.14^{b}	81.22 ± 0.16^{a}

Note: Different superscripts within the same row indicate a highly significant difference (p<0.01).

Table 4. Yield and secondary metabolite composition of *Acanthophora spicifera* macroalga

Variables	Values
Yield (% DM)	5.49 ± 0.05
Total Flavonoids (mg QE/g)	43.12 ± 12.87
Total Phenol (mg GAE/g)	7.60 ± 0.27
PRPs (mg PGE/g)	1.94 ± 0.19
Saponins (mg SE/g)	364.60 ± 7.64

Note: DM: dry matter; QE: quercetin equivalents; GAE: gallic acid equivalents; PRPs: phloroglucinol-reactive phenolics; PGE: phloroglucinol equivalents; SE: saponin equivalents.

at the 9-h mark, treatments P2 and P3, containing *A. spicifera* extract, inhibited *H. contortus* motility by more than 64% (p<0.05), with no significant difference from the positive control, albendazole. In contrast, the 0.5 mg/mL extract (P1) did not exhibit notable bioanthelmintic activity at this time point. The bioanthelmintic efficacy of the ethanolic extract at 1–1.5 mg/mL increased with time, reducing motility to 40% by the 12th h. This represented a 56.52% improvement compared to the negative control (p<0.05), while remaining comparable to albendazole.

Albendazole, a commercially available anthelmintic, exhibited LD_{100} by completely eliminating *H. contortus* within 15 h of observation, leaving 0% motility, which was significantly different from all other treatments (p<0.05). This finding indicates that the efficacy of *A. spicifera* extract at concentrations of 0.5–1.5 mg/mL remains inferior to that of albendazole. Nevertheless, treatments P2 and P3 consistently reduced *H. contortus* motility up to 18 h (p<0.05). Beyond this time point, a natural decline in *H. contortus* viability was observed across all treatment groups.

A significant interaction was observed between extract concentration and observation time (p<0.05), indicating that higher extract concentrations accelerated the decline in *H. contortus* motility. A significant reduction in *H. contortus* motility was observed at the 9th h in treatments P2, P3, and albendazole, whereas P1 did not exhibit a significant difference compared to P0, with a gradually declining curve, despite showing an initial downward trend. The motility curve for P1 remained statistically similar to P0 between the 11th and 18th h of observation. All treatments eventually reached the critical point of natural *H. contortus* mortality starting at the 20th h.

Egg Hatch Inhibition Test

The egg hatch inhibition assay revealed that the ethanolic extract of *A. spicifera* at concentrations ranging from 0.5–1.5 mg/mL significantly inhibited (p<0.01) *H. contortus* egg hatching by more than 90% within 24 hours of observation (Figure 3**)**. Under normal conditions, *H. contortus* eggs hatch within 24 hours after fertilization in the abomasum (Sakti *et al*., 2024d). In this study, the inhibitory effect of *A. spicifera* extract was approximately 195% more effective than the negative control group, with fewer than 15% of the eggs successfully hatching.

The inhibition of *H. contortus* egg hatching by *A. spicifera* extract, on the other hand, exhibited no significant differences in efficacy among the three tested concentrations and was statistically comparable to that of albendazole. This finding highlights the potential of this red macroalgal extract as a promising candidate to replace albendazole in disrupting the *H. contortus* life cycle at the embryonic development stage within the

Table 5. *Haemonchus contortus* motility in response to anthelmintic treatment at different incubation periods

