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Research article

In vitro anthelmintic activity of *Chaetomorpha vieillardii* ethanolic extract against adult worm motility and egg-hatching of *Haemonchus contortus* from sheep

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Abstract

This study aimed to evaluate the potential of macroalgae *Chaetomorpha vieillardii* as an *in vitro* candidate for bio-anthelmintic. Adult Worm Motility Test (AWMT) was conducted at concentration levels of 2 mg/mL, 4 mg/mL, and 6 mg/mL of *C. vieillardii* ethanol extract on 150 female *H. contortus* worms, randomly divided into 6 treatments with 5 replications, using a two way completely randomized factorial design. 0.9% sodium chloride was used as a negative control, and albendazole at 0.5 and 2 mg/mL served as positive controls. The observation of worm motility was conducted at time intervals of 0, 0.25, 0.5, 1, and 2 hours, and subsequently at hourly intervals until 100% of the worms were deceased. The identical treatment was applied to the Egg Hatching Inhibiting Test (EHIT) using a completely randomized design in a one-way pattern by observing the number of eggs at 0 and 24 hours. The results showed that the concentration level of *C. vieillardii* ethanol extract and the observation time significantly influenced ($P<0.01$) the motility of *H. contortus*, and there was an interaction between them ($P<0.01$). All three levels were able to decrease the motility of *H. contortus*, but they were unable to match albendazole. Despite this result, the three levels were equally effective ($P<0.01$) as albendazole in terms of inhibiting the egg-hatching of *H. contortus*. The 2 mg/mL level was sufficient to inhibit the motility and egg-hatching of *H. contortus*. These findings indicated that the ethanol extract of *C. vieillardii* has the potential to be developed as a bio-anthelmintic for ruminants.

Keywords: Bio-anthelmintic, *Chaetomorpha vieillardii*, *Haemonchus contortus*, Macroalgae

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INTRODUCTION

The development of ruminant farming faces various challenges in terms of feed, genetics, diseases, and socio-cultural factors. Concerning feed and disease factors, the inefficiency of feed digestion and animal productivity caused by gastrointestinal parasite infestation has been a significant problem that researchers worldwide have paid attention to for at least the past decade (Taki et al., 2020; Flay et al., 2022; Ocampo-Gutierrez et al., 2022). One of the gastrointestinal tract disturbances is caused by nematodes that grow, reproduce, and suck the blood of their host animals on the mucosal membrane of the gastrointestinal tract, from the stomach (abomasum) to the intestine. Parasitic infestation by the nematode *Haemonchus contortus*, which is specifically found in the abomasum, especially in small ruminants, causes a condition called haemonchosis, which is a parasitic disease that causes anaemia in livestocks, with reduced appetite and body weight, a lack of digestible nutrients, decreased performance and immunity, and can result in animal death, which eventually gives a bad economic impact (Ali et al., 2021; Kuisue et al., 2021; Politi et al., 2021; Adduci et al., 2022).

Control of gastrointestinal nematodes in ruminant livestock is generally carried out using anthelmintics, which are mostly synthetic, as well as the utilization of terrestrial and marine forages that contain secondary metabolites (Kustantinah et al., 2010; Kustantinah et al., 2014; Sakti et al., 2018; Sakti et al., 2020; Politi et al., 2021; Widiarso et al., 2021). The use of herbal with antiparasitic properties are considered appropriate, as the continuous administration of broad-spectrum commercial anthelmintics such as albendazole is known to have caused parasite resistance to drugs, making treatment ineffective and increasing farm operational costs (Nixon et al., 2020; Maestrini et al., 2021; Widiarso et al., 2021; Niciura et al., 2022; Palevich et al., 2022). This occurrence is also a common case in Indonesia (Widiarso et al., 2021). In addition, problems have been found related to synthetic anthelmintic residues left in livestock products, such as meat and milk, which can become a source of chemical anthelmintic contamination in animal and human bodies, soil, and water. This problem increases the likelihood of parasite and other biota resistance in contaminated environments (Nixon et al., 2020).

Regarding the biodiversity of feed from the sea, Indonesia is the world's second-largest producer of macroalgae (38.7%) after China (47.9%) (Ferdouse et al., 2018). Macroalgae, commonly known as seaweed, besides its biomass benefits as food, also contains bioactive compounds such as terpenoids (Arrieche et al., 2022; González-Andrés et al., 2022), phlorotannins (Fraga-Corral et al., 2021), polysaccharides (Fauziee et al., 2021), carotenoids, sterols/steroids, phenolic acids, phenols, pheromones, xanthophylls, chlorophylls, phloroglucinols, alkaloids, vitamins, amino acids, and fatty acids (Lever et al., 2020; Bonde et al., 2021; Arrieche et al., 2022; Samar et al., 2022). These bioactive compounds are recognized to have antimicrobial, antiparasitic, anti-inflammatory, antioxidant, anesthetic agent, and even antitumor activities (Ghania et al., 2017; Abu-Khudir et al., 2020; Dhara and Chakraborty, 2020; Saraswati et al., 2020; Purbosari et al., 2022; Samar et al., 2022).

