Phytochemical analysis and anthelmintic activity of *Andrographis paniculata*, *Azadirachta indica,* and *Litsea elliptica* leaves extracts against *Caenorhabditis elegans*

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Abstract

Despite the challenges posed by multi-drug resistance in synthetic anthelmintics, the potential of plant extracts as an alternative helminth control option offers a ray of hope. The current study, conducted with meticulous care and thoroughness, delves into the anthelmintic activity of crude water and ethanol extracts of three plant species, *Andrographis paniculata* (Hempedu Bumi), *Azadirachta indica* (Neem), and *Litsea elliptica* (Pawas), against the nematode, *C. elegans*. The preparation of water and ethanol extracts from the three plants involved maceration and ultrasonic methods, respectively. Phytochemical screening was conducted to identify the active compounds in both extracts, followed by determining the total phenolic content (TPC) and total flavonoid contents (TFC) using the corresponding Folin-Ciocalteau and aluminum colorimetric methods. The percentage yields of water extracts were 15.10%, 13.11%, and 9.55%, and ethanol extracts were 9.78%, 2.94%, and 15.50% for *A. paniculata*, *A. indica*, and *L. elliptica*, respectively. All extracts tested positive for alkaloids, steroids, terpenoids, protein, and carbohydrates. In water extract, *A. indica* exhibited the highest total phenolic (TPC) and total flavonoid (TFC) contents of 58.38 ± 0.005 mg GAE/g and 18.68 ± 0.005 mg QE/g, respectively. In contrast, in ethanol extract, *L. elliptica* exhibited the highest TPC and TFC of 122.67 ± 0.398 mg GAE/g and 96.69 ± 0.027 mg QE/g, respectively. *L. elliptica* ethanol extract appeared to induce the most pronounced effect on reducing head thrashing activity compared to other extracts in *C. elegans*. Both water and ethanol extracts of *A. indica* and *L. elliptica* exhibited significant mortality in all examined concentrations with maximum mortality.
Keywords: egg-laying, head thrashing, nematicide activity, parasitic infection, plant secondary metabolite, toxicity

Introduction

Helminthiasis remains one of the most important animal diseases worldwide, causing a major threat to the health and welfare of livestock, especially in small ruminants (Maphosa, 2010; Ahmed *et al.*, 2020). This results in huge economic losses to the farmers as it leads to a decrease in milk, meat, and wool production, retarded growth, and even the death of animals (Baihaqi *et al.*, 2019; Castagna *et al.*, 2024; Kasah *et al.*, 2024). The prevalence of helminthiasis is high in developing countries, mainly due to warm temperatures, associated with poor management practices and inadequate control measures (Baihaqi *et al.*, 2019). In Brunei, the occurrence of helminthiasis is considered endemic, with a ratio of 1:1 between the number of farms and the number of positive helminth cases. Annual surveillance conducted by the Division of Livestock Industry and Veterinary Services (LIVS) from the Department of Agriculture and Agrifood, Brunei Darussalam (DoAA) revealed that every fecal sample subjected to fecal flotation from farms across Brunei tested positive for helminth eggs (DoAA, 2023). The parasites that often infect small ruminants are from the Family Trichostrongylidae (*Trichostrongylus spp.*, *Haemonchus contortus*, *Ostertagia spp.*) and Family Strongylidae (*strongyloides sp.*).

Prophylactic vaccines are the ideal primary line of defense against parasitic helminths in livestock farming. However, due to the unavailability of most vaccines, the treatment relies heavily on commercial anthelmintic drugs and anthelmintics like albendazole and ivermectin that are commonly used to fight against parasitic infection (DoAA, 2023; Belga *et al.*, 2024). They have succeeded in reducing intestinal parasitic infections, but none have been able to diminish the reinfestation of diseases (Gunathilaka *et al.*, 2018). Recurrence events in cases of helminthiasis usually occur three months after the treatment (DoAA, 2023). This leads to the overuse of these drugs, contributing to the development of extensively drug-resistant parasites. Other factors that contribute to the development of resistance are poor efficacy of anti-parasitic agents, inadequate dose level, low protein diet, and environmental toxicity (Rizwan *et al.*, 2021).

The increased resistance of parasites to modern anthelmintics, combined with the wish for a more sustainable way of farming, has geared towards the effort to find alternative parasite control options (Rahmann & Seip, 2007; Kasah *et al.*, 2024). This has led to the resurgence of other alternative sources to control parasitic infection and to improve public health. Medicinal plants are among the natural products being explored for their anthelmintic properties (Widaad *et al.*, 2022; Kļaviņa *et al.*, 2023; Castagna *et al.*, 2024). Not only is natural anthelminthic more sustainable, but the cost of production could also be less than conventional drugs, with the chances of

developing resistance meager (Kļaviņa *et al.*, 2023). Among the plant species with anthelmintic properties are *Azadirachta indica* (Priscilla *et al.*, 2014; Hellawi & Ibrahim*,* 2020), A*ndrographis paniculata* and *Litsea sp.* (Park *et al.*, 2007; Chastity *et al.*, 2015; Banerjee *et al.*, 2019; Mohammad, 2021) Although studies have been done to validate the potential of these plants for their anthelmintic action, yet no studies have been conducted using the species found and grown in Brunei. Therefore, the present study aimed to evaluate the anthelminthic activity of three selected Medicinal plants. The objectives of the study were (i) to screen the phytochemical constituents present in the selected plants, (ii) to determine the total phenolic content (TPC) and total flavonoid content (TFC) in the plant extracts, and (iii) to determine the anthelmintic activities of these plant extracts. The nematode C*aenorhabditis elegans* was used as a model species. *C. elegans* has been used as a model to screen plant extracts globally (Katiki *et al.*, 2012; Widaad *et al.*, 2022). Nematodes have also been utilized to determine the mode of action of new anthelmintics, and the results were replicated in sheep infected with drug-resistant *H. contortus.*