Treatments	*H. contortus* motility (%) at hour									
	3	6	9	12	15	18	21	24	SEM	p-value
P0	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	92.00 ± 8.00^{Aa}	92.00 ± 8.00^{Aa}	60.00 ± 6.32^{Ba}	16.00 ± 7.48^{C}	12.00 ± 4.90^{Ca}	7.00	0.00***
P1	100.00 ± 0.00^{A}	96.00 ± 4.00^{A}	76.00 ± 11.66^{AB}	64.00 ± 11.66^{Bab}	60.00 ± 10.95^{BCb}	36.00 ± 7.48^{CDb}	20.00 ± 10.95^{DE}	8.00 ± 4.90^{Eab}	6.57	0.00***
P2	100.00 ± 0.00^{A}	96.00 ± 4.00^{A}	64.00 ± 11.66^{B}	40.00 ± 10.95^{Cbc}	24.00 ± 7.48^{CDcd}	8.00 ± 4.90^{DEc}	0.00 ± 0.00^{E}	0.00 ± 0.00^{Eb}	7.81	0.00***
P3	100.00 ± 0.00^{A}	96.00 ± 4.00^{A}	68.00 ± 12.00^{B}	40.00 ± 10.95^{Cbc}	28.00 ± 12.00^{CDc}	12.00 ± 8.00^{DEc}	4.00 ± 4.00^{DE}	0.00 ± 0.00^{Eb}	7.68	0.00***
Palb	100.00 ± 0.00^{A}	96.00 ± 4.00^{A}	64.00 ± 7.48^{B}	32.00 ± 8.00^{Cc}	0.00 ± 0.00^{Dd}	0.00 ± 0.00^{Dc}	0.00 ± 0.00^{D}	0.00 ± 0.00^{Db}	8.41	0.00***
SEM	-	1.47	4.74	5.96	7.29	5.02	2.99	1.60		
p-value	-	0.91^{ns}	0.08^{ns}	0.00**	0.00***	0.00***	0.09^{ns}	0.03*		

Note: Different uppercase superscripts within the same row and different lowercase superscripts within the same column indicate significant differences (p<0.05). ns denotes no significant difference (p>0.05). P0: 0.9% physiological NaCl (negative control); P1: 0.5 mg/mL *Acanthophora spicifera* extract; P2: 1 mg/mL *A. spicifera* extract; P3: 1.5 mg/mL *A. spicifera* extract; Palb: 0.5 mg/mL albendazole; SEM: standard error of the mean.

egg, even at the lowest tested concentration of 0.5 mg/mL. However, the efficacy of *A. spicifera* extract has yet to surpass that of albendazole.

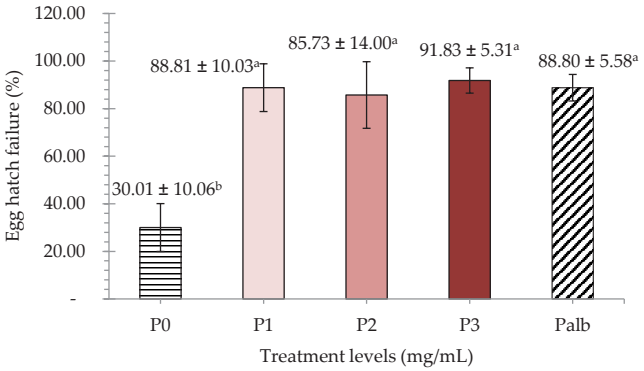


Figure 3. Egg hatch inhibition test of ethanolic *Acanthophora spicifera* extract on *Haemonchus contortus* eggs. Different subscripts indicate a highly significant difference ($p<0.01$); P0: 0.9% physiological NaCl (negative control); P1: 0.5 mg/mL ethanolic *A. spicifera* extract; P2: 1 mg/mL ethanolic *A. spicifera* extract; P3: 1.5 mg/mL ethanolic *A. spicifera* extract; Palb: 0.5 mg/mL albendazole (positive control); ⊥: Negative standard deviation; T: Positive standard deviation. ▬ P0; ▬ P1; ▬ P2; ▬ P3; ▨ Palb.