Flavonoid and phenolic compounds have the potential to exhibit antiparasitic properties. Haq et al. (2019) reported the total flavonoid and phenol

contents of ethanolic extracts from *Chaetomorpha* sp. as 189.14 mg QE/g and 21.92 mg GAE/g, respectively. These extracts have been recognized for their antioxidant and anticancer properties. However, the antiparasitic activity of *Chaetomorpha* sp. remains largely unexplored. The green macroalgae *Chaetomorpha vieillardii* found on the southern coast of Java Island has not been widely published as an antiparasitic agent against the egg stage and adult worms of *Haemonchus contortus* in ruminants. Does this local tropical *C. vieillardii* from Indonesia have antiparasitic activity for sheep? This study attempts to address this question.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the National Research and Innovation Agency, Jakarta, Indonesia (No. 005/KE.02/SK/01/2023). The decision was deliberated on January 13th, 2023, and declared to fulfil the ethical clearance requirements with a research period from February to December 2023.

Sample collection and identification

A collection of fresh macroalgae samples was obtained from the southern coast of Gunungkidul Regency, DIY Province during low tide conditions at coordinates 8°08'14.9" SL and 110°34'07.9" EL. The fresh samples were washed with seawater as an initial cleaning step to remove sand, marine animals, and other impurities. The samples were then brought to the laboratory and washed with fresh water three times to ensure they were free from contaminants, then rinsed with running tap water to remove salt on the surface (Ramin et al., 2019). The samples were drained for 1 hour, then cut into 1-2 cm pieces, packed in polypropylene plastic, and frozen at -20°C for 24 hours. The frozen samples were then dried using a freeze dryer (Roque et al., 2019) at -50°C for approximately 30 hours and repeated at least twice until a dry sample was obtained and could be ground. The resulting macroalgae powder was sieved using an 80 mesh sieve shaker.

DNA was extracted from 0.5 g of apical talus samples of fresh macroalgae using a modified Chelex method (Zuccarello and Lokhorst, 2005; Zuccarello and Paul, 2019). The extracted DNA was amplified using a 20 µL PCR solution containing: buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5% bovine serum albumin (BSA), 1 U Taq polymerase (Vivantis, Selangor Darul Ehsan, Malaysia), and 7.5 pmols of the Nuclear Large Subunit Ribosomal (LSU) primer set C'1_for (ACCCGCTGAATTTAAGCATAT) and D2_rev (TCCGTGTTTCAAGACGG) (Hassouna et al., 1984; Leliaert et al., 2003). The PCR products were assessed for quality and quantity using 1% agarose gel electrophoresis and then sequenced. Several sequences targeting the 28S rRNA gene from different haplotypes and species were downloaded from the National Center for Biotechnology Information, USA (https://www.ncbi.nlm.nih.gov/) and added to the MEGA X software (Tamura et al., 2013). DNA diversity measurement and phylogenetic analysis were performed using the maximum-likelihood method implemented in IQ-tree (Trifinopoulos et al., 2016), with 500 bootstrap replicates and the Kimura 2-parameter model.

Extraction process

The extract of green macroalgae *C. vieillardii* was obtained by maceration using 96% ethanol as a solvent with a ratio of 1:10 for 24 hours, followed by exposure to ultrasonic waves for 45 minutes. The filtrate was collected, and the yield was subjected to maceration and ultrasonication up to three times. The entire filtrate was evaporated using a vacuum evaporator to obtain a concentrated extract, followed by freeze-drying at -50°C for 30 hours to produce a dry ethanolic extract of *C. vieillardii.* The dried macroalgae extract samples were dissolved in an *in vitro* test medium consisting of 0.9% sodium chloride solution (Medeiros et al., 2020) to obtain concentrations of 2 mg/mL, 4 mg/mL, and 6 mg/mL, following the preparation methods of Alvarez-Mercado et al. (2015), Sakti et al. (2018), and Bonde et al. (2021).

Analysis of chemical composition, minerals, and color determination

The proximate analysis was conducted following the AOAC method (AOAC, 2012). The mineral composition was measured using X-ray fluorescence (XRF) and presented in the form of elements. To determine the effect of drying methods on the physical quality of macroalgae, considering color determination, three types of samples were used: fresh condition, freeze-dried at -50°C, and oven-dried at 40°C. Color determination was determined by its coordinates using a Chromameter (Konica Minolta Sensing, Inc. Japan).