Material and Methods

Plant identification and collection

Three selected plants, *Andrographis paniculata* (Hempedu Bumi), *Azadirachta indica* (Neem), and *Litsea elliptica* (Pawas), were purchased from Rimba Horticulture Centre in December 2022. Samples of the plant were then authenticated, each with respective Brunei National Herbarium number (BRUN): *A. paniculata* (BRUN number B 045 279), *A. indica* (BRUN number B 045 277) and *Litsea elliptica* (BRUN number B 045 280). The samples were then deposited at BRUN with their voucher number: *A. paniculata* (FOA 0003), *A. indica* (FOA 0001) and *Litsea elliptica* (FOA 0004). The leaves of each plant were collected and washed with distilled water before proceeding with drying the leaves in the drying cabinet (Genlab, UK) at 60°C for three days. The dried leaves were then ground well using a laboratory blender (Waring 800s, USA) and filter-sieved using a mechanical siever (0.3 mm mesh hole size). The resulting powders were put in well-labele,d airtight containers and stored at room temperature, awaiting extraction.

Preparation of plant extracts

The powdered leaves of three plant species were extracted using two solvents, water and ethanol, following the protocol of Shahlehi *et al.* (2020). A maceration technique was utilised for water extraction. Briefly, the plant powder samples were soaked with distilled water at a ratio of 1:10 (50g of powder in 500mL of distilled water) and kept in 60°C water bath (Stuart SBS40, UK) for 3 days. Water was then drained, and the remaining powder samples were macerated for the second

time under the same condition. On the final day of maceration, the suspensions were filtered, and the filtrate were centrifuged (Jouan BR4i, UK) at 3000 rpm for 20 minutes. The supernatants were recovered and stored at -80°C overnight before freeze-drying (Labconco Freeze Dry System, USA) the samples for three days, yielding brown powder extract (Kamaruzaman & Noor, 2017). The water extracts were stored in universal bottles and refrigerated at 4°C to maintain their freshness until use.

For the ethanol extraction, the ultrasonic extraction (UE) method was employed using 95% ethanol as the solvent with the help of a 300W ultrasonic bath (Branson Ultrasonics, USA). The plant powder was mixed with 95% ethanol in a 1:20 solute-to-solvent ratio in a beaker (50g of powder in 1000mL of ethanol) and was subjected to ultrasonic treatment at 25°C for 30 minutes. The mixture was then filtered through a filter paper, and the remaining plant residues were subjected to a second extraction cycle with ethanol at the same ratio. The collected filtrates from both cycles were then evaporated using a rotary evaporator (Yamato RE301, Japan) at 63°C and left to dry in a 60°C drying cabinet for five days. This yielded a dark green solid ethanol extract stored in universal bottles and kept at 4°C until needed. The following equation calculated the extraction yield of the selected plants:

$$\text{Yield (\%)} = (X_1 * 100)/X_0$$

where X_1 refers to the weight of the extract after evaporation of the solvent, and X_0 refers to the dry weight of the plant powder before extraction.

Phytochemical screening

Qualitative tests to detect the presence of secondary metabolites in water and ethanolic leaves' extracts of *A. paniculata, A. indica and L. elliptica* were carried out using standard phytochemical screening procedures. The screening was done to determine the presence of alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, glycosides, proteins, and carbohydrates. Visual examination of the appearance of color or frothing was used to indicate the presence or absence of a phytochemical group (Table 1).

Table 1. Phytochemical screening methods for the three plant species extracts: *A. paniculata, A. indica* and *L. elliptica.*

Active compound	Phytochemical test	Chemicals and Method	Indicator for presence	Reference
Alkaloids	Mayer's test	Mayer's reagent	Cream-coloured precipitate	Iqbal *et al.,* 2015
Alkaloids	Dragendorff's test	Dragendorff's reagent	Orange red precipitate	Sadaqat Shah *et al.,* 2018
Flavonoids	Alkaline test	1M NaOH + 1M HCl	Intense yellow to colourless	Raman *et al.,* 2018
Tannins	Potassium Dichromate test	1% $K_2Cr_2O_7$	Solution develops a precipitate	Iqbal *et al.,* 2015
Saponins	Foam test	Heat	Persistent white foam on surface	Raman *et al.,* 2018
Steroids	Salkowski's test	$CHCl_3$ + 80% H_2SO_4	Red colouration in chloroform layer	Iqbal *et al.,* 2015
Terpenoids	Liebermann-Burchard test	$CHCl_3$ + $(CH_3CO)_2O$ + conc. H_2SO_4	Reddish violet colour	Iqbal *et al.,* 2015
Glycosides	Glycoside test	5% H_2SO_4 + $CHCl_3$ + 25% NH_3	Rose pink to Red	Iqbal *et al.,* 2015
Proteins	Xanthoproteic test	H_2O (distilled) + HNO_3	Yellow colouration	Raman *et al.,* 2018
Proteins	Biuret test	1% NaOH + 1% $CuSO_4$	Blue or purple colouration	
Carbohydrates	Benedict's test	Benedict's solution + Heat	Blue green (low conc.), Yellow, Orange and Red (high conc.)	Deshmukh & Theng, 2018

Note: $(CH_3CO)_2O$ = Acetic anhydride; $CHCl_3$ = methyl chloroform; Conc. = Concentrated; $CuSO_4$ = Copper sulphate; HCl = Hydrochloric acid; H_2SO_4 = Sulphuric acid; HNO_3 = Nitric acid; $K_2Cr_2O_7$ = Potassium dichromate; NaOH = Sodium Hydroxide; NH_3 = Ammonia.

