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Research Article

In Vitro Anthelmintic Activity of *Tectona grandis* Linn. Leaves Nanoemulsion

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Abstract | Frequent use of pharmaceutical anthelmintic has resulted in a marked increase in anthelmintic resistance. This resistance is characterized by the inherited reduction in sensitivity of a parasite population to an anthelmintic that was previously effective against it. This study aimed to evaluate the potential of *Tectona grandis* (teak) leaf nanoemulsion as a bioanthelmintic. The nanoemulsion was prepared using leaves of *T. grandis* grown in Gunungkidul, Yogyakarta. The anthelmintic effect was assessed through an *in vitro* mortality test on *Haemonchus* sp. worms and analyzed using scanning electron microscopy. Observations were made at 0.5 hours, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 8 hours, and 12 hours during the *in vitro* mortality test of *Haemonchus* sp. worms. A one-way analysis of variance (ANOVA) was performed, followed by Duncan's test to determine significant differences. The results of the *in vitro* study indicated that concentrations of 3%, 5%, and 7% of *T. grandis* Linn. leaf nanoemulsion inhibited *Haemonchus* sp., with the 7% concentration causing the death of all adult *Haemonchus* sp. worms by the 4th hour. Scanning electron microscopy analysis of the nanoemulsion revealed damage to the cuticle structures of the worms. The inclusion of up to 7% *T. grandis* leaf nanoemulsion may serve as a promising alternative for combating *Haemonchus* sp.

Keywords | Anthelmintic, *Haemonchus* sp, Nanoemulsion, *Tectona grandis* linn, Secondary metabolite, Mortality

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INTRODUCTION

Helminths are a diverse group of parasites that represent a significant health challenge for animals across the globe. Gastrointestinal worm infections are prevalent in ruminants and represent a significant barrier to their productivity (Hou *et al.*, 2022). Among these infections, haemonchiasis stands out as a critical concern for livestock. The nematode *Haemonchus* sp. is recognized as the most pathogenic species affecting the gastrointestinal tract of sheep and goats globally. According to the Food and Agriculture Organization (FAO, 1991), the prevalence of haemonchiasis in goats in Indonesia is reported to be 89.4%, resulting in annual economic losses estimated at USD 1 million. The primary approach to managing helminth infections has been the use of pharmaceutical anthelmintics. However, the widespread and frequent use of these drugs has resulted in a marked increase in anthelmintic resistance. This resistance is characterized by the inherited reduction in sensitivity of a parasite population to an anthelmintic that was previously effective against it (Fissiha and Kinde, 2021).

The management and control of gastrointestinal nematodes in livestock production primarily rely on the application of macrocyclic lactone anthelmintics (Yanuartono *et al*., 2020). Consequently, this practice fosters selective pressure within practice populations, leading to the development of resistance against macrocyclic lactones (Gasbarre *et al*., 2009). Notably, widespread reports have documented resistance among ruminant livestock, particularly towards ivermectin (Macedo *et al*., 2015). Recent research has identified numerous phytochemical properties of various plants that can be utilized to address health issues in livestock. Plant-derived natural anthelmintics have gained popularity regarding the management of nematode infections in ruminant animals and are anticipated to mitigate the development of resistance to conventional anthelmintics in the future (Miro *et al*., 2020). Multiple studies have highlighted the potential of active compounds obtained from plant-based agro-industrial waste for alternative treatments in livestock. These compounds have been reported to act as bio-anthelmintics, bio-antibacterials, methane reduction agents, and productivity enhancers (Baihaqi *et al*., 2020; Sakti *et al*., 2024; Rokana *et al*., 2024; Prasetyo *et al*., 2024; Lokapirnasari *et al*., 2024; Prayudi *et al*., 2023; Lisnanti *et al*., 2023). Additionally, Baihaqi *et al*. (2023, 2024) also supported these findings.