Analysis of total flavonoid

Total flavonoid analysis was conducted using the colorimetric-$AlCl_3$ method (Chang et al., 2002). A total of 3 mg of dry macroalgae extract sample was dissolved in 3 mL of methanol to obtain a sample concentration of 1,000 ppm. Then, the sample solution and quercetin standard solution with various concentrations were added to a microplate in 10 µL and mixed with 60 µL of methanol, 10 µL of 1 M KCH_3COO reagent, 10 µL of $AlCl_3$, and 120 µL of aquades. After incubation for 30 minutes, the mixture was read using a microplate reader at a wavelength of 415 nm. Standard solutions at various concentration points were used to form the standard curve. The total flavonoid content was expressed as mg equivalent of quercetin standard per gram of extract (mg QE/g extract).

Analysis of total phenol

The determination of total phenolic content was carried out using a colorimetric approach according to Baek et al. (2021) method. A total of 15 mg of the sample was dissolved in 3 mL of 80% methanol to obtain a sample with a concentration of 5,000 ppm. Then, 10 µL of the sample solution (5,000 ppm) and a standard solution of gallic acid with various concentration points were added to a microplate, followed by the addition of 130 µL of aquades and 10 µL of Folin-Ciocalteu reagent. The mixture was vortexed and incubated for 6 minutes. Afterward, 100 µL of 7% Na_2CO_3 reagent was added, vortexed, and incubated for 90 minutes. The sample was analyzed by using a microplate reader at a wavelength of 750 nm. Standard gallic acid solutions at various concentration points were used to construct the standard curve. The total phenolic content was determined as mg equivalent of gallic acid per gram of extract (mg GAE/g extract).

In vitro adult worm motility test (AWMT)

The AWMT followed the method of Eguale et al. (2007) with modifications according to Sakti et al. (2018) applying a completely randomized design (CRD) factorial pattern. The first factor was the concentration level, while the second factor was the observation time. In addition to the significance of each factor, interactions between them were also analyzed. *Haemonchus contortus* worms for *in vitro* AWMT were collected from the abomasum of thin-tailed sheep aged less than 12 months, which were slaughtered at a local slaughterhouse in Yogyakarta, Indonesia. Adult female *H. contortus* worm samples were collected from the abomasum based on the methodology described by Barbosa et al. (2023), as homogeneous size and maturity of the samples were readily attainable and did not require culture, unlike typical studies involving worm larvae (Gives et al., 2022). Adult female *H. contortus* worms are distinguished from males by their characteristic red and white spiral appearance, which is unique to females (Adduci et al., 2022), and larger size in adult females (24-27 mm vs. 16-18 mm) compared to males (Alborzi et al., 2023). A total of 150 worms were randomly assigned into 6 treatments at concentrations of 2 mg/mL, 4 mg/mL, and 6 mg/mL. Sodium chloride of 0.9% was used as a negative control, and albendazole of 0.5 mg/mL and 2 mg/mL as a positive control. There were 5 replicates with each replicate consisting of 5 worms. The number of live and dead worms was observed at 0, 15, and 30 minutes, and hourly thereafter until all worms were dead. Worms were considered completely dead when they were stiff and did not move when touched for at least 10 seconds. AWMT values were obtained by comparing the number of live worms with the initial number of worms at each observation time at room temperature. Descriptive imaging using a binocular optical microscope will be performed to observe the morphological responses of the worms to the treatments.

In vitro egg hatch inhibiting test (EHIT)

The EHIT test was conducted using a one-way completely randomized design with three different concentration levels of a total volume of 3 mL solution poured into the reaction tubes. Albendazole (Eguale et al., 2007) was used as a positive control at doses of 0.5 mg/mL and 2 mg/mL, while 0.9% sodium chloride was used as a negative control. Each treatment was replicated three times. A total of 54 female *H. contortus* worms collected from the abomasum were randomly divided into 18 reaction tubes of six different treatments, each containing three worms. The female worms were then ground with a blunt glass spatula and vortexed, allowing the eggs in their reproductive tract to dissolve in the test solution. Samples from the test solution were taken at 0 and 24 hours after incubation at room temperature to count the number of worm eggs under a binocular microscope according to the method described in Sakti et al. (2018) at a magnification of 100 times. The number of *H. contortus* eggs counted from adult female worms was determined based on their maturity characterized by morphological shape, dark brown blastomeres, and average egg dimensions of 70 ± 10 µm in length and 45 ± 5 µm in width (Ljungström et al., 2018). The number of eggs observed and counted in the first 24 hours was compared to the number at 0 hours and expressed as the percentage of eggs that

failed to hatch.

Data analysis

Statistical analysis was performed using factorial for AWMT and one-way ANOVA for EHIT analysis. A Duncan's multiple range test was conducted for the post-hoct test after the results showed a significant effect. All the calculations were performed by CoStat Statistical Software Version 6.451 (Cohort, 2022) at a 0.01 value of significance level.