Analysis of total phenolic (TPC) and total flavonoid (TFC) contents

The total phenolic content was determined using the Folin-Ciocalteau assay (Shahlehi *et al.,* 2020). 20 µL of extracts or each standard solution of gallic acid (50, 100, 150, 200, 250, and 300 µg/mL) was added into separate wells of 96-well plate, followed by 100 µL of Folin-Ciocalteu reagent (10% v/v). After 8 minutes, 80 µL of sodium carbonate solution (7.5% w/v) was added to the mixture. A blank was prepared in the same manner where 20 µL of distilled water was used instead of the sample or standard, and the equivalent amount of Folin-Ciocalteu reagent (10% v/v) and sodium carbonate solution (7.5% w/v) was added to the mixture. The plate containing the mixtures was then allowed to incubate in the dark at room temperature for two hours. The absorbance was measured at 765 nm using a microplate reader (BioTek Epoch, USA). The total phenolic content of the plant extract was calculated based on the standard curve generated from the absorbance readings of the gallic acid standards. All experiments were conducted in triplicates, and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight of the plant extract.

Total flavonoid content was determined using the aluminum chloride colorimetric method (Iqbal *et al.,* 2015). 20 µL of extracts or each standard solution of quercetin (50, 100, 150, 200, 250, and 300 µg/mL) was added into separate wells of the 96-well plate, followed by 90 µL of aluminum chloride solution (10% w/v), and 90 µL of potassium acetate (10% w/v). A blank was prepared in the same manner where 20 µL of distilled water was used instead of the sample or standard, and the equivalent amount of aluminum chloride (10% w/v) and potassium acetate (10% w/v) was added to the mixture. The plate was then incubated at room temperature for 30 minutes before measuring the absorbance at 415 nm using a microplate reader. The total flavonoid content of the plant extract was calculated based on the standard curve generated from the absorbance readings of the quercetin standards. The assay was repeated in triplicate, and the results were expressed as milligrams of quercetin equivalents (QE) per gram of dry weight of the plant extract.

C. elegans Maintenance and Synchronisation

The wild-type Bristol (N2) strain of *Caenorhabditis elegans* used in this study was provided by the Caenorhabditis Genetics Center (CGC) at the University of Minnesota, facilitated by the Institute of Health Sciences (IHS) at Universiti Brunei Darussalam. Wild-type worms were grown on Modified Youngren's Only Bactopeptone (MYOB) agar plates seeded with *Escherichia coli* (*E. coli*) OP50 bacteria at 20°C. Worm synchronization was performed, as previously explained by Widaad *et al.* (2022), with few modifications.

Agar plates with adequate populations of eggs or hermaphrodite worms were washed with M9 buffer and transferred into a 15 mL falcon tube. The tube was centrifuged for 1 minute at 1500 rpm to pellet the worms, and the supernatant was removed. 5 mL of bleaching solution (a mixture of sodium hypochlorite and 2 M NaOH at a ratio of 1:1 (v/v)) was then added to the tube to kill the adult worms and isolate the eggs for growth synchronization. The tube was shaken vigorously for 3 min before adding 10 mL of M9 buffer to stop the bleaching effect. The tube was then centrifuged again for 1 minute at 1500 rpm before discarding the supernatant, followed by adding 10 mL of M9 buffer. This step was repeated until the solution cleared bacteria or dead worm debris. 1 mL of M9 buffer was added for the final centrifugation, and the supernatant was discarded, leaving only approximately 100 µL of egg pellets, which were transferred onto unseeded MYOB plates and grown to the L1 stage.

Head thrashing assay

The assay was carried out according to a method Nawa *et al.* (2012) described, with slight modifications. At increasing concentrations, 20 µL of both extracts (5, 10, 15, 20, 25, and 30

mg/mL) were added into allocated wells in a 96-well plate. M9 buffer (in water extract) or 1% DMSO (in ethanol extract) and 100 μM (Lev) acted as the negative and positive controls, respectively. Five synchronized L4 worms were placed in each well and incubated for 2 h at 22°C. The number of head thrashes for five nematodes per treatment was then counted for 30 seconds and doubled to obtain the thrashing activity of 1 minute. The assay was performed in three replicates.

Egg-laying Assay

The assay was done using the method described by Lee and Kang (2017), with minor adjustments. 8 plates of 35 mm unseeded MYOB were prepared, and five synchronized gravid hermaphrodites were added onto each plate before being treated with the following: both extracts with concentrations of 5, 10, 15, 20, 25, and 30 mg/mL, M9 buffer (in water extract) or 1% DMSO (in ethanol extract) (negative control) and 100 μM lev (positive control). The plates were incubated at 22°C for 24 h to allow the laying of eggs. After 24 hours, the hermaphrodites were transferred into the new plates, and the number of eggs laid on the previous plates was counted. This procedure was repeated until no more eggs were laid, and the total number of eggs laid from each treatment was then calculated. The assay was performed in triplicate.

Toxicity assay

The toxicity assay followed the protocol described by Qiao *et al.* (2014), with minor adjustments. 20 μL of the following were added into allocated wells in a 96-well plate: both extracts in increasing concentrations (5, 10, 15, 20, 25, and 30 mg/mL) prepared in M9 buffer (for water extract) or 1% DMSO (for ethanol extract), respectively (negative control), and using 100 μM of levamisole (Lev) (positive control) (Parodi *et al.*, 2015). Subsequently, 10 synchronized L4-staged worms were added into each treatment and incubated for 24 hours at 22°C. Following exposure, the activity of the worms in each well was observed under a dissecting microscope, where inactive ones were scored. Worms were judged dead if they did not respond to stimulus using a small metal wire. The assay was conducted in triplicates.