Tectona grandis (*T. grandis*) is recognized for its medicinal significance, with numerous claims regarding its efficacy in treating severe illnesses according to traditional Indian medicine. The survey indicated that the plant is utilized for the treatment of urinary discharge, bronchial disorders, cold, and headaches. Additionally, it is employed as a laxative, sedative, diuretic, and anti-diabetic agent. The flowers of *T. grandis* are utilized in the management of bronchitis, biliousness, and urinary discharge. Both the flowers and seeds serve as diuretics, while the wood exhibits expectorant, anti-inflammatory, anti-bilious, and anthelmintic properties. The bark functions as a potent astringent, particularly in cases of bronchitis. The roots are employed for managing anuria and urinary retention. Additionally, nut oil is used in the treatment of scabies and other skin conditions, as well as for promoting hair growth (Asdaq *et al*., 2022). Ogunmefun *et al*. (2017) stated that *T. grandis* leaves contain secondary metabolites, namely alkaloids, flavonoids, tannins, saponins, cardiac glycosides, total phenolics, and carotenoids as listed in Table 1. According of Gururaja *et al*. (2011) the anthelmintic activity of the ethanolic extract derived from the fruits of *T. grandis* preliminary phytochemical analysis of the crude ethanolic extract indicated the presence of flavonoids. At a concentration of 50 mg/ml, the ethanolic extract induced paralysis in *Pheretima posthuma* within 21 minutes and resulted in mortality within 32 minutes (Gururaja *et al*., 2011).

Table 1: Secondary metabolite of Tectona grandis Leaves.

Phytochemical name	Presence in T. grandis leaf
Alkaloid (mg per 100 g)	1778.30
Flavonoids (mg per 100 g)	666.60
Tannins (mg per 100 g)	1583.00
Saponins (mg per 100 g)	406.60
Cardiac glycosides (mg per 100 g)	20.00
Total phenolics (GAE per g)	69.50
Carotenoids (μg per 100 g)	1268.33

adopted from Ogunmefun *et al* (2017).

Nanoemulsion is a thermodynamically stable lipid-based drug delivery system composed of oil, surfactants, cosurfactants, and water, characterized by droplet sizes in the nanometer range (Ahmad *et al*., 2019). Nanoemulsion have been reported to possess several advantageous properties, including high drug solubility, substantial protective capabilities, thermodynamic stability, enhanced oral bioavailability, and a droplet size smaller than 100 nm (Vatsraj *et al*., 2014). Currently, anthelmintics formulated as nanoemulsion are not widely available. Therefore, this study aims to determine the potential of *T. grandis* leaf nanoemulsion as an alternative anthelmintic for *Haemonchus* sp. worms in small ruminants.

MATERIALS AND METHODS

Ethical Clearance

The Institutional Ethics Committee of the Faculty of Veterinary Medicine at Universitas Gadjah Mada in Yogyakarta, Indonesia, has granted approval for the study under the reference number: 70/EC-FKH/int./2024

Area of Study and Collection of Samples

The study was carried out from July to November, 2024. The *in vitro* anthelmintic experiments were conducted at the Animal Parasitology Laboratory in the Department of Internal Medicine, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Scanning electron microscopy were carried out at the Research Center for Animal Husbandry, National Research and Innovation Agency (BRIN), Indonesia. *T. grandis* leaves samples were collected from community forests located in Gunungkidul, Yogyakarta. Concurrently, adult *Haemonchus* sp. nematodes were collected from the abomasum of sheep that exhibited clinical symptoms, including thin, dull hair, and pale. The sheep selected for this study were sourced from the Mutiara Domba Slaughterhouse in Godean, Yogyakarta.The procedure involved carefully opening the abomasum with scissors and extracting the worm using tweezers, which were then placed in a sodium chloride (NaCl) solution for preservation. The collected specimens were specifically identified as female worms, characterized by alternating red and white coloration on their bodies (Victor *et al*., 2014). A total of 100 female worms were required for this experiment, sourced from seven different abomasums.