RESULTS

Species identification, chemical and metabolite compounds composition, and color determination of *C. vieillardii*

The Maximum-likelihood phylogenetic tree based on DNA sequences from the samples and several *Chaetomorpha* genera obtained from GenBank NCBI is shown in Figure 1. The identification results based on the 28S rRNA gene indicate that the sample originating from Gunungkidul Regency, DIY Province is *Chaetomorpha vieillardii* (LT969743.1) with a similarity level of 100%. The data on the chemical composition, color determination, minerals, and secondary metabolite composition of the green macroalgae *C. vieillardii* are presented in Tables 1, 2, 3, and 4. The chemical composition (Table 1) is displayed on a fresh, dried, and 100% dry matter basis. The sample of green macroalgae *C. vieillardii* used in this study has a moisture content of more than 86%, making it susceptible to quality changes during processing, which can be maintained by referring to the color determination results. The chromameter analysis (Table 2) results showed that the drying process affected the color determination values, which could have caused chemical quality changes. There were significant differences in color determination values between fresh macroalgae samples, freeze-dried at -50°C, and oven-dried at 40°C. Both drying methods were found to have increased the values of L*, a*, b*, and C*, and decreased the value of h^o compared to the fresh condition. This green macroalgae species contains more than 19% potassium, which is the dominant mineral, followed by levels of chlorine, calcium, sulphur, and magnesium (Table 3). The ethanolic extract of *C. vieillardii* contains total flavonoids and phenols as shown in Table 4, expressed in mg quercetin equivalents and mg gallic acid equivalents per gram of extract, respectively.

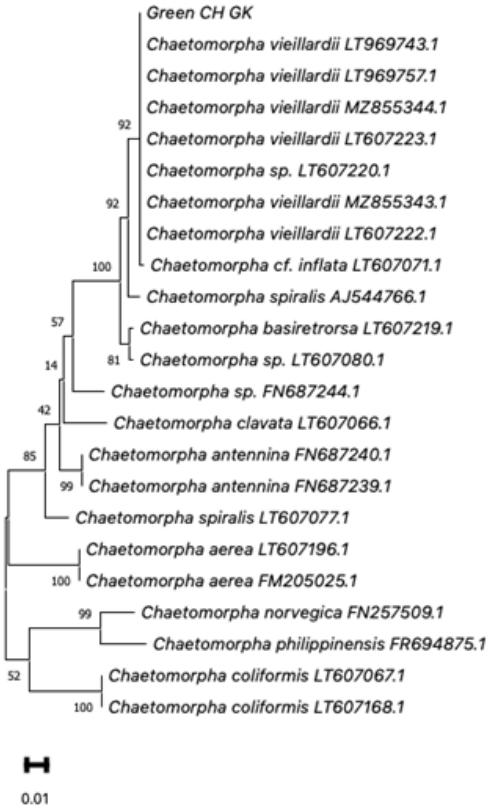

Figure 1 The Maximum-likelihood phylogenetic tree based on DNA sequences from the samples of *Chaetomorpha* sp.

Table 1 Chemical compositions of macroalgae *C. vieillardii*

Specimen	Chemical compositions (%)					
	DM	Ash	CP	EE	CF	NFE
Fresh	13.36	3.27	2.16	0.19	2.90	4.83
Freeze dried	94.39	23.14	15.27	1.37	20.52	34.10
100% DM	100.00	24.52	16.18	1.45	21.74	36.12

DM: Dry Matter; CP: Crude Protein; EE: Ether Extract; CF: Crude Fiber; NFE: Nitrogen Free Extract

Table 2 Color determination of macroalgae *C. vieillardii*

Variables	Sample specimen		
	Fresh	Freeze dried	Oven 40°C
Degree of lightness (L*)	21.47 ± 0.07^{c}	44.17 ± 0.02^{b}	46.59 ± 0.02^{a}
Green to red degree (a*)	-6.95 ± 0.05^{c}	-2.86 ± 0.02^{b}	-1.66 ± 0.04^{a}
Blue to yellow degree (b*)	16.90 ± 0.06^{c}	19.67 ± 0.06^{b}	20.62 ± 0.02^{a}
Chroma value (C*)	18.28 ± 0.05^{c}	19.88 ± 0.06^{b}	20.69 ± 0.02^{a}
Hue value (h°)	112.37 ± 0.18^{a}	98.26 ± 0.04^{b}	94.60 ± 0.10^{c}

± Standard Deviation; Different superscripts within the same line indicate highly significant differences (P<0.01).

Table 3 Macro and micromineral composition of macroalgae *C. vieillardii*

Mineral compositions (element; compound)	Value
Macrominerals (%)	
Calcium (Ca; CaO)	3.89
Magnesium (Mg; MgO)	0.31
Sulphur (S; SO_3)	2.44
Potassium (K; K_2O)	19.84
Phosphor (P; P_2O_5)	0.18
Chlorine (Cl; Cl)	9.57
Aluminium (Al; Al_2O_3)	0.06
Silicon (Si; SiO_2)	0.15
Microminerals (ppm)	
Titanium (Ti; TiO_2)	120.80
Tin (Sn; Sn_2O_3)	46.30
Cesium (Cs; Cs_2O)	4.50
Palladium (Pd; PdO)	1.80
Antimony (Sb; Sb_2O_3)	13.70
Iodine (I; I)	55.80
Barium (Ba; BaO)	15.30
Tellurium (Te; TeO_2)	33.60
Molybdenum (Mo; MoO_3)	1.00
Rhodium (Rh; Rh)	0.40
Technetium (Tc; Tc)	0.30
Ruthenium (Ru, RuO_2)	0.10

