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Molecular Identification, Chemical Composition, and *In Vitro* Anthelmintic Activity of *Sargassum duplicatum* Against *Haemonchus contortus*

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ABSTRACT

The resistance of the *Haemonchus contortus*, a parasite causing severe anemia in ruminants, to commercial anthelmintics emphasizes the need for alternative bio-anthelmintics. This study aimed to identify the molecular, chemical composition, and *in vitro* anthelmintic activity of *Sargassum duplicatum* against *H. contortus*. Molecular identification employed the Chelex method, with DNA diversity and phylogeny assessed using maximum likelihood in IQ-tree. The analyzed chemical composition included proximate, total flavonoid, and total phenols. Adult worm motility test (AWMT) and egg hatch inhibiting test (EHIT) were conducted at concentrations of 2, 4, and 6 mg/mL of *S. duplicatum* ethanolic extract. AWMT followed a completely randomized factorial design (5 replications, each with 5 worms), while EHIT used a completely randomized one-way design (5 replications, each with *H. contortus* egg batches from 3 adult female worms). The cox1 gene sequence revealed the *Sargassum* sample as *S. duplicatum* (KP101270.1) with 99.83% similarity. The results indicated that the identified concentrations of *S. duplicatum* ethanolic extract, and the observation time significantly influenced motility and egg hatchability ($p<0.05$). Both factors exhibited a significant interaction ($p<0.05$). Concentrations of 4-6 mg/mL reduced worm motility by up to 50% (LD_{50}) within 6-8 hours, while concentrations of 2-6 mg/mL inhibited egg hatchability by more than 87% during 24 hours of incubation. In conclusion, *S. duplicatum* holds significant potential as a bio-anthelmintic agent.

Keywords: anthelmintic; Haemonchus contortus; Sargassum duplicatum; sheep

INTRODUCTION

One of the inhibiting factors of ruminants' performance is parasitic infection in the digestive tract, such as *Haemonchus contortus* in the abomasum (Velázquez-Antunez *et al.*, 2023). The parasite sucks the host's blood, causing anemia symptoms, and leading to nutrient deficiencies in the body. Inefficiency in feed utilization is unavoidable, resulting in economic losses (Adduci *et al.*, 2022). The occurrence of parasite resistance to commercial antiparasitic agents further exacerbates *haemonchosis* cases as gastric worms become more resistant to previous dosages (Jabbar *et al.*, 2022).

This condition attracts researchers' attention to search for effective and non-resistance-inducing antiparasitic alternatives. One breakthrough is by exploring macroalgae known to contain secondary metabolite compounds such as flavonoids, phenols (Castillo *et al.*, 2023), phlorotannins (Fraga-Corral *et al.*, 2021), terpenoids (Arrieche *et al.*, 2022), and bromoforms, which have proven effectiveness as antibacterial, antioxidant (Castillo *et al.*, 2023), rumen modifier, antiprotozoal (Sofyan *et al.*, 2022), and even anticancer agents (Abu-Khudir *et al.*, 2020). Generally, several tropical macroalgae species from Indonesia are commonly utilized for food, cosmetics, fertilizers, and mineral resources, including brown macroalgae, *Sargassum duplicatum*, which is commonly found on the southern coast of Java Island (Charles *et al.*, 2020; Winarni *et al.*, 2022).

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In previous studies, the methanol extract from *Sargassum polycystum* has demonstrated efficacy in eradicating parasites in leech (Haron *et al.,* 2022). Similarly, other macroalgae, such as *Palisada tenerrima* and *Laurencia* sp., exhibit anthelmintic activity against *Cylicocyclus* sp. parasites in donkeys (Maestrini *et al.,* 2021). Additionally, *Corallina* sp. and *Ulva* sp., in the form of ethanol extracts, have been found effective in eliminating *Meloidogyne incognita* parasites in eggplant roots (Ghareeb *et al.,* 2019). Interestingly, the utilization of macroalgae as antiparasitic agents for eradicating parasites in ruminant livestock worldwide is still underdeveloped. On the other hand, Indonesia possesses abundant marine vegetation and is capable of producing more than 38% of the world's macroalgae (Ferdouse *et al.,* 2018). Domestic use of *Sargassum* is still very low, both for consumption and other uses, despite its abundant distribution (Pramesti *et al.,* 2019). *Sargassum* populations are spread throughout almost all coastal areas of Indonesia (Wouthuyzen *et al.,* 2016). Therefore, evaluating antiparasitic activity from tropical macroalgae with high populations, such as *S. duplicatum,* is necessary and considered to have high novelty as an antiparasitic agent against *H. contortus* in ruminant livestock.