Extraction and Nanoemulsion

Collected and selected *T. grandis* leaves were first cut into smaller pieces and then dried in an oven at 55°C until fully desiccated. The dried leaves were subsequently ground into a fine powder. Ethanol (70%) was gradually added during the evaporation process using a rotary evaporator until the desired extract was obtained (Charoensit *et al*., 2021). The extract was then formulated into an oil-in-water nanoemulsion, where the oil phase is dispersed within the aqueous phase. The nanoemulsion was produced using a high-energy method with an ultrasonic homogenizer, resulting in smaller and more stable particle sizes (Sadeq, 2020). The *T. grandis* leaf nanoemulsion was prepared at three different concentrations: 3%, 5%, and 7%, with formulation details provided in Table 2. This formulation was adapted from the study by Sreeja and Poulose (2017). The formulation consists of leaf extract, virgin coconut oil (VCO) as the oil phase, Tween 80 as the surfactant, polyethylene glycol 400 (PEG 400) as a cosurfactant, chitosan as an antiparasitic agent, and distilled water as the aqueous phase.

Table 2: Formulation of Tectona grandis Leaves nanoemulsion.

Formulation of nanoemultion (100 ml)	Material*					
	T. grandis Extract (gram)	VCO (ml)	Tween 80(ml)	PEG 400 (ml)	Chitosan (ml)	Distilled water (ml)
Concentration 3%	3	1	3	1	1	91
Concentration 5%	5	1	3	1	1	89
Concentration 7%	7	1	3	1	1	87

***VCO:** virgin coconut oil; **PEG 400:** polyethylene glycol 400.

In vitro Test for the Mortality of Adult Worm

Adult female *Haemonchus sp.* were obtained from an abattoir in Yogyakarta. In vitro anthelmintic experiments were conducted using a combination of methodologies (Mondal *et al*., 2015). *Haemonchus sp.* worms were divided into three treatment groups: nanoemulsion at concentrations of 3%, 5%, and 7%; a negative control group with 0.9% sodium chloride; and a positive control group with 2 mg/ml albendazole, with 20 worms per group. Each group consisted of four repetitions. The worms were placed in 50 mm Petri dishes containing nanoemulsion, sodium chloride, or albendazole solution and incubated at 37°C for 0.5, 1, 2, 3, 4, 6, 8, and 12 hours. The Petri dishes were covered with a black cloth. The incubation period was recorded, and the number of surviving worms was noted. Mortality was confirmed by applying pressure to the body of the worm using tweezers. Worms that exhibited no movement were classified as deceased. A worm was considered dead when it lost its motility and showed a noticeable fading of color.

Scanning Electron Microscopy Analysis

Specimens of *Haemonchus* sp. collected from the *in vitro* assay studies were preserved in a 2% glutaraldehyde solution within a 0.1 M sodium cacodylate buffer for 4 hours at 4°C (Baihaqi *et al*., 2023). The worms were subsequently dehydrated using a gradient of ethanol from 30% to absolute concentration, critically dried with an EMSCOPE CPD 750 (Ashford, Great Britain), and coated with gold-palladium for 5 minutes. The parasites were then examined using a Hitachi S450 scanning electron microscope.

Statistical Analysis

The worm mortality data were evaluated using a one-way analysis of variance (ANOVA). Statistical variations among the means were evaluated using Duncan's multiple-range test. Result were deemed statistically significant at $p<0.05$. The analyses were conducted using IBM Statistical Package for the Social Sciences (SPSS) version 30.0.

RESULTS

In vitro Test for the Mortality of Adult Worm

Table 3 presents the results of the mortality test on *Haemonchus* sp. The nanoemulsion of *T. grandis* leaves was found to be effective in causing the death of *Haemonchus* sp.

Table 3: *In vitro Haemonchus* sp. mortality test with *Tectona grandis* leaves nanoemulsion.