Table 4 Total flavonoids and phenols content of macroalgae *C. vieillardii* ethanolic extract

Secondary metabolites compositions	Value
Total flavonoids (mg QE/g extract)	117.96 ± 1.67
Total phenols (mg GAE/g extract)	5.17 ± 0.09

QE: Quercetine Equivalent; GAE: Gallic Acid Equivalent

In vitro adult worm motility test (AWMT)

The level of treatment concentration (b/v) and observation time (hours) in AWMT showed a significant effect in reducing the viability or motility (%) of *H. contortus* worms (P<0.01) (Figure 2 and Table 5). The factors of treatment concentration level and observation time showed an interaction between them (P<0.01). Ethanol extract of tropical macroalgae *C. vieillardii* at concentration levels of 2 mg/mL to 6 mg/mL showed significant anthelmintic activity against *H. contortus* worms during 29 hours of observation, which was significantly different from the negative control (P<0.01). Meanwhile, albendazole at levels of 0.5-2 mg/mL showed the most optimal efficacy compared to other treatments, and reached an LD_{50} value after 8 hours and 9 minutes of incubation, and an LD_{100} after 12 hours of observation.

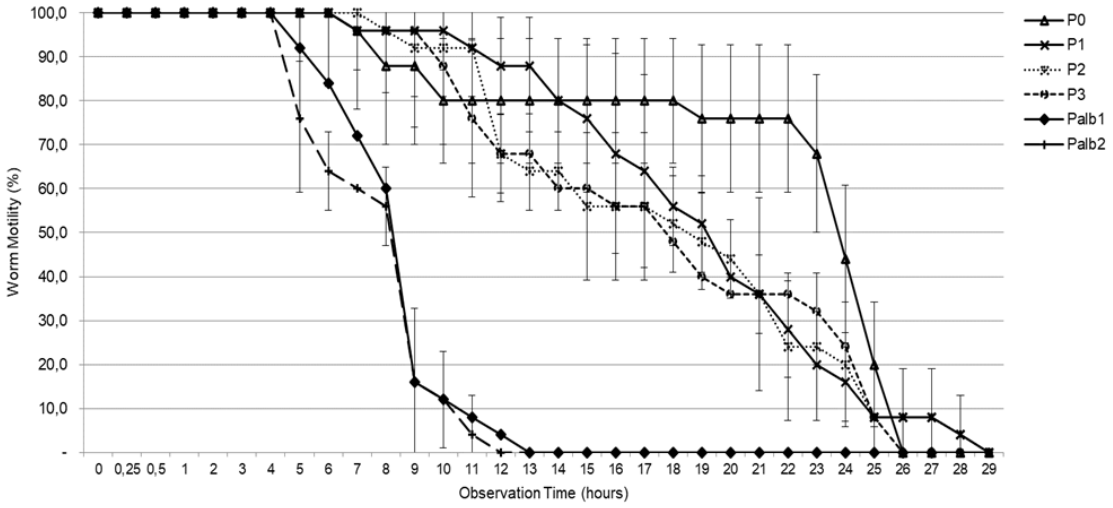

Figure 2 AWMT from the ethanol extract of *C. vieillardii* against *H. contortus* at levels of 2 mg/mL, 4 mg/mL, and 6 mg/mL *in vitro*. P0: 0.9% physiological NaCl (negative control); P1: 2 mg/mL ethanol extract of *C. vieillardii*; P2: 4 mg/mL ethanol extract of *C. vieillardii*; P3: 6 mg/mL ethanol extract of *C. vieillardii*; Palb1: Albendazole 0.5 mg/mL (positive control 1); Palb2: Albendazole 2 mg/mL (positive control 2); ⊥: Negative Standard Deviation; T: Positive Standard Deviation.

Table 5 Motility of *H. contortus* (%) due to treatments during observation

Treatments	Motility (%)	Observation hours	
		LD_{50}	LD_{100}
P0	72.77[a]	23 hours 45 minutes	26 hours
P1	66.13[b]	19 hours 10 minutes	29 hours
P2	62.88[c]	18 hours 30 minutes	26 hours
P3	61.88[c]	17 hours 45 minutes	26 hours
Palb1	32.75[d]	8 hours 13.64 minutes	13 hours
Palb2	30.88[d]	8 hours 9 minutes	13 hours

P0: 0.9% physiological NaCl (negative control); P1: 2 mg/mL ethanol extract of *C. vieillardii*; P2: 4 mg/mL ethanol extract of *C. vieillardii*; P3: 6 mg/mL ethanol extract of *C. vieillardii*; Palb1: Albendazole 0.5 mg/mL (positive control 1); Palb2: Albendazole 2 mg/mL (positive control 2); LD_{50} and LD_{100}: the Lethal Dose (LD) of a level concentrations that is lethal to 50% and 100% of the worm population, respectively; Different superscripts within the same column indicate highly significant differences ($P<0.01$).