Statistical analysis

Statistical analysis was performed using R statistical software. All results were expressed as mean ± standard error of means (SEM). The data between the experimental treatments and the control in toxicity, head thrashing, and egg-laying assays were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's posthoc. LC_{50} values were calculated using probit

analysis to determine the concentration at which 50% mortality occurred. Statistical significance was recorded as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and **** $p \leq 0.0001$.

Results

Extraction yield and phytochemical analysis

For water extracts, *A. paniculata* exhibited the highest yield at 15.10%, followed by *A. indica* at 13.11%, and *L. elliptica* at 9.55%. Conversely, the percentage yield of ethanol extract was the highest in *L. elliptica* at 15.50%, followed by *A. paniculata* at 9.78% and *A. indica* at 2.94%. All water extracts of the selected plant species produced a light brown fluffy solid, whereas ethanol extracts produced a sticky dark-green solid, except *L. elliptica* exhibited a non-sticky dark-green solid. The phytochemical screening of all plant extracts revealed the presence of most secondary metabolites (Table 2), which were evident with remarkable color changes. A qualitative study revealed the presence of alkaloids, steroids, terpenoids, proteins, and carbohydrates in all extracts. Flavonoids were primarily detected in ethanol extracts of *A. indica* and *L. elliptica*. Tannins were prominent in all ethanol extracts of the three plants, whereas glycosides were found in all plant water extracts. Saponins were absent in all plant extracts except *A. paniculata* water extract.

Table 2. Qualitative phytochemical screening of water and ethanol extracts of the target species.

Phytochemical Test	*A. paniculata* water extract	*A. paniculata* ethanol extract	*A. indica* water extract	*A. indica* ethanol extract	*L. elliptica* water extract	*L. elliptica* ethanol extract
Alkaloids (Mayer's Test)	+	+	++	++	+++	+++
Alkaloids (Dragendorff's test)	+	-	-	-	++	+
Flavonoids	-	-	-	+	-	+
Tannins	-	++	-	++	-	+++
Saponins	+	+	-	-	-	-
Steroids	+	++	++	+++	++	++
Terpenoids	+	++	++	+++	++	++
Glycosides	++	-	+++	-	+++	-
Protein (Xanthoproteic test)	-	+	++	++	+++	++
Protein (Biuret test)	++	+	++	+	++	+
Carbohydrates (Benedict's test)	+	+	+++	+	+++	+

Note: Relative abundance of phytochemicals in plant extracts, indicated as follows: +++ signifies highly present, ++ signifies moderately present, + signifies mildly present, and -signifies absence.

Total phenolic and flavonoid contents

Table 3 demonstrates the total phenolic and total flavonoid contents of both extracts of *A. paniculata, A. indica and L. elliptica* stated as mg GAE/g extract and mg QE/g extract, respectively. The ethanol extracts of the studied plants were observed to have higher total phenolic content than water extracts, except for *A. indica.* The ethanol extracts of *L. elliptica* exhibited the highest total phenolic content (122.67 ± 0.398 mg GAE/g), followed by *A. paniculata* (73.59 ± 0.014 mg GAE/g) and *A. indica* (50.67 ± 0.073 mg GAE/g). On the other hand, *A. indica* exhibited the highest total phenolic content in water extracts (58.38 ± 0.005 mg GAE/g), followed by *L. elliptica* (54.40 ± 0.022 mg GAE/g) and lastly *A. paniculata* (6.10 ± 0.004 mg GAE/g). The total phenolic content of ethanol and water extracts was obtained based on the standard gallic acid curve obtained at $y = 4.809x + 0.0528$ ($r^2=0.99$), and $y = 4.7751x + 0.0762$ ($r^2=0.99$), respectively.

Similar to total phenolic content, the total flavonoid content also varied with the type of solvent used for extraction in the studied plant species. The ethanol extract of *L. elliptica* showed the highest total flavonoid content (96.69 ± 0.027 mg QE/g), followed by *A. paniculata* (40.45 ± 0.032 mg QE/g), and *A. indica* (52.48 ± 0.038 mg QE/g). In water extracts, the total flavonoid content of A. indica was found to be the highest (18.68 ± 0.005 mg QE/g), and *A. paniculata* showed the lowest (4.91 ± 0.0007 mg QE/g), with *L. elliptica* being the latter (13.13 ± 0.004 mg QE/g). Overall, ethanol extracts demonstrated higher total flavonoid content than the water extracts of the plants. The total flavonoid content of both ethanol and water was obtained based on standard quercetin curve obtained at $y = 3.5317x + 0.1113$ ($r^2=0.99$) and $y = 0.9018x + 0.0543$ ($r^2=0.99$), respectively.

Table 3. Total phenolic (TPC) and total flavonoid (TFC) content of *A. paniculata, A. indica* and *L. elliptica* water and ethanol extracts. Values are expressed as mean of mg of GAE or QE per gram of extract ± SEM; n=3.

Species	Solvent	TPC (mg GAE/g)	TFC (mg QE/g)
A. paniculata	Ethanol	73.59 ± 0.014	40.45 ± 0.032
	Water	6.10 ± 0.004	4.91 ± 0.0007
A. indica	Ethanol	50.67 ± 0.073	52.48 ± 0.038
	Water	58.38 ± 0.005	18.68 ± 0.005
L. elliptica	Ethanol	122.67 ± 0.398	96.69 ± 0.027
	Water	54.40 ± 0.022	13.13 ± 0.004

Note: GAE: Gallic acid equivalent, QE: Quercetin equivalent, SEM: Standard error of the mean.