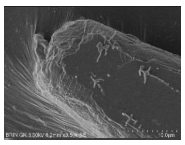
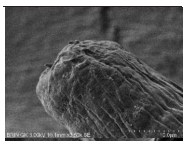
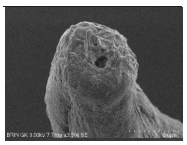
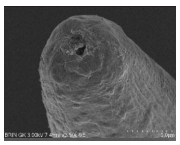
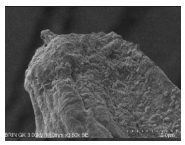
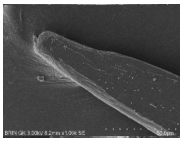
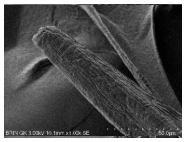
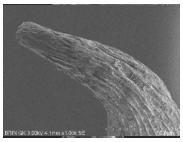
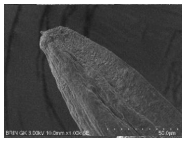
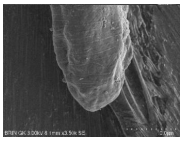
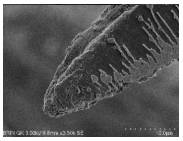
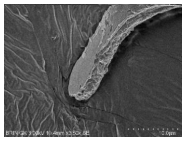
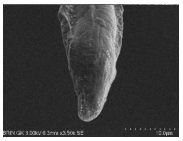
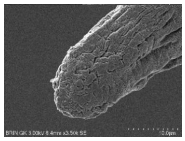
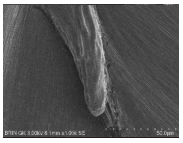
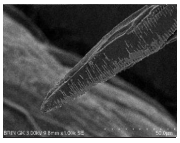
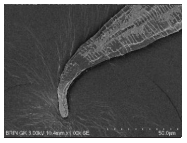
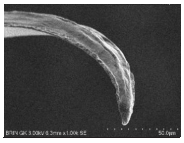
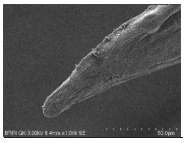
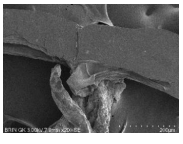
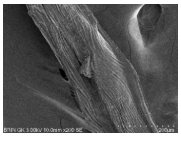
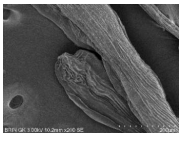
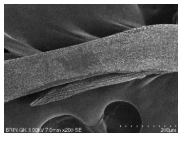
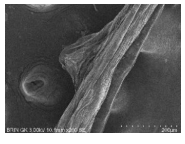
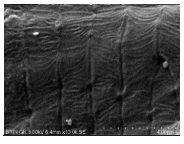
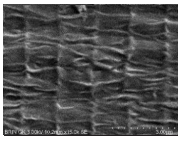
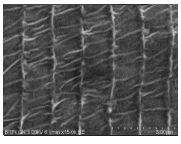
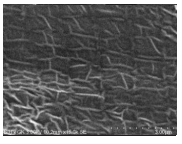
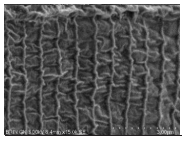
Morphological Response of *H. contortus*

Observations focused on the anterior region (buccal area), posterior region, genital organs, and overall cuticle morphology. The images (Table 6) illustrate that the physical condition of *H. contortus* in treatment P1 appeared fresh, with no visible differences compared to P0, despite a statistically significant improvement in adult worm motility being observed in the assay. The dark reddish coloration of worms in P1 dan P0 indicated the presence of fresh blood, distinguishing them from those in treatments P2 and P3. At doses of 1 and 1.5 mg/mL (P2 and P3), the worms exhibited a pale-whitish appearance, similar to those in the positive control group (Palb), suggesting a reduced blood content within their tissues. The buccal area of worms in P2 and P3 appeared wrinkled, with visible damage at the distal end. Structural deterioration was also observed in the cuticular layer of the parasite's skin, where higher extract doses led to drier, more wrinkled cuticles with reduced moisture content.

DISCUSSION

One of the macroalgae species found in tropical waters is *Acanthophora spicifera*, which is distributed

Table 6. Morphological response of *Haemonchus contortus* cuticle after exposure to ethanolic *Acanthophora spicifera* extract, physiological NaCl 0.9%, and albendazole

Morphological feature	Mag.	Treatments				
		P0	P1	P2	P3	Palb
Anterior, buccal area	3,500					
	1,000					
Posterior	3,500					
	1,000					
Genital organs	200					
Cuticle condition	15,000					

Note: Mag.: Magnification; P0: 0.9% physiological NaCl (negative control); P1: 0.5 mg/mL ethanolic *A. spicifera* extract; P2: 1 mg/mL ethanolic *A. spicifera* extract; P3: 1.5 mg/mL ethanolic *A. spicifera* extract; Palb: 0.5 mg/mL albendazole (positive control).

in the Asia, it is located in the waters of Banda Aceh, Indonesia (Akbar & Hasan, 2024); Tamil Nadu, India (Sivakumar *et al*., 2024); and Phetchaburi, Thailand (Samrit *et al*., 2024); and in the Americas, such as Santa Elena, Ecuador (Guillén *et al*., 2024); Hawaii, USA (Gibson *et al*., 2024); Trinidad and Tobago (Ali *et al*., 2021).