Sargassum is a taxonomically difficult genus with populations that are widely distributed throughout the world, and it is the genus with the richest species diversity (Mattio & Payri, 2009). The *Sargassum* genus contains approximately 400 species distributed in tropical and subtropical regions of the world (Johnson *et al.,* 2019). The high plasticity of each species in response to its environment leads to morphological and chemical composition changes, thus rendering morphological identification less convincing without a molecular DNA approach (Phetcharat *et al.,* 2023). Accordingly, a preliminary analysis regarding molecular identification and chemical composition is required to determine the species and characteristics of the macroalgae. This research aims to molecularly identify the species, analyze the chemical composition, and assess the anthelmintic activity of *S. duplicatum* against *H. contortus in vitro*.

MATERIALS AND METHODS

Ethical Clearance

The entire research protocol has obtained an ethical clearance certificate from the Directorate of Management for Research and Innovation Permit and Scientific Authorities, National Research and Innovation Agency, Indonesia, under certificate number 005/KE.02/SK/01/2023, approved on January 20th, 2023. All research activities have been conducted in accordance with the granted ethical clearance.

Sample Preparation and Chemical Composition Analysis of Macroalgae

Brown macroalgae *Sargassum duplicatum* was collected from the Sepanjang Beach, Gunungkidul Regency, Yogyakarta Special Region Province, Indonesia, at coordinates 8°08'14.9" S and 110°34'07.9" E, during low tide. Fresh macroalgae were transported to the laboratory and rinsed with flowing freshwater to remove contaminants and debris. A fresh sample of macroalgae was identified molecularly to determine the species. The samples were dried using a freeze dryer (Lyovapor L-200 Buchi) at ice condenser temperature and pressure of -50 °C and 0.60-0.75 mbar, respectively, for 30 hours. The dried samples were then ground and sieved to a size of 80 *mesh*.

Molecular DNA analysis. The apical tips of the macroalgae were extracted using the Chelex method with modifications to obtain DNA extracts (Zuccarello & Lokhorst, 2005; Zuccarello & Paul, 2019). The subsequent process involved amplifying the DNA extract with a PCR solution containing buffer, $MgCl_2$, dNTPs, bovine serum albumin, Taq polymerase (Vivantis, Selangor Darul Ehsan, Malaysia), and mitochondrial primers (Cytochrome oxidase subunit 1 (cox1)) for the brown algal group, namely GazF2 (CAACCAYAAAGATATWGGTAC) and GazR2 (GGATGACCAAARAACCAAAA) (Lane *et al.,* 2007). Gel electrophoresis was employed to assess the quality and quantity of the PCR products before sequencing at 1st BASE (Singapore). DNA diversity and phylogeny were evaluated using the maximum likelihood feature in IQ-tree (Trifinopoulos *et al.,* 2016). Edited sequence data from the samples were aligned with a dataset of cytochrome c oxidase subunit 1 (cox1) genes from the genus *Sargassum*, which were subsequently blasted and classified based on the highest identification (%) and query cover (%) values. Several sequence databases were utilized, such as *Sargassum* sp., *S. dotyi*, *S. ilicifolium*, *S. polycystum*, *S. sandaei*, and *S. duplicatum*, retrieved from NCBI GenBank (https://www.ncbi.nlm.nih.gov/). The aligned dataset had a length of approximately 550-700 base pairs, and the molecular evolution model used was maximum likelihood (Kimura 2-parameter model).