Treatment	Mortality of Haemonchus sp.								P value
	0.5 h	1 h	2 h	3 h	4 h	6 h	8 h	12 h	
3%	0±0.00	0±0.00	$0±0.00^a$	$0±0.00^a$	$15±1.0^a$	$30±2.58^b$	$60±1.63^b$	$75±1.91^b$	0.001
5%	0±0.00	0±0.00	$5±1.0^a$	$10±1.15^a$	$20±2.82^a$	$55±1.0^c$	$65±1.0^b$	$90±1.15^b$	0.001
7%	0±0.00	0±0.00	$20±0.00^b$	$45±1.0^b$	$100±0.00^b$	$100±0.00^d$	$100±0.00^c$	$100±0.00^b$	0.001
NaCl	0±0.00	0±0.00	$0±0.00^a$	$0±0.00^a$	$0±0.00^a$	$0±0.00^a$	$10±2.0^a$	$15±3.0^a$	0.001
Albendazole	0±0.00	0±0.00	$50±1.15^c$	$100±0.00^c$	$100±0.00^b$	$100±0.00^d$	$100±0.00^c$	$100±0.00^b$	0.001
P value	-	-	0.001	0.001	0.001	0.001	0.001	0.001	-

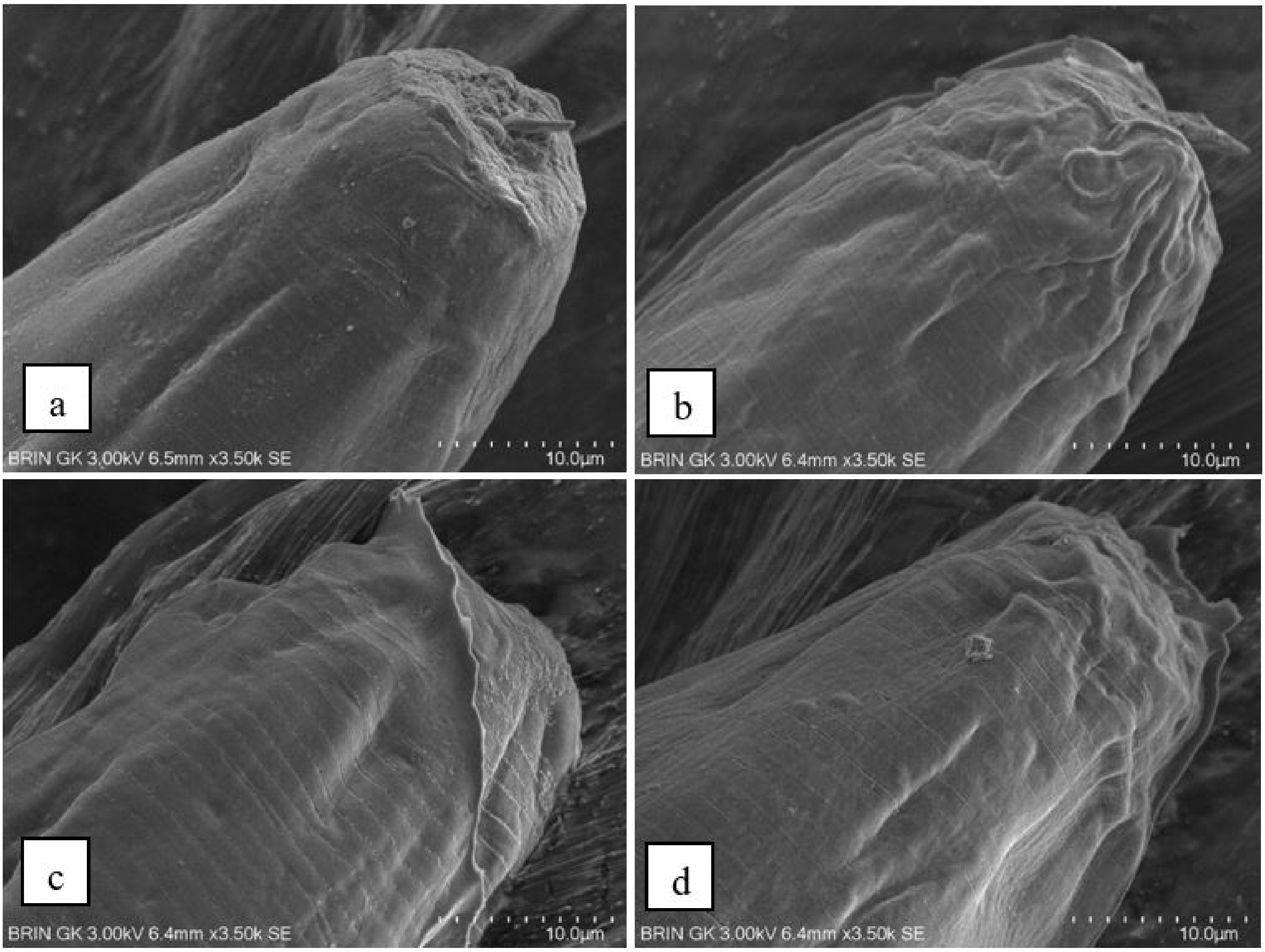

Figure 1: Scanning electron microscopy of *Haemonchus sp* buccal area. (a) negative control, *Tectona grandis* leaves nanoemulsion (b) 3%, (c) 5%, and (d) 7%.

Significant differences (P = 0.001) were recorded between various treatment groups for the mortality of *Haemonchus* sp. which was also affected by the incubation time (P = 0.001). At a concentration of 3%, the nanoemulsion starts to kill the worms after 4 hours. At concentrations of 5% and 7%, the nanoemulsion begins to cause worm mortality after 2 hours. The positive control, albendazole, kills all the worms within 3 hours. Compared to the positive control, the 7% *T. grandis* leaf nanoemulsion shows a similar effect, with all worms dying by the 4th hour.

Scanning Electron Microscopy