Nutrient content of *A. spicifera* includes 10.12% dry matter (Hidayah *et al*., 2023), 18.9% crude protein, and 2.1% crude fat (Rameshkumar *et al*., 2013). Compared to these compositions, our study found a 30.32% higher crude protein content and a 20.95% lower crude fat content. The NDF fiber fraction obtained in this study was also lower (52.96%) and the ADF fiber fraction was slightly higher (4.42%) than in the study by Hidayah *et al*. (2023). Differences in the chemical composition of macroalgae are generally caused by several factors, including environmental factors, such as climate, temperature, and salinity (Vinuganesh *et al*., 2022), and handling processes, such as drying method (Suwignyo *et al*., 2020).

In this study, the mineral composition of *A. spicifera* was dominated by potassium, chlorine, and calcium. These results align with research reported by Hidayah *et al*. (2023) that found potassium to be the dominant mineral in *Acanthophora* genera macroalgae, accounting for approximately 8%. In this study, heavy metals such as cadmium or plumbum are not found in the macroalgae biomass. Variations in mineral concentrations between samples and species may be due to differences in analytical methods and instrumentation, as well as marine environmental factors closely related to the nutrient contents and active compounds of macroalgae (Vinuganesh *et al*., 2022).

The high crude protein content of *A. spicifera* (CP > 20%) makes it a potential source of protein for animal feed. Furthermore, its relatively high inorganic content (ash content > 30%) makes a highly mineralized marine plant compared to terrestrial or freshwater plants (El-Adl *et al*., 2022). The use of macroalgae in ruminant feed formulas improves meat and milk quality (Harahap *et al*., 2024). The provision of macroalgae does not negatively impact ruminant performance and maintains nutrient balance with the application in an appropriate dosage (Sofyan *et al*., 2022). Based on its chemical composition, red macroalgae contain high levels of soluble carbohydrates and organic minerals, making them a promising source of feed nutrients. However, the potential for accumulation of potentially hazardous heavy metals requires regular monitoring and further processing to prevent potential risk to animal health (Kustantinah *et al*., 2022).

Characteristics of *A. spicifera* which contains high minerals and low organic matter, decrease the degradation of easily soluble fractions in the rumen, indicating the less value of *in vitro* gas production accounted for 30 mL per 100 mg dry matter sample (Hidayah *et al*., 2023). High levels of inorganic fraction or minerals are the main factors inhibiting the ruminal degradation and feed digestion in ruminants (Sakti *et al*., 2024d). Therefore, preparation macroalgae sample by applying the extraction technique showed minimizing the excess macro-nutrients and minerals, while improving active compound availability (Dewi *et al*., 2024). Then, these compounds can be explored as potential bioanthelmintic agents for ruminants.

In a previous study, the drying process alters the color profile of *A. spicifera*, indicating the alteration of chemical composition (Charles *et al*., 2020). In food and agro-industrial products, color profiles have been widely implemented as a parameter assessment of product quality (Sitanggang *et al*., 2023). The *L** value denotes brightness, which scales from 0 (dark) to 100 (light). The *a** value denotes the green-red axis, with negative values (-*a*: -80 to 0) showing a green hue, and positive values (+*a*: 0 to 100) showing a red hue. The *b** value shows a spectrum from blue (-) to yellow (+) (Baycar *et al*., 2022; Sitanggang *et al*., 2023). In this study, all color variables increased following both freeze-drying at -50 °C using a freeze dryer and oven drying at 40 °C, except for *a**, which decreased following oven drying at 40 °C.