Sample extraction. Macroalgae powder was extracted using a combination of methods: maceration and ultrasonication (Hodhodi *et al.,* 2022; Cikos *et al.,* 2022). The sample was macerated with 96% ethanol in a 1:10 ratio for 24 hours with a single manual stirring. The macerated solution was subjected to ultrasonic waves at 20-25 kHz frequency (Biostellar BSD-900W). The ultrasonic exposure settings were 2 seconds on and 3 seconds off, for a total of 10 minutes. The filtrate was obtained by filtering through filter paper, while the residue was re-macerated and re-ultrasonicated three times (3 x 24 hours). The total filtrate collected was evaporated using a vacuum evaporator (Rotavapor R-300 Buchi). The extract concentrate was subsequently dried using a freeze dryer at a temperature of -50 °C and a pressure of 0.60-0.75 mbar to obtain a drier *S. duplicatum* extract. The concentrated extract was weighed to determine the percentage yield and stored at a temperature of 4 °C for further analysis.

Chemical composition analysis. The chemical composition of macroalgae was analyzed using the proximate analysis method (AOAC, 2012). The fiber fractions analyzed were neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin using the Gravimetric method by Goering & Van Soest (1970).

Bioactive compound analysis. The colorimetric method using $AlCl_3$ (Chang *et al.,* 2002) was employed to measure the total flavonoid content of the macroalgae. A volume of 10 µL of the 1,000 ppm macroalgae extract sample solution in 80% methanol was added to a microplate. Subsequently, 60 µL of methanol, 10 µL of 1 M KCH_3COO reagent, 10 µL of $AlCl_3$, and 120 µL of distilled water were added, followed by a 30-minute incubation period. The absorbance was then measured at a wavelength of 415 nm. Quercetin was used as the standard, and the total flavonoid content was expressed as mg equivalent of quercetin standard per g of extract (mg EQ/g extract). Meanwhile, the total phenol content was determined using a colorimetric approach based on the method by Baek *et al.* (2021). A volume of 10 µL of the 5,000 ppm macroalgae extract sample solution in 80% methanol was added to a microplate, along with 130 µL of distilled water and 10 µL of Folin-Ciocalteu reagent. The mixture was vortexed and incubated for 6 minutes, followed by the addition of 100 µL of 7% Na_2CO_3 reagent. After vortexing and a 90-minute incubation period, the absorbance was measured at a wavelength of 750 nm. The gallic acid standard solution was used as the standard, and the total phenol content was expressed as mg equivalent of gallic acid standard per g of extract (mg EAG/g extract).

Haemonchus contortus Preparation

Haemonchus contortus adult worms were collected from the abomasum of a thin-tailed sheep aged less than 12 months, slaughtered at a local abattoir in Yogyakarta Special Region, Indonesia. We used one animal to ensure the worms originated from the same host livestock. Female *H. contortus* worms were chosen (Baihaqi *et al.*, 2023) and identified by their red and white twisted body pattern, a characteristic absent in males. The body length averaged around ± 20 mm with a diameter of ± 2 mm. Worms meeting the criteria were individually transferred manually using a fine needle to Petri dishes pre-filled with 0.9% sodium chloride solution. Worms were cleansed of adhering debris by gently rinsing them with a 0.9% sodium chloride solution. The samples were ready for use in the *in vitro* Adult Worm Motility Test (AWMT) and Egg Hatch Inhibiting Test (EHIT).

Adult Worm Motility Test (AWMT)

The *in vitro* AWMT followed the method outlined by Sakti *et al*. (2018). We employed female worms to match the samples used in the EHIT test. Besides their role in egg production, female worms also exhibit a larger body size, measuring 24-27 mm compared to 16-18 mm in length for males (Alborzi *et al.*, 2023). This size difference is believed to lead to a more significant absorption of the host's blood, justifying the necessity for eradication. A total of 125 adult female *H. contortus* were randomly allocated to 5 treatments with 5 replications each. A volume of 5 mL of *S. duplicatum* ethanol extract was poured into each petri dish of the respective replication. Each petri dish containing 5 worms according to the treatments C: negative control (0.9% sodium chloride); SA-1: 2 mg/mL extract; SA-2: 4 mg/mL extract; SA-3: 6 mg/mL extract; ALB: positive control (0.5 mg/mL albendazole). The albendazole, sourced from the commercial anthelmintic drug Wormzol-B (Medion, Indonesia), was dissolved in 5 mL of 0.9% physiological NaCl to serve as a positive control, with a concentration of 2.5 mg. The experiment utilized a complete randomized factorial design. The first factor was the concentration level, while the observation time served as the second factor. Observations of the number of live worms (motility) were conducted at 0 minutes, 15 minutes, and 30 minutes after the start, and then hourly until all worms were deceased. Worms were considered dead if they remained motionless for at least 10 seconds when touched.