Effects of ethanol and water extracts of *A. paniculata, A. indica,* and *L. elliptica* on head thrashing activity of *Caenorhabditis elegans*

The result of head thrashing activity reveals varying degree of locomotion behaviour upon exposure to both water and ethanol extracts of *A. paniculata, A. indica* and *L. elliptica*. From the observation, there is an apparent dose-dependent reduction in head thrashing activity when worms were exposed to both extracts after two hours of incubation. Exposure to both extracts of *L. elliptica* in increasing concentrations up to 30 mg/mL caused a significant decrease in the head thrashing movement in a dose-dependent manner, from 43.73 ± 5.47 at 5 mg/mL to 2.67 ± 2.07 thrashes per min at 30 mg/mL in water extract, and from 3.86 ± 4.41 at 5 mg/mL to 0.00 ± 0.00 thrashes per min at 30 mg/mL in ethanol extract, indicating complete immobility and even death of the worms, which was comparable to levamisole (Table 4, Fig. 1). A similar reduction was observed when *C. elegans* were treated with *A. indica* extracts, whereby the number of head thrashes significantly decreased from 18.13 ± 9.07 at 5 mg/mL to 0.00 ± 0.00 thrashes per min (complete immobility and death of worms) at 30 mg/mL in water extract, and from 60.33 ± 10.97 at 10 mg/mL (Table 4, Fig. 1) to 0.00 ± 0.00 thrashes per min (total immobility and death of worms) at 30 mg/mL in ethanol extracts. In contrast, treatment with *A. paniculata* in each water and ethanol extract in worms only demonstrated significant reduction at higher concentrations, with 33.47 ± 3.27 thrashes per min at 30 mg/mL in water extract. With ethanol extract, the reduction was observed at 15 mg/mL (75.87 ± 4.93 thrashes per min) to 30 mg/mL (52.07 ± 7.4 thrashes per min) (Table 4; Figure 1). Overall, *L. elliptica* ethanol extract appeared to induce more pronounced effects on head thrash reduction as complete immobility and death of the worms were shown at 20 mg/mL among all extracts of the studied plant species.
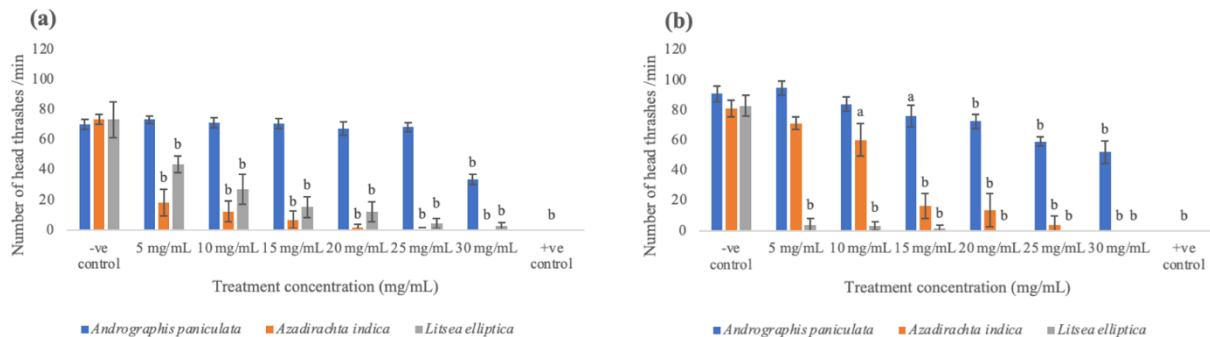
Figure 1. Effect of *A. paniculata, A. indica* and *L. elliptica* extracts on head thrashing activity in *C. elegans* in increasing concentration (5, 10, 15, 20, 25 and 30 mg/mL). (a) The number of head thrashes per min in water extract (b) The number of head thrashes per min in ethanol extract. Data are presented as means ± SEM and are compared to M9 buffer (in water extract) or 1% DMSO (in ethanol extract) as the negative control. Statistical significance was calculated using a one-way ANOVA with Tukey's post hoc multiple comparisons of means, denoted by superscripts a and b. Superscripts a and b denote significance at ***$p \leq 0.001$ and **** $p \leq 0.0001$, respectively. n = 5 worms. Statistical significance was shown by comparing the means between each extract concentration's negative control and treatment.

Table 4. Mean of head thrashing activity of water and ethanol extracts of *A. paniculata, A. indica and L. elliptica* and controls. (Mean ± SEM).

Treatments	*A. paniculata* water extract	*A. paniculata* ethanol extract	*A. indica* water extract	*A. indica* ethanol extract	*L. elliptica* water extract	*L. elliptica* ethanol extract
M9 buffer or 1% DMSO (negative control)	70.1 ± 3.33	91 ± 5.26	73.5 ± 3.48	81.13 ± 5.32	73.2 ± 12.1	83 ± 6.85
Levamisole (positive control)	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
5 mg/mL	73.2 ± 2.57	94.73 ± 4.55	18.13 ± 9.07 [b]	71.27 ± 4.13	43.73 ± 5.47 [b]	3.86 ± 4.41 [b]
10 mg/mL	71.27 ± 3.35	84.07 ± 4.46	12.33 ± 6.82 [b]	60.33 ± 10.97 [a]	26.93 ± 9.97 [b]	2.93 ± 2.72 [b]
15 mg/mL	70.73 ± 3.41	75.87 ± 4.93 [a]	6.73 ± 5.80 [b]	16.47 ± 8.42 [b]	15.2 ± 6.9 [b]	1.733 ± 2.14 [b]
20 mg/mL	67.33 ± 4.63	72.53 ± 4.92 [b]	1.73 ± 1.99 [b]	13.73 ± 11.18 [b]	12.00 ± 6.65 [b]	0.00 ± 0.00 [b]
25 mg/mL	68.33 ± 3.08	59.2 ± 3.26 [b]	0.60 ± 0.97 [b]	3.80 ± 5.8 [b]	4.40 ± 3.32 [b]	0.00 ± 0.00 [b]
30 mg/mL	33.47 ± 3.27 [b]	52.07 ± 7.41 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	2.67 ± 2.07 [b]	0.00 ± 0.00 [b]