In vitro egg hatch inhibiting test (EHIT)

The results of EHIT are shown in Figure 3. Consistent with the *in vitro* test results of AWMT, the ethanol extract of *C. vieillardii* macroalgae also significantly inhibited the hatching of *H. contortus* eggs at levels of 2-6 mg/mL for 24 hours of observation ($P<0.01$). The concentration levels of the ethanol extract of *C. vieillardii* at the three different levels were distinct, and even at 2 mg/mL, it was able to match the efficacy of albendazole as a positive control, although no level could surpass it. This is different from the negative control, which could not inhibit hatching up to 50%. In the positive control level, there was no significant difference between concentrations of 0.5 mg/mL and 2 mg/mL, and numerically it still tended to be superior compared to other treatments.

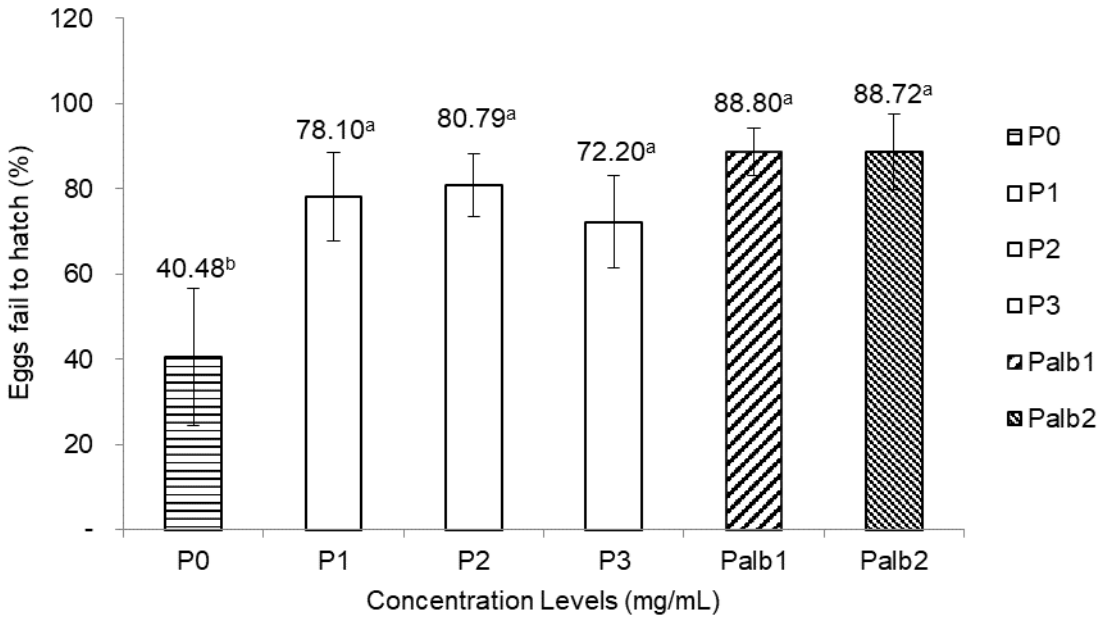

Figure 3 EHIT from the ethanol extract of *C. vieillardii* against *H. contortus* at levels of 2 mg/mL, 4 mg/mL, and 6 mg/mL *in vitro*. P0: 0.9% physiological NaCl (negative control); P1: 2 mg/mL ethanol extract of *C. vieillardii*; P2: 4 mg/mL ethanol extract of *C. vieillardii*; P3: 6 mg/mL ethanol extract of *C. vieillardii*; Palb1: Albendazole 0.5 mg/mL (positive control 1); Palb2: Albendazole 2 mg/mL (positive control 2); ⊥: Negative Standard Deviation; T: Positive Standard Deviation. Different superscripts indicate highly significant differences (P<0.01).