Egg Hatch Inhibiting Test (EHIT)

The *in vitro* EHIT was also conducted following the method outlined by Sakti *et al*. (2018). Seventy-five adult female *H. contortus* worms were randomly assigned to 5 treatments identical to those mentioned in AWMT. Each treatment comprised 5 replications, with each replication containing 3 worms in a reaction tube, with 3 mL of test medium. The experiment utilized a completely randomized one-way design. Female worms were ground and homogenized in each reaction tube until all eggs were dissolved. The samples were incubated at laboratory room temperature within the range of 27-28 ^{o}C. The number of worm eggs was confined to match the quantity of adult female donor worms used, specifically, three adult females per replication. Consequently, the initial count of eggs per replication may vary. To assess hatching failure, we employed the ratio of the number of eggs at 24 hours to those at 0 hours, calculated using the McMaster method. *H. contortus* eggs were identified based on their morphology, distinguished by dark brown blastomeres, with an average size of 70 mm in length and 45 mm in width (Ljungström *et al.*, 2018). The number of eggs was observed using a binocular microscope (Olympus BX53 Upright) at 10x10 magnification. The comparison of enumerated worm eggs at 24 and 0 hours was expressed as a percentage of hatching failure (Sakti *et al.*, 2024).

Data Analysis

The AWMT data were analyzed using a two-way analysis of variance (ANOVA), while EHIT data were analyzed using a one-way ANOVA. Mean comparisons were conducted using Duncan's test at a significance level of 0.05. Data analysis was performed using CoStat Software Version 6 (Cohort, 2022).

RESULTS

DNA Molecular Identification of Macroalgae

The results of DNA molecular identification (Figure 1) based on the cox1 gene sequence revealed that the *Sargassum* sample originating from Gunungkidul Regency belongs to the species *Sargassum duplicatum* (KP101270.1), with a similarity level of 99.83%. Based on the sample's blasting results, there was also a similarity between the species *S. duplicatum* and *S. dotyi* (OP270179.1), with an identification percentage exceeding 99.80%. However, *S. duplicatum*, which exhibits a higher similarity, was chosen for further analysis.

Chemical Composition of *Sargassum duplicatum*

The results of the proximate analysis are presented in Table 1. As a marine vegetation, the dry matter content of *S. duplicatum* in this study was only below 10%. The results indicate that *S. duplicatum* is a vegetation with a high ash content exceeding 36%. Meanwhile, the results of fiber fraction analysis indicate that *S. duplicatum* has NDF, ADF, and lignin contents of 10.61%, 27.24%, and 8.68%, respectively. The ethanol extract yield of *S. duplicatum* was 2.25 ± 0.28% on a dry matter basis. After extraction, the levels of total flavonoids and total phenols were obtained as 30.33 ± 3.31 mg QE/g extract and 5.15 ± 0.33 mg GAE/g extract, respectively.

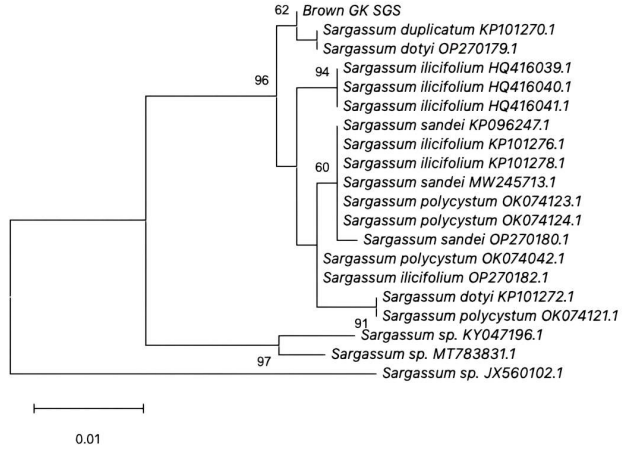

Figure 1. Phylogenetic tree of *Sargassum duplicatum* species based on molecular DNA analysis