Data are presented as means ± SEM and are compared to M9 buffer (in water extract) or 1% DMSO (in ethanol extract) as the negative control. Statistical significance was calculated using a one-way ANOVA with Tukey's post hoc multiple comparison of means and is denoted by superscripts a and b. Superscripts a and b denote significance at ***$p \le 0.001$ and **** $p \le 0.0001$, respectively. n = 5 worms. Statistical significance was shown by comparing the means between each extract concentration's negative control and treatment.

Effects of ethanol and water extracts of *A. paniculata, A. indica* and *L. elliptica* on Egg-laying activity of *Caenorhabditis elegans*

Exposure of *C. elegans* to water and ethanol extracts of *A. paniculata, A. indica and L. elliptica* significantly decreases the mean number of eggs laid. In water extract, the mean number of eggs laid in *A. paniculata, A. indica* and *L. elliptica* reduced substantially to 142.67 ± 53.04, 77.00 ± 5.51, and 54.00 ± 9.17 as the concentration increased to 30 mg/mL when compared to the negative control, which had around 311.67 ± 8.35 to 316.3 ± 10.41 eggs. A significant decrease was also observed when the worms were exposed to the ethanol extracts of the studied plant species, whereby the highest concentration of 30 mg/mL reduced the number of eggs laid to 220.67 ± 7.69 for *A. indica,* 227.00 ± 9.17 for *A. paniculata* and 76.33 ± 8.41 for *L. elliptica.* The results are tabulated in Table 5. Both extracts of *L. elliptica* exhibited the most potent egg-laying reduction activity in *C. elegans*. Figure 2 shows the mean number of eggs laid in the three plant species' water and ethanol extracts, respectively.

Table 5. Mean of egg-laying activity of water and ethanol extracts of *A. paniculata, A. indica and L. elliptica* and controls. (Mean ± SEM).

Treatments	*A. paniculata* water extract	*A. paniculata* ethanol extract	*A. indica* water extract	*A. indica* ethanol extract	*L. elliptica* water extract	*L. elliptica* ethanol extract
M9 buffer or 1% DMSO (negative control)	316.3 ± 10.41	372.67 ± 20.03	314.00 ± 4.58	363.33 ± 8.69	311.67 ± 8.35	374.67 ± 21.98
5 mg/mL	311.67 ± 10.87	385.33 ± 35.97	186.00 ± 4.16 [b]	341.00 ± 6.51	231.0 ± 12.29 [b]	215.67 ± 5.78 [b]
10 mg/mL	278.67 ± 14.17 [a]	264.33 ± 11.68 [b]	148.67 ± 11.17 [b]	327.33 ± 16.37	204.0 ± 5.51 [b]	170.00 ± 12.86 [b]
15 mg/mL	221.33 ± 8.84 [b]	223.00 ± 7.51 [b]	102.67 ± 6.96 [b]	313.33 ± 10.14	163.67 ± 9.84 [b]	165.67 ± 19.72 [b]
20 mg/mL	220.00 ± 22.91 [b]	198.00 ± 10.44 [c]	75.33 ± 5.90 [b]	265.33 ± 9.91 [a]	112.67 ± 4.33 [b]	97.67 ± 2.96 [b]
25 mg/mL	202.33 ± 18.21 [b]	227.33 ± 8.76 [a]	65.33 ± 8.84 [b]	270.33 ± 10.53 [a]	105.33 ± 5.84 [b]	87.33 ± 26.34 [b]
30 mg/mL	142.67 ± 53.04 [b]	227.00 ± 9.17 [b]	77.00 ± 5.51 [b]	220.67 ± 7.69 [b]	54.00 ± 9.17 [b]	76.33 ± 8.41 [b]

Data are presented as means ± SEM and are compared to M9 buffer (in water extract) or 1% DMSO (in ethanol extract) as the negative control. Statistical significance was calculated using a one-way ANOVA with Tukey's post hoc multiple comparison of means and is denoted by superscript a and b. Superscript a and b denotes significance at, *** $p \leq$ 0.001, $p \leq$ ****0.0001 respectively. n = 5 worms. Statistical significance shown compared the means between the negative control and treatment of each concentration.
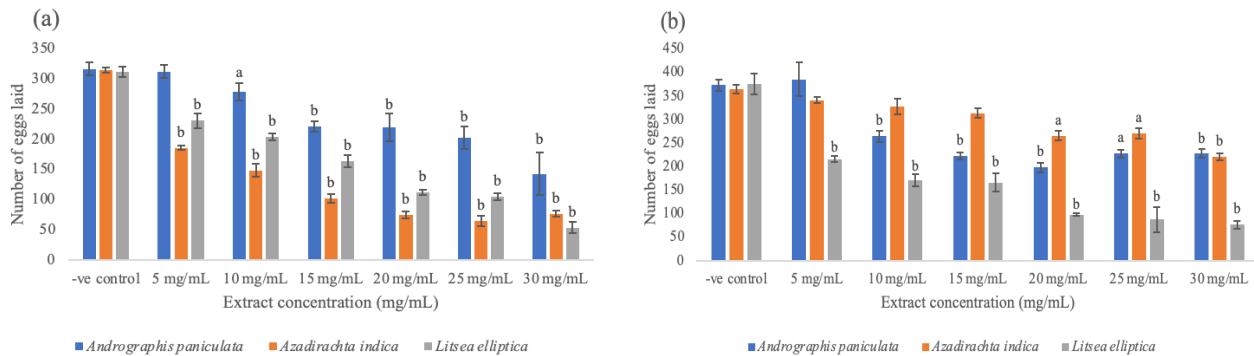
Figure 2: Effect of *A. paniculata, A. indica and L. elliptica* extracts on the egg-laying activity in *C. elegans* in increasing concentration (5, 10, 15, 20, 25 and 30 mg/mL). (a) The number of eggs laid in water extract (b) The number of eggs laid in ethanol extract. Data are presented as means ± SEM and are compared to M9 buffer (in water extract) or 1% DMSO (in ethanol extract) as the negative control. Statistical significance was calculated using a one-way ANOVA with Tukey's post hoc multiple comparisons of means, denoted by superscripts a and b. Superscripts a and b denote significance at ***$p \leq$ 0.001 and ****$p \leq$ 0.0001, respectively. n = 5 worms. Statistical significance was shown by comparing the means between each concentration's negative control and treatment.