Freeze-drying technique increases brightness intensity, causing water loss throughout the drying process. The *a** value is consistently positive, indicating the presence of red pigmentation in *A. spicifera* naturally, with only a slight green hue. While freeze-drying affects convinced color, it is commonly considered more effective in maintaining the quality of macroalgae compared to the conventional direct sunlight and oven drying (Charles *et al*., 2020).

Furthermore, the hue coordinate (h°) in this study ranged between 62° and 68°, consistent with the typical color profile of red macroalgae, where yellow tones appear around 90° and green tones above 100° (Sakti *et al*., 2024d; Sitanggang *et al*., 2023). The hue coordinate value exceeded 80 °C occurred during the oven drying process at 40 °C. This indicates that the drying technique alters the proportions of chemical components compared to conventional drying techniques. Shift in color variables is related to the availability of bioactive compounds (β-carotene, chlorophyll, fucoxanthin, and xanthophyll), which function as prominent pigments of macroalgae (Amoriello *et al*., 2021).

In this study, the ethanolic extract yield of *A. spicifera* exceeded 5%, which is relatively high when compared with other tropical macroalgae from Gunungkidul Regency, such as *Sargassum duplicatum*, which yielded only 2.25% under similar conditions (Sakti *et al*., 2024c). The macroalgal extract yield percentage is influenced by species, biological age, solvent type, particle size, maceration time, and even centrifugation speed during extraction (Prasedya *et al*., 2021; Sakti *et al*., 2019). The ethanolic extract of *A. spicifera* in our study was identified to contain flavonoids, phenols, PRPs, and saponins. A related species from the same area, *A. muscoides*, was also reported to contain flavonoids, tannins, and phenols (Hidayah *et al*., 2023). In contrast, *A. spicifera* from Banda Aceh contains flavonoids and polyphenols but lacks tannins and saponins (Akbar & Hasan, 2024), while samples from Mandapan, India, contained flavonoids, alkaloids, and tannins but lacked saponins (Kumar *et al*., 2023).

The variability in secondary metabolite levels is influenced by factors such as species differences, heat stress in the habitat (Urrea-Victoria *et al*., 2022), extraction techniques (Alara *et al*., 2021; Castillo *et al*., 2023), and the type of solvent used during extraction (Abu-Khudir *et al*., 2020). Among these metabolites, flavonoids and phenols are commonly found in both terrestrial and marine vegetation, playing essential roles as antioxidants (Samrit *et al*., 2024; Winarni *et al*., 2022) and antiparasitic agents (Olmedo-Juárez *et al*., 2022). In this study, the total phenol content of *A. spicifera* ethanolic extract was 75.12% higher than that reported for *samples* from Banda Aceh, which contained 4.34 ± 0.07 mg GAE/g extract (Akbar & Hasan, 2024). This difference is likely attributed to variations in the environmental conditions between the two habitats.

Acanthophora spicifera extract has a promising bioanthelmintic activity. In this study, the efficacy of treatments P2 and P3 was 11.11% lower than the 36% efficacy reported by Sakti *et al*. (2024a) for *Acanthophora* sp. at 1 mg/mL within the first 12 hours of observation. Nevertheless, these treatments were 60% more effective than the corresponding results with *Acrocystis* sp., a red macroalga from a similar habitat (Sakti *et al*., 2024a). Flavonoids, phenols, and tannins are present in different macroalgae, such as green macroalgae like *Chaetomorpha vieillardii* (Sakti *et al*., 2024d) or *Ulva* sp. (Sakti *et al*., 2024b), brown macroalgae, for example, *Sargassum duplicatum* (Sakti *et al*., 2024c), and red macroalgae, like *Acrocystis* sp. and *Acanthophora sp.* (Sakti *et al*., 2024a), are regarded as major contributors to the inhibition of egg hatching and the reduction of *H. contortus* motility at different concentration levels. One proposed mechanism for egg hatching inhibition involves the disruption of embryonic growth and development within parasite eggs due to the activity of phenolic compounds (Velázquez-Antunez *et al*., 2023). The diverse biological activities of macroalgal secondary metabolites remain an active area of research, particularly regarding their modes of action (Kalasariya *et al*., 2021).