Adult Worm Motility Test (AWMT)

The concentration levels of *S. duplicatum* extract significantly affected the motility of *H. contortus* worms ($p<0.05$), as did the observation time ($p<0.05$). Moreover, an interaction existed between the concentration levels and observation time ($p<0.05$), as shown in Figure 2, which determined the percentage of parasite motility at LD_{50} and LD_{100} points (Table 2). The *S. duplicatum* extract at a concentration of 2 mg/mL was unable to reduce the motility of adult *H. contortus in vitro*. However, 4 and 6 mg/mL levels exhibited anthelmintic activity by reducing motility to half the population between hours 6 and 8. Despite its strength, its efficacy could not match albendazole, which achieved LD_{50} in less than 4 hours and killed all *H. contortus* within less than 10 hours of observation.

Table 1. Chemical composition of brown macroalgae *Sargassum duplicatum* originating from Gunungkidul Regency, Yogyakarta Special Region, Indonesia

Composition	Specimen		
	Fresh	Freeze dried	Dry matter (100%)
Dry matter (%)	9.80	93.94	100.00
Ash (%)	3.85	36.65	39.20
Crude protein (%)	1.08	7.60	7.17
Ether extract (%)	0.14	1.38	1.48
Crude fiber (%)	0.46	4.40	4.72
Nitrogen free extract (%)	4.26	43.3	47.44

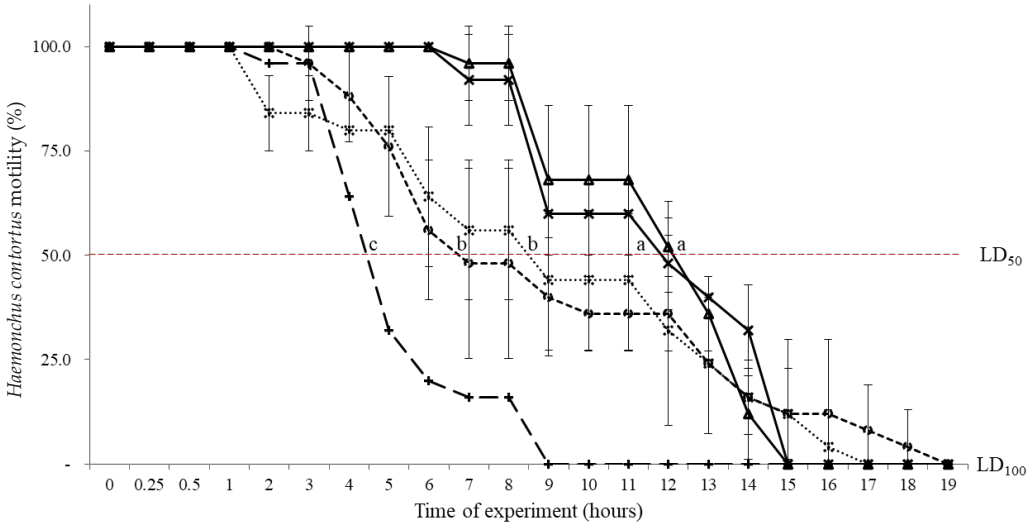

Figure 2. Adult worm motility test (AWMT) data of *Haemonchus contortus* motility due to administration of *Sargassum duplicatum* extract at different concentration levels. Different superscripts indicate significant differences ($p<0.05$). C (—▲—)= negative control (0.9% sodium chloride), SA-1 (—✕—)= 2 mg/mL extract, SA-2 (···✕···)= 4 mg/mL extract, SA-3 (- - ✕ - -)= 6 mg/mL extract, ALB (—|—)= positive control (0.5 mg/mL albendazole).