Figure 1 and 2 display scanning electron microscopy images of adult female *Haemonchus* sp. of the front region and cuticle. *Haemonchus* sp. has damage to the buccal area and cuticle. Figure 1 illustrates that worms immersed in sodium chloride (NaCl) solution, serving as the negative control, exhibit a smooth cuticle surface without any signs of damage. In contrast, those treated with albendazole (positive control) display damage in the buccal region, accompanied by the presence of aggregates. Furthermore, *Haemonchus* sp. worms subjected to *T. grandis* leaves nanoemulsion treatment at concentrations of (b) 3%, (c) 5%, and (d) 7%, demonstrated damage to the buccal area. Figure 2 shows worms immersed in sodium chloride (NaCl) solution, serving as the negative control, which exhibit smooth cuticles devoid of aggregates and display an undamaged reticular pattern on their longitudinal dorsal surfaces. In contrast, following treatment with teak leaf nanoemulsion at concentrations of 3%, 5%, and 7%, *Haemonchus* sp. worms exhibited wrinkling in the annular cuticle. Additionally, worms treated with albendazole (positive control) displayed numerous wrinkles in both the annular and dorsal longitudinal regions.

DISCUSSION

Secondary metabolites derived from plants are crucial in the realm of drug discovery and development. These compounds are extracted from plant materials through various extraction processes, which involve the separation of desired substances from their mixtures using specific solvents