Effects of ethanol and water extracts of *A. paniculata, A. indica And L. elliptica* on mortality Rate of *Caenorhabditis elegans*

The lethality of both extracts towards *C. elegans* was determined, and the results are shown in Table 6. The study showed a dose-dependent increase in the percentage mortality of *C. elegans* 24 hours after exposure to both water and ethanol extracts (Figure 3). In water extracts, both *A. indica* and *L. elliptica* exhibited significant mortality in all examined concentrations, with maximum mortality of 90.00 ± 0.0 % and 100.00 ± 0.0 %, respectively, when compared to the negative

control, which did not cause any worm death (Fig. 3). On the other hand, water extract of *A. paniculata* did not cause a significant effect on L4 mortality rates of *C. elegans* in any tested concentration. The LC_{50} for *A. indica* and *L. elliptica* were estimated from linear regression equations and were calculated to be 10.71 mg/mL, and 11.53 mg/mL, respectively. *L. elliptica* water extract was the most toxic, although *A. indica* water extract demonstrated higher mortality rates in the first five concentrations. The increasing toxicity order is: *A. paniculata < A. indica < L. elliptica.*

In ethanol extracts, *L. elliptica* showed significant effectiveness of 100.0 ± 0.0 mortality rates starting at concentrations of 15 mg/mL to 30 mg/mL post 24 h of exposure to the extract (Table 6). Treatment with *A. indica* ethanol extract revealed significant differences in the concentrations of 25 mg/mL and 30 mg/mL with L4 mortality rates of 56.7 ± 1.2 % and 66.7 ± 1.45 %, respectively. Conversely, *A. paniculata* ethanol extract only demonstrated significant mortality at the highest concentration (30 mg/mL) with a mortality rate of 50.00 ± 1.0 (Fig. 3). The LC_{50} for *A. indica, A. paniculata, and L. elliptica* were 23.74 mg/mL, 66.86 mg/mL, and 1.01 mg/mL, respectively. Like water extract, *L. elliptica* ethanol extract was the most toxic, and the order of increasing toxicity is demonstrated as: *A. paniculata < A. indica < L. elliptica.*
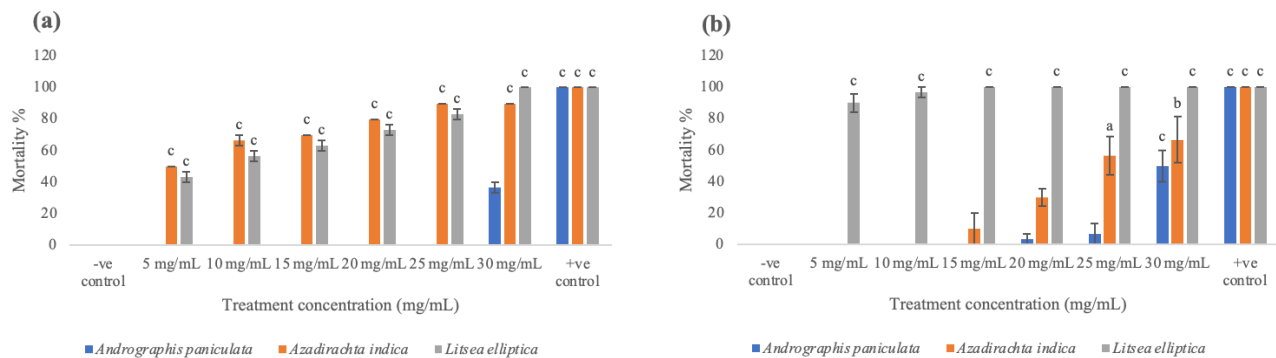
Figure 3. Toxicity effect of *A. paniculata, A. indica* and *L. elliptica* extracts in *C. elegans* in increasing concentration (5, 10, 15, 20, 25 and 30 mg/mL). (a) Percentage mortality of *C. elegans* in water extract (b) Percentage mortality of *C. elegans* in ethanol extract. Data are presented as means ± SEM and are compared to M9 buffer (in water extract) or 1% DMSO (in ethanol extract) as the negative control. Statistical significance was calculated using a one-way ANOVA with Tukey's post hoc multiple comparisons of means and is denoted by superscripts a, b, and c. Superscript a, b, and c denote significance at **$p \leq 0.01$, ***$p \leq 0.001$, **** $p \leq 0.0001$, respectively. n = 10 worms. Statistical significance was shown by comparing the means between the negative control and treatment of each concentration.

Table 6. The percentage mortality rate of water and ethanol extracts of *A. paniculata*, *A. indica*, and *L. elliptica* (Mean ± SEM) and their estimated LC_{50} values, representing the lethal concentration at which 50% mortality occurred, was determined through probit analysis.