Table 2. Observation times for lethal dose treatment of *Sargassum duplicatum* on *Haemonchus contortus*

Treatments	Motility (%)	Observation times	
		LD_{50}	LD_{100}
C	62.33±3.18[a]	12 h 7.5 min	15 h
SA-1	61.83±1.00[a]	11 h 50 min	15 h
SA-2	51.00±6.38[b]	8 h 30 min	17 h
SA-3	51.67±8.29[b]	6 h 45 min	19 h
ALB	40.17±1.44[c]	4 h 26.25 min	9 h

Note: Means (±standard deviation) in the same column with different superscripts differ significantly ($p<0.05$). C= negative control (0.9% sodium chloride), SA-1= 2 mg/mL extract, SA-2= 4 mg/mL extract, SA-3= 6 mg/mL extract, ALB= positive control (0.5 mg/mL albendazole), h= hours, min= minutes.

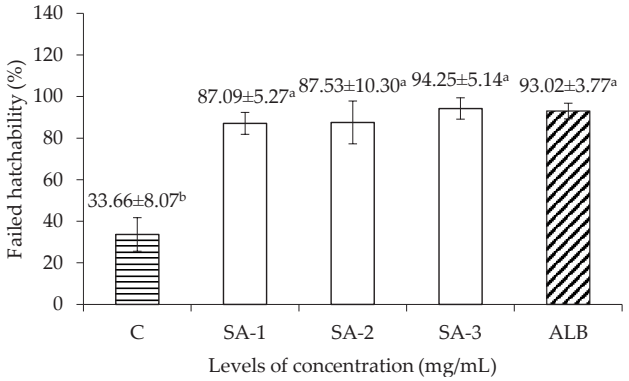

Figure 3. Egg hatch inhibiting test (EHIT) data (±standard deviation) of *Haemonchus contortus* egg hatch failure due to administration of *Sargassum duplicatum* extract at different concentration levels. Different superscripts indicate significant differences ($p<0.05$). C (▤)= negative control (0.9% sodium chloride), SA-1 (□)= 2 mg/mL extract, SA-2 (□)= 4 mg/mL extract, SA-3 (□)= 6 mg/mL extract, ALB (◪)= positive control (0.5 mg/mL albendazole).

Egg Hatch Inhibiting Test (EHIT)

The effect of *S. duplicatum* extract concentration levels on the hatchability of *H. contortus* eggs is depicted in Figure 3. The research results demonstrated that concentration levels of 2-6 mg/mL significantly inhibited the hatchability of *H. contortus* eggs *in vitro* compared to the control ($p<0.05$). Disparate from AWMT, this study revealed an efficacy of S. duplicatum extract that could match albendazole's ability to inhibit egg hatching, albeit not surpass it. These findings indicate that the 2 mg/mL concentration level was already effective in demonstrating its anthelmintic activity and did not differ significantly from the two levels above it.

DISCUSSION

The brown macroalgae *S. duplicatum* (KP101270.1 in Figure 1) has been discovered and investigated in various tropical regions in South and Southeast Asia, particularly in Indonesia. While we utilized samples collected from the coastal waters of southern Java Island for ruminant anthelmintic purposes, similar species have also been explored on Madura Island, Indonesia (Charles *et al.,* 2020) for functional food development, and from the southeastern coast of Tamil Nadu, India (Johnson *et al.,* 2019) as antioxidants. Other *S. duplicatum* specimens from Indonesia have been tested as agents to enhance the healing of open wounds in diabetes patients (Winarni *et al.,* 2022).

The freeze-drying technique successfully decreased the moisture content of macroalgae to below 10% (Table 1). However, the differences in the chemical composition of macroalgae can depend on species variations even within the same genus (Freile-Pelegrin *et al.,* 2020). The dry matter of *S. duplicatum* in this study was lower compared to the *Sargassum* genus collected from Malaysian waters (Nazarudin *et al.,* 2021), which was reported to be less than 90%. This was followed by lower ether extract, crude protein, and crude fiber. However, *S. duplicatum* in this study contained ash and nitrogen-free extracts 45.46% and 22.96% higher, respectively. Besides differences in ecosystem growth habitat, seasonal variations might also be responsible for the biochemical composition differences in macroalgae. The NDF content in this study was higher than ADF, consistent with the findings of Lee-Rangel *et al*. (2022) in *Ulva* sp. (green algae) and Kelp (brown algae). The crude protein and NDF levels in *S. duplicatum* were lower compared to the other *Sargassum* genera, as reported by Park *et al*. (2022) in *S. horneri* at 11.80% and 19.02% on a DM basis, respectively, indicating that *S. duplicatum* may be less suitable for use as ruminant feed in raw material form. This is also associated with its high ash content. High ash content can reduce feed digestibility (Hidayah *et al.,* 2023); hence, we employed extracts in its application, where minerals can be removed and bioactive compounds captured during the extraction process (Sakti *et al.,* 2024).