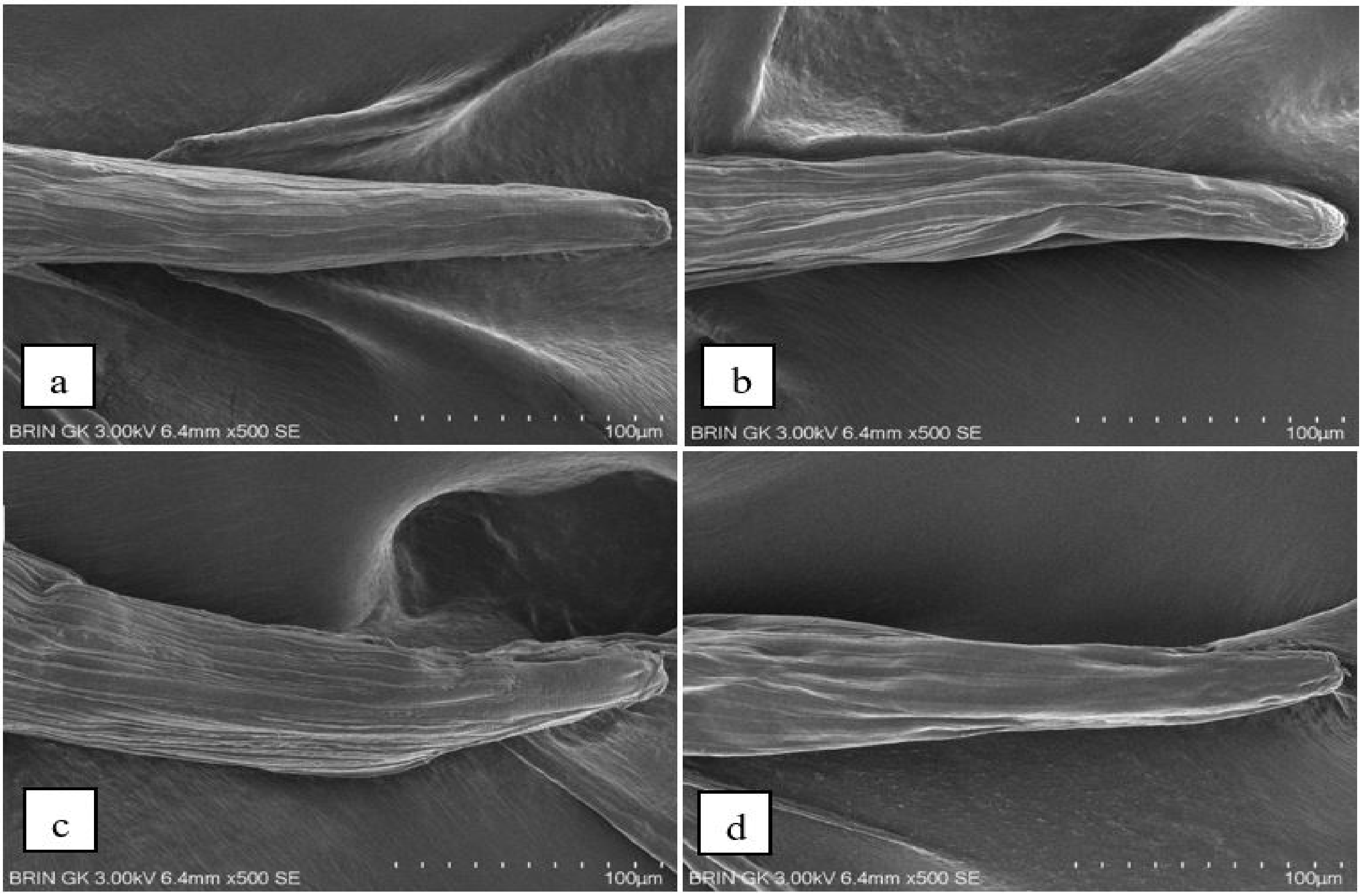

Figure 2: Scanning electron microscopy results of *Haemonchus sp* surface. (a) negative control, *Tectona grandis* leaves nanoemulsion (b) 3%, (c) 5%, and (d) 7%

(Mukhriani, 2014). In the case of teak leaves, a 70% ethanol solution is employed for extraction. The choice of solvent in the extraction process is primarily determined by its polarity, which influences the solubility of the target compounds (Hikmawanti *et al*., 2020). Ethanol is a polar solvent that is able to extract flavonoid compounds, which are also polar so they are easily soluble (Markham, 1988). Moreover, ethanol is favored for its safety profile, making it suitable for the extraction of phytochemicals and bioactive compounds from plants. It is less susceptible to microbial contamination by fungi and bacteria, does not introduce toxins, remains inert with respect to the extracted substances, and exhibits favorable absorption characteristics (Sa'adah and Nurhasnawati, 2017).