Phenolic compounds, including flavonoids, phlorotannins, phloroglucinol-reactive phenolics, and saponins, are highly soluble in polar solvents such as 96% ethanol. Consequently, both their extraction yield and biological activity are strongly dependent on the solvent used (Alara *et al*., 2021). Flavonoids, a class of structurally heterogeneous natural polyphenols, are composed of at least one aromatic ring possessing one or more hydroxyl groups (Phang *et al*., 2023; Suwignyo *et al*., 2023; Tarahovsky *et al*., 2014). Their capacity to form complex molecular interactions with metal ions plays a crucial role in biological processes such as membrane adhesion, membrane fusion, and protein-membrane binding (Tarahovsky *et al*., 2014).

Covalent interactions between phenolic compounds and cell membranes can alter membrane conformation and fluidity, leading to structural disruption and membrane depolarization. This depolarization facilitates the passive diffusion of phenolic compounds into parasite cells (Ali *et al*., 2021). Once inside, these compounds interfere with transcription and translation processes, disrupt enzymatic activity, and inhibit ribonucleic acid and protein synthesis, thereby arresting cellular metabolism and inducing cell death (Ali *et al*., 2021; Khan *et al*., 2022). In addition, the suppressive activity on the egg hatching by macroalgae extracts on *H. contortus* could be explained by the inhibition of α-amylase and α-glucosidase, which are essential for the larval development (Lima *et al*., 2021). This disruption was pronounced for *H. contortus*, for both nervous and muscular cells (Sakti *et al*., 2024d).

Despite being a notable physical feature of adult female *H. contortus* (Mohamed *et al*., 2024), under light microscopy (SEM), no discernible or consistent changes were seen in this structure across the treatment groups. Because significant structural disturbances were seen in the buccal area and cuticular surface, the morphological investigation in this study concentrated on these more obviously impacted areas. The buccal portion of *H. contortus* showed signs of wrinkles and structural deterioration, especially at the front end, in groups P1 and P3. The parasite's capacity to adhere its anterior section to the abomasal mucosa and consume the host's blood is jeopardized by this degradation. Phenolic chemicals like tannins and PRPs are probably to blame for this structural damage. These substances' primary anthelmintic action is linked to their capacity to disturb the buccal region, which causes *H. contortus* cuticular aggregation (Baihaqi *et al*., 2020). The parasite's ability to move, feed, and reproduce may all be impacted by these structural alterations. Both condensed and hydrolyzable tannins demonstrate anthelmintic activity, with prodelphinidin-type tannins showing higher efficacy (Engström *et al*., 2016).

The parasite's cuticular layer also showed structural changes, with increasing extract concentrations causing more dryness, wrinkles, and dehydration, findings consistent with those reported by Barbosa *et al*. (2023). Phenolic chemicals cause cellular damage, especially in the intestine and cuticle cells, by interacting with parasite proteins. This inhibits motility and ultimately results in death (Borges & Borges, 2016). By causing both structural disturbance and mortality, plant-derived phenolics, particularly tannins and flavonoids, have shown strong anthelmintic effects against *H. contortus*. These substances cause oxidative stress, which damages and alters the worms' intestinal, muscular, and hypodermal cells physically (Martínez-Ortiz-De-Montellano *et al*., 2019). The ensuing structural degradation weakens the parasite's defenses, hinders its mobility, and interferes with its digestive system's metabolic functions, all leading to death (Barbosa *et al*., 2023).

CONCLUSION

The ethanol extract of the red macroalga *Acanthophora spicifera* at 0.5–1.5 mg/mL exhibits anthelmintic activity against *Haemonchus contortus*. This efficacy is reflected in its ability to inhibit the motility and hatching of *Haemonchus contortus* eggs. We consider that *A. spicifera* has great potential to be explored as a promising bioanthelmintic candidate for ruminants,

especially sheep. However, further validation through *in vivo* studies is necessary to confirm its effectiveness and safety under practical conditions.

CONFLICT OF INTEREST

The authors confirm that they have no competing interests regarding this research. No financial, personal, or professional affiliations with any organizations or individuals have influenced the study's content or findings.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT in order to improve readability and language of the work. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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