DISCUSSION

Significant differences exist in the chemical composition of red, brown, and green seaweeds or even among species within the same genus (Freile-Pelegrin et al., 2020). Therefore, the biochemical characterization of each species is crucial for identifying their potential. The chemical composition of *C. vieillardii* in this study (Table 1) was found to be similar to the chemical composition of the *Chaetomorpha* genus as reported in the study by Freile-Pelegrin et al. (2020), which were 13.3% protein, 27.9% carbohydrates, 1.6% lipids, and 20.6% ash. More precisely, the crude protein content of *C. vieillardii* in this study is 15% higher, which suggests its potential as a source of crude protein for ruminant livestock feed. On the other hand, the main constituents in dried macroalgae are minerals, proteins, and crude fiber (Uribe et al., 2020). The first mentioned constituent, minerals, is generally higher in macroalgae compared to terrestrial plants and to macroalgae collected from freshwater pond (El-Adl et al., 2022). The high mineral content in macroalgae (23.42-65.65%), accompanied by low organic matter, indeed leads to low degradation of easily degraded fractions during *in vitro* ruminant digestion, resulting in gas production of less than 60 mL/200 mg dry matter, as reported in the study by Hidayah et al. (2023). That's why the high mineral content in raw macroalgae materials can be a factor inhibiting feed digestibility. Extraction is considered one of the solutions to harness the untapped bioactive potential without disrupting the rumen ecosystem. Hence, extracting a wide variety of products not only minimizes waste but also adds value to seaweed biomass (Freile-Pelegrin et al., 2020). Furthermore, raw macroalgae materials can be utilized as a source

of minerals for ruminants. The potassium mineral content of *C. vieillardii* in this study (Table 3) was higher by more than three-fold compared to the that of *Chaetomorpha crassa* at 6.91% (Kalasariya et al., 2021). The sequence of the top five mineral contents in this study, which were K > Cl > Ca > S > Mg, differed from the top five sequences reported by Kalasariya et al. (2021), which were Si > K > Ca > Fe > Mg. It is worth noting that seasonal variations could potentially influence the physiological and biochemical composition of *Chaetomorpha* sp. (Vinuganesh et al., 2022).

In color determination (Table 2), the possible changes in the quality of macroalgae samples were identified from the values of L*, a*, b*, C*, and h^o coordinates. The L* value represents the degree of lightness, while the degree of green to red and blue to yellow color is represented by the a* and b* values, respectively (Baycar et al., 2022). As time goes on, the redness and yellowness values significantly decrease (Hamzaoui et al., 2020). Drying that involves heat causes a greater loss of phycoerythrin, phycocyanin, and total phenolic content than freeze-drying. Although drying causes changes in color determination, the freeze-drying method produces the highest quality dried sample compared to other drying methods, including maintaining protein quality (Uribe et al., 2020).

To date, no previous studies have been found that observe the antiparasitic activity of *Chaetomorpha* sp. against *H. contortus*. In this study, the ethanol extract of *C. vieillardii* at levels of 2, 4, and 6 mg/mL demonstrated its anthelmintic activity against *H. contortus* and was able to eradicate 50% of *H. contortus* worms (LD_{50}) respectively more than 4, 5, and 6 hours faster than the negative control (Table 5). Typically, *H. contortus* worms can survive for more than 6 hours in 0.9% sodium chloride (Laut et al., 2022), in fact, 24 hours in this study, as well as other control media such as Phosphate Buffer Saline (PBS) and distilled water (Medeiros et al., 2020). However, our study findings indicated a unique observation that at P1, the LD_{100} lasted longer compared to P0 (Figure 2). The efficacy of albendazole, as a commonly used synthetic anthelmintic for positive control in *in vitro* studies (Sakti et al., 2018; Laut et al., 2022) was observed to emerge after the fifth hour of observation. This is different from the study by Sakti et al. (2018), where albendazole was observed to begin decreasing the motility of *H. contortus* from the second hour of observation, consistent with the findings of Sakti et al. (2020) at the same concentration of 2 mg/mL and Laut et al. (2022) at the 1% (b/v). The longer time for the efficacy of albendazole may be attributed to the increasing severity of resistance, low drug availability in plasma, and the high occurrence of resistant genes (Coles et al., 2006; Jabbar et al., 2022), although from the perspective of the host animal, genetic selection can be performed to obtain a more parasite-resistant breed (Niciura et al., 2022).

The efficacy of *C. vieillardii* ethanol extract was not only effective against adult worms but also in inhibiting egg hatching. Normally, *H. contortus* eggs require about 24 hours to hatch and grow into larvae outside the host animal's body. Inhibiting egg hatching was one of the efforts to break the *H. contortus* parasite cycle, besides killing adult worms in the abomasum. The results of this study showed that all concentration levels of *C. vieillardii* ethanol extract exhibited anthelmintic activity by inhibiting more than 80% of *H. contortus* worm eggs (Figure 3). These results were comparable to the efficacy of albendazole in inhibiting egg hatching within the first 24 hours of observation and superior by almost twice as much as the negative control.

The inhibition of *H. contortus* egg hatching in this study may be attributed to the disruption of embryonic development within the parasite eggs caused by the activity of phenolic compound extracts (Velázquez-Antunez et al., 2023). Nevertheless, the mechanism underlying the ovicidal activity still requires clarification.