The ethanol extract of teak leaves is formulated into an oil-in-water (o/w) nanoemulsion, which consists of an oil phase dispersed within a water phase. The production of this nanoemulsion employs a high-energy technique utilizing an ultrasonic homogenizer, which is known to yield nanoemulsions with smaller and more stable particle sizes (Sadeq, 2020). The oil phase in this formulation is VCO, which serves as a solvent for lipophilic active substances (Puspitasari, 2022). In the emulsification process, the oil phase forms droplets within the dispersion medium, facilitated by surfactants and cosurfactants. Tween 80, a non-ionic surfactant characterized by its sorbitan ester bond resulting from the reaction between sorbitol and fatty acids with ethylene oxide, is utilized in this formulation (Amin and Das, 2019). The cosurfactant employed is PEG 400, a colorless and viscous liquid that is soluble in water. The role of the cosurfactant is to enhance the emulsification process and improve the stability of the nanoemulsion (Sadeq, 2020). Additionally, chitosan is incorporated into the formulation to inhibit the growth of bacteria and fungi in the nanoemulsion preparations (Chaudhary *et al*., 2020). Preliminary phytochemical analysis *T. grandis* Linn has revealed the presence of glycosides, alkaloids, carbohydrates, proteins, tannins, and flavonoids in the ethanolic extracts. *T. grandis* leaves methanol extract contains Sinapic, gallic, p-hydroxybenzoic, ferulic, p-coumarate, chlorogenic, cinnamic, vanillic acid (Murukan and Kumara, 2018). *T. grandis* leaves aqueous extract also contains Sinapic, gallic, p-hydroxybenzoic, ferulic, p-coumarate, chlorogenic, cinnamic, vanillic acid (Koffi *et al*., 2015).

T. grandis is recognized for its medicinal significance, with numerous reports indicating its efficacy in treating various diseases according to traditional Indian systems of medicine. Surveys indicate that this plant is utilized for the management of conditions such as urinary discharge, bronchitis, colds, and headaches. Additionally, it is employed in the treatment of scabies and is known for its laxative and sedative properties, as well as its diuretic, anti-diabetic, analgesic, and anti-inflammatory effects (Asdaq *et al*., 2022). The leaves of *T. grandis* are rich in secondary metabolites, which include alkaloids, tannins, saponins, flavonoids, anthraquinones, and carotenoids (Ogunmefun *et al*., 2017). Many studies have been undertaken to assess the efficacy of tannins as anthelmintics, both *in vitro* and *in vivo*. *T. grandis* leaves exhibits significant anthelmintic activity; however, the time required for inducing mortality in the worms is relatively prolonged. According to Javalgikar at al. (2019) *T. grandis* Linn caused paralysis within 19 to 32 minutes and resulted in death within 30 to 49 minutes. In comparison, the standard drug induced paralysis within 20 minutes and led to mortality at 48 minutes. These findings suggest that the test drug demonstrates a potent effect relative to the standard treatment. Toklo *et al.* (2021) reported that the evaluation of anthelmintic activity demonstrated that the extracts at various doses, along with all tested compounds at a concentration of 150 mg/ml, significantly inhibited larval migration and the motility of adult parasitic worms when compared to the negative control, which consisted of a phosphate buffer solution. The study conducted by Handayanta *et al.* (2023) demonstrated that a 12% of *Acacia auriculiformis* leaves achieved 100% mortality of *Haemonchus contortus* within six hours. This finding suggests that *T. grandis* leaves nanoemulsion exhibits a higher efficacy in eliminating *Haemonchus* sp. Another study consucted by Mumed *et al.* (2022) demonstrated that a 500 mg/ml extract of *Croton macrostachyus* leaves achieved 100% mortality of *Haemonchus contortus* within four hours. In the present study, 7% *T. grandis* leaves nanoemulsion exhibited a similar ability to eliminate *Haemonchus* sp. within the same timeframe. Generally, the capacity of tannins to bind proteins enables them to penetrate various structures of nematodes. The cuticle, in particular, is a structure that is easily penetrated by macromolecules like tannins. A series of microscopic analyses have demonstrated detrimental effects on the cuticle that also impact the underlying tissues across various nematode species (Greiffer *et al*., 2022).