The ethanol extract yield of *S. duplicatum* in this study is higher than the yield of *Sargassum* sp. reported by Gazali *et al*. (2018) at 0.57%, but falls within the range of *Sargassum cristaefolium* yield as reported by Prasedya *et al*. (2021), which is 1.03 to 4.07%, influenced by species, solvent type, and particle size (Prasedya *et al.,* 2021). In this extract yield, phenolic compounds were identified. Flavonoids and phenols are secondary metabolites of terrestrial and marine plants that play essential roles as antioxidants (Winarni *et al.,* 2022) and anthelmintics (Olmedo-Juárez *et al.,* 2022). The brown macroalgae *S. polycystum*, as investigated by Johnson *et al*. (2019), was found to contain total flavonoids (429.0-953.33 mg GAE/g extract) and total phenols (17.46-33.49 mg GAE/g extract), which hold potential biological activity in petroleum ether, chloroform, acetone, and methanol. The total flavonoid and total phenols content of *Sargassum* in this study is lower than the data reported by Johnson *et al*. (2019). Furthermore, when converted to fresh conditions, *S. duplicatum* in this study exhibited total flavonoid and total phenol contents of 2.99 mg QE/g sample (fresh weight) and 0.27 mg GAE/g sample (fresh weight), respectively. The total flavonoid and total phenols content on a fresh weight basis in this study surpassed the *S. duplicatum* using the freeze-drying method reported by Charles *et al*. (2020), which was 0.11 mg QE/g fresh weight and 0.13 mg GAE/g fresh weight, respectively. The diversity in metabolite

compound levels is influenced by various factors such as species, environmental heat stress (Urrea-Victoria *et al.*, 2022), extraction techniques (Castillo *et al.*, 2023; Alara *et al.*, 2021), and solvent types (Abu-Khudir *et al.*, 2020). Flavonoids and phenols are considered as metabolites that are responsible for acting as antiparasitic agents in livestock, including interrupting the life cycle of *H. contortus* at the egg, larval, and adult stages (Rehman *et al.*, 2023; Olmedo-Juárez *et al.*, 2022).

In the *in vitro* AWMT study, the intersection of the graphs depicting the percentage of *H. contortus* motility in Figure 2 indicates an interaction with the observation time. This is consistent with previous research on AWMT studies involving macroalgae (Sakti *et al.*, 2024) as well as terrestrial plants containing phenolic compounds (Sakti *et al.*, 2018). Researchers aimed to determine the shortest time to achieve LD_{50} and LD_{100}. Figure 2 illustrates that SA-2 and SA-3 could demonstrate their anthelmintic activity, similar to albendazole, starting from the 2nd hour of incubation. However, as time progressed, their respective LD_{50} values were reached at different intervals within the 4-8 hours range. The ethanol extract of *S. duplicatum* at a concentration range of 4-6 mg/mL was able to kill 50% of the population (LD_{50}) in a time that was 44.33% shorter than the untreated group, as evidenced by the levelling of the graph in Figure 2 approaching the positive control graph (albendazole). Although slower by 34.26% compared to albendazole, which is generally recognized as an effective treatment (Jabbar *et al.*, 2022), there is no doubt about the anthelmintic activity of the ethanol extract of *S. duplicatum* at this level, similar to the anthelmintic activity of the other brown macroalgae such as *S. polycystum* (Haron *et al.*, 2022), *Bifurcaria bifurcata* (Miclon *et al.*, 2023), and *Caulocystis cephalornithos* (Taki *et al.*, 2020) against *H. contortus*.