The widespread application of seaweed extract is based on its valuable bioactive compounds and potential bioactivity (Kalasariya et al., 2021). Secondary metabolite compounds from *C. vieillardii* that were soluble in polar ethanol solvents included flavonoids and phenols which were found in this study (Table 4). The reaction of phenols varied based on the specific solvent employed (Alara et al., 2021). The total flavonoid and phenol content of the ethanol extract from macroalgae *C. vieillardii* in this study were lower than those reported in a previous study (Haq et al., 2019) on *Chaetomorpha* sp. from the Arabian Gulf, which were 189.14 ± 0.99 mg QE/g extract and 21.92 ± 0.43 mg GAE/g extract, respectively, although they exhibited the same pattern. However, they were higher compared to the aqueous solvent (Haq et al., 2019). Extraction using ethanol as a solvent exhibited a higher bioactivity potential of polyphenols and flavonoids compared to water (Ghareeb et al., 2019; Vinuganesh et al., 2022). Flavonoids, which are considered one of the extensively studied and diverse classes of natural polyphenols, contribute polarity and weak acidic properties to the molecules due to the presence of one or more aromatic rings bearing hydroxyl groups (Tarahovsky et al., 2014; Phang et al., 2023; Suwignyo et al., 2023). The formation of molecular assemblies is facilitated by flavonoid–metal complexes, which play a role in membrane adhesion and fusion, as well as protein–protein and also protein–membrane binding (Tarahovsky et al., 2014). At low levels of extract concentration in our study, this complex is strong enough to protect the cell membrane from external disturbances. Therefore, this mechanism is utilized in the utilization of macroalgae for skin health cosmetics (Anis et al., 2017). However, at high levels of concentration, in this study at 4 and 6 mg/mL, the covalent bonds formed between the active groups of phenolic compounds and the cell membrane changed its conformation and fluidity. The double-layer structure of the cell membrane was disrupted, causing depolarization (Ali et al., 2021). This depolarization provided an opportunity for phenolic compounds to enter the intracellular fluid through diffusion (Ali et al., 2021) and interfere with cell metabolism in the cytoplasm and mitochondria, with responses such as inhibition of transcription-translation and secretion of enzymes for metabolism (Khan et al., 2022). In addition to the diffusion of bioactive compounds from macroalgae, membrane damage also resulted in the leakage of cellular proteins and ribonucleic acid (RNA) outside the cell, leading to metabolic failure and cell death (Khan et al., 2022). This mechanism could occur in any *H. contortus* worm cell, such as in its muscle and peripheral nerves. Further investigation is required to determine the extent of damage caused by the bioactive compounds of macroalgae on *H. contortus* cells and tissues. Additionally, their efficacy in addressing parasitic infections in sheep needs to be evaluated *in vivo*. *Chaetomorpha* sp. has been extensively studied for its biological activity as an anticancer (Haq et al., 2019; Acharya et al., 2020), antioxidant (Haq et al., 2019), anticoagulant (Qin et al., 2020), bioremediation (Aquilino et al., 2020), antibacterial, antifungal, anti-inflammation, immunostimulatory benefits to the

skin (Kalasariya et al., 2021), alternatives to pull through antibiotic resistance (Bhowmick et al., 2020) and enhanced growth performance, immunity, and the haematological response of fish challenged with bacteria (Sattanathan et al., 2020). However, scientific publications regarding *Chaetomorpha* sp. as an anthelmintic agent are still very limited. Therefore, the findings of this study provide a new insight that *C. vieillardii* has the potential to be developed as a future bio-anthelmintic, contributing to sustainable and environmentally-friendly farming practices.

CONCLUSIONS

The ethanol extract of *Chaetomorpha vieillardii* has the potential as a bio-anthelmintic agent to eradicate gastrointestinal worms, specifically *Haemonchus contortus* in ruminants. This is evident from its ability to inhibit the motility and hatching of *Haemonchus contortus* eggs. Further research is needed to identify the specific metabolites responsible for the anthelmintic activity of this tropical macroalgae. Additionally, its effects on rumen fermentation should be evaluated to obtain further data before *in vivo* testing on ruminants can be conducted.

AUTHOR CONTRIBUTIONS

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AUTHOR CONTRIBUTIONS

All authors were involved in all research activities and in writing this scientific article. In the final stage, all authors have read and agreed to the submitted draft. Awistaros Angger Sakti: designed the study, collected data, analyzed data, drafted the manuscript framework, and finalized the manuscript. Bambang Suwignyo: supervised and contributed to manuscript drafting. Ahmad Sofyan: designed the study, supervised, and contributed to manuscript drafting. Chusnul Hanim: contributed to manuscript drafting. Hendra Herdian: collected data and contributed to manuscript drafting. Jasmadi Jasmadi: collected data and contributed to manuscript drafting. Tiurma Pasaribu: contributed to manuscript drafting. Hardi Julendra: contributed to manuscript drafting. Gunawan Gunawan: contributed to manuscript drafting. Pustika Ratnawati: molecular identification. Lilis Hartati: contributed to manuscript drafting. Syifa Adisa Eminita Tarigan: collected data and contributed to manuscript drafting. Kustantinah Adiwimarta: designed the study, supervised, and contributed to manuscript drafting.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this research.

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