In nematodes, saponins have been linked to the formation of complexes with components of cellular membranes at various stages of the nematode life cycle. This interaction results in increased membrane permeability, ultimately leading to the mortality of these parasites (Maestrini *et al*., 2019). Exposure to medicinal ethnoveterinary plant extracts induces characteristic alterations in the surface morphology of worms. This hypothesis is supported by numerous prior reports documenting the anthelmintic activity of plant extracts against helminths. The process induces membrane destabilization, alters membrane permeability, and results in the loss of membrane potential, ultimately leading to cellular lysis and damage to the cuticle. Flavonoids exhibited the most pronounced activity, with flavones showing particularly strong effects, suggesting their potential for further development into derivatives that may enhance anthelmintic efficacy (Balqis *et al*., 2017). Alkaloids possess neurotoxic properties by inhibiting acetylcholinesterase, leading to acetylcholine accumulation, which induces spasms in the body wall muscles, followed by flaccid paralysis and eventual death of gastrointestinal parasites (Hrckova and Velebny, 2013).

Nematodes are the sole group of parasites that have a robust protective outer layer called the cuticle. This cuticle is made up of a syncytium of proteins, primarily composed of collagen. The mortality of these worms is likely attributed to damage sustained in various body parts, including the buccal area and cuticle, as a result of contact with metabolites present in the nanoemulsion derived from *T. grandis* leaves. This structure functions not only as a protective barrier but also as the structural framework for the organism's body (Lalthanpuii and Lalchhandama, 2020). In nematodes, it is evident that anthelmintic drugs disrupt the structural integrity of the cuticle primarily by targeting cuticular proteins, which serves as their main mechanism of action (Page *et al*., 2014). Martínez-Ortíz-de-Montellano et al. (2013) reported that alterations occur in adult *Haemonchus* sp. worms following in vitro exposure to tannin-rich extracts. In contrast to the control worms, which exhibited a smooth cuticle surface, the parasites treated with both tannin-rich plant extracts displayed significant morphological changes, including longitudinal and transverse folds as well as thicker ridges on the cuticle. These lesions were observed across the entire body of the nematodes or in patches, affecting both the cephalic region and other parts of the body, including the distal ends of the. Notably, the most pronounced changes were observed as aggregates surrounding the buccal capsule, female vulva, or anus. Barone *et al.* (2018) observed that the buccal region of *Haemonchus* sp. exposed to condensed tannins (CTs) from cranberry vines exhibited damage and the presence of aggregats. In contrast, the worms that were not subjected to exposure to these active compounds appeared normal. Tresia *et al.* (2016) reported that the anthelmintic activity of active compounds in plants is exerted synergistically through the damage to the cuticle and alterations in the form and permeability of the worm's cuticle.

CONCLUSIONS AND RECOMMENDATIONS

The 7% teak leaf nanoemulsion demonstrated anthelmintic activity against *Haemonchus* sp. This study establishes a foundation for the use of nanoemulsions as an anthelmintic agent. However, additional *in vivo* studies are necessary to validate the potential application of this medicinal plant in ruminants.

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NOVELTY STATEMENTS

The study on the anthelmintic activity of Tectona grandis leaves nanoemulsion against haemonchus in ruminants has never been done before.

AUTHOR'S CONTRIBUTIONS

Dian Indriati, Yulita Nadzifa Erista, Irkham Widiyono, Zein Ahmad Baihaqi and Yanuartono: Designed the study, collected samples, and conducted laboratory examinations; Dian Indriati: composing the initial draft. All authors contributed to drafting and revising the manuscript. All authors have read, evaluated, and given their approval for the final manuscript.

Conflict of Interest

There were no conflicts of interest reported.

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