Although *S. duplicatum* extract demonstrated its efficacy during a 19-hour observation period and provided significant LD_{50} data, it did not exhibit the same trend for LD_{100}. All levels showed maximum mortality time, similar to the untreated level. This condition may be attributed to two factors. Firstly, the active substance administered is in the form of crude extract, necessitating periodic administration, unlike albendazole, which is a single compound given as a single dose. This phenomenon is observed in some *in vivo* studies where the efficacy of crude extract containing phenolic compounds needs to be administered periodically rather than as a single dose (Sakti *et al.*, 2018). Further *in vitro* research related to the frequency of administration differentiation is also needed, given the discovery of *H. contortus* resistance (Babják *et al.*, 2023). Secondly, parasitic resistance factors may alter the efficacy pattern of a tested substrate, even though the parasite samples were collected from the same animal, with the same morphological size and maturity levels used in this study, 24-27 mm adult female worms (Adduci *et al.*, 2022; Alborzi *et al.*, 2023). Resistant nematodes, over time, may consume the substrate for survival (Lima *et al.*, 2021) and maintain the integrity of their skin since the extract protects it from damage, as seen in the use of macroalgae extract in cosmetics (Anis *et al.*, 2017). However, further research on this resistance is needed.

The EHIT data in Figure 3 indicates efficacy that is somewhat different from AWMT. The 2 mg/mL extract level in SA-1 has been proven to possess anthelmintic abilities that are not significantly different from the other levels, and it can inhibit hatching 1.5 times higher than the untreated group. The efficacy of *Sargassum* extract has been reported by Miclon *et al*. (2023), indicating that aqueous extract from the brown macroalgae *B. bifurcata* at 5 mg/mL was able to eliminate 67% of the larvae and inhibit 28% of egg hatching in the murine parasite *Heligmosomoides polygyrus*.

The efficacy of brown macroalgae *Sargassum* spp. was also reported by Khan *et al*. (2015) on the species *Sargassum tenerrimum*, *Sargassum bindarri*, and *Sargassum wightii* against root-knot nematode. Reports on the anthelmintic activity of *S. duplicatum* against *H. contortus* are not widely available, thus, this finding contributes to the parasitological knowledge towards sustainable farming by discovering novel bio-anthelmintics (Sakti *et al.*, 2018). Haron *et al*. (2022) reported the anthelmintic activity of another tropical *Sargassum* species, *S. polycystum*, against marine parasitic leech, where further LC-MS/MS analysis revealed the presence of flavonoids. The presence of these active compounds increased parasite mortality.

Phenolic compounds with affinity-based activity are capable of binding to the protein structures of parasites, leading to changes in cuticular structure, muscle degeneration, and intestinal cell degradation that can reduce nematode motility (Borges & Borges, 2016). Velázquez-Antunez *et al*. (2023) indicated the biological action of phenolic compound extracts that inhibit the hatching of *H. contortus* eggs, suppressing development and impairing embryogenesis within the eggs. The active compounds, flavonoids, and phenols in this study are strongly suspected of damaging the cuticle layer, degrading membranes, and chitin in the eggshell (Ali *et al.*, 2021). Alara *et al*. (2021) indicated that the solvent type and extraction method influence the quantity of metabolites. Nevertheless, a primary challenge in bioanthelmintic research is that promising *in vitro* test results may not necessarily translate to the same effects when tested *in vivo* on livestock (Borges & Borges, 2016). The key findings in this study, indicating that the motility of adult worms and egg hatching is inhibited due to the toxicity of *S. duplicatum* ethanolic extract, highlight the potential benefits of this tropical macroalga species as a candidate bioanthelmintic that necessitates further in-depth exploration.

CONCLUSION

Molecular identification of the Sargassum species determines the highest similarity to *Sargassum duplicatum*. Ethanolic extract of *S. duplicatum* contains metabolites that exhibit anthelmintic activity against the motility response and egg hatchability of *Haemonchus contortus* abomasum worms in sheep. This indicates that the brown macroalgae *S. duplicatum* holds potential for development as an anthelmintic agent, although its

efficacy *in vivo* compared to albendazole still requires further investigation.

CONFLICT OF INTEREST

We confirm the absence of any conflict of interest pertaining to financial, personal, or other affiliations with individuals or organizations relevant to the subject matter covered in the manuscript.

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