Treatments	*A. paniculata* water extract	*A. paniculata* ethanol extract	*A. indica* water extract	*A. indica* ethanol extract	*L. elliptica* water extract	*L. elliptica* ethanol extract
M9 buffer or 1% DMSO (negative control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Levamisole (positive control)	100.0 ± 0.0[c]	100.00 ± 0.0[c]	100.00 ± 0.0[c]	100.00 ± 0.0[c]	100.00 ± 0.0[c]	100.0 ± 0.0[c]
5 mg/mL	0.00 ± 0.00	0.00 ± 0.00	50.00 ± 0.0[c]	0.00 ± 0.00	43.3 ± 0.33[c]	90 ± 0.577[c]
10 mg/mL	0.00 ± 0.00	0.00 ± 0.00	66.70 ± 0.33[c]	0.00 ± 0.00	56.70 ± 0.33[c]	96.7 ± 0.33[c]
15 mg/mL	0.00 ± 0.00	0.00 ± 0.00	70.00 ± 0.0[c]	10.00 ± 1.0	63.3 ± 0.33[c]	100.0 ± 0.0[c]
20 mg/mL	0.00 ± 0.00	3.33 ± 0.33	80.00 ± 0.0[c]	30.00 ± 0.58	73.3 ± 0.33[c]	100.0 ± 0.0[c]
25 mg/mL	0.00 ± 0.00	6.67 ± 0.67	90.00 ± 0.0[c]	56.70 ± 1.2[a]	83.3 ± 0.33[c]	100.0 ± 0.0[c]
30 mg/mL	36.7 ± 0.33	50.00 ± 1.0[c]	90.00 ± 0.0[c]	66.7 ± 1.45[b]	100.00 ± 0.0[c]	100.0 ± 0.0[c]
LC_{50} value	n/a	66.86 mg/mL	10.71 mg/mL	23.74 mg/mL	11.53 mg/mL	1.01 mg/mL

Data are presented as means ± SEM and are compared to M9 buffer (in water extract) or 1% DMSO (in ethanol extract) as the negative control. Statistical significance was calculated using a one-way ANOVA with Tukey's post hoc multiple comparisons of means, denoted by superscripts a, b, and c. Superscript a, b, and c denote significance at **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, respectively. n = 10 worms. Statistical significance compared the means between each concentration's negative control and treatment. LC_{50} for *Andrographis paniculata* water extract could not be determined due to its low toxicity within the concentration range tested.

Discussion

The emergence of anthelmintic resistance to parasitic worms has led to a renewal of interest in using plant-based drugs. This study investigated the anthelmintic activity of three plant species: *A. paniculata*, *A. indica*, and *L. elliptica* tested in the nematode *C. elegans*. This nematode is free-living, easy to culture in the lab, and has a short life cycle that enables rapid experimentation and high-throughput screening. Its transparency allows easy visualization of internal organs and processes, facilitating the observation of secondary metabolites' effects on anatomy and physiological changes, thus providing valuable insights into the mode of action of anthelmintic compounds (Kasah *et al.*, 2024). *C. elegans* has also proven an effective model for screening and discovering lead compounds in various pharmacological research areas, including studies on anthelmintic compounds (Widaad *et al.*, 2022).

Our study revealed the variation in the percentage yield for both extracts in all studied plants. For water extracts, the percentage yields were comparable to other studies ranging from 3.6% to

36.76% for *A. paniculata* (Isha *et al.*, 2013; Kaur and Gupta, 2017), 6.2% to 22.22% for *A. indica* (Raphael, 2012; Hikaambo *et al.*, 2022) and 1.57% to 23.53% for *L. elliptica* (Ji *et al.*, 2012; Sharma *et al.*, 2019), respectively. Similarly, yields for ethanol extracts correspond to previous studies ranging from 4.7% to 11% % for *A. paniculata* (Bardi *et al.*, 2014; Umbara *et al.*, 2016), 1.8% to 5.6% for *A. indica* (Costa *et al.*, 2008; Mahabub *et al.*, 2009) and 10.42% to 13.62% for *L. elliptica* (Pradeepa *et al.,* 2011; Mariani *et al.*, 2021). The observed variations in the extraction yields are due to several factors, such as the nature of the plant species, solvent polarity, extraction methods, and chemical composition differences of the extracts. Additionally, environmental conditions might contribute to the differences in yield, including geography, general health, and the harvest time of the plant species during collection (Tewari *et al.*, 2010; Mumed *et al.*, 2022).

The phytochemical screening of *A. paniculata*, *A. indica*, and *L. elliptica* revealed the presence of alkaloids, steroids, terpenoids, proteins, and carbohydrates in all extracts. Other active compounds were also present in some extracts, including tannins and glycosides in all ethanol and water extracts, respectively, flavonoids in ethanol extracts of *A. indica* and *L. elliptica*, as well as saponins in *A. paniculata* water extract. These secondary metabolites are thought to contribute to the broad therapeutic activities of medicinal plants, including their implication for motility and ovicidal and larvicidal activities against helminths (Cabardo & Portugaliza, 2017). The reported secondary metabolites with anthelmintic activity are tannins, alkaloids, saponins, and flavonoids (Zenebe *et al.*, 2017; Tagoe *et al.*, 2021). The present study estimated the total phenolic and flavonoid contents of both extracts of the plants, whereby *A. indica* and *L. elliptica* exhibited the highest total phenolic and flavonoid contents in water and ethanol extracts, respectively. Phenolic compounds are secondary metabolites often related to antiparasitic effect (Kļaviņa *et al.*, 2023). Some phenolic compounds (flavonoids and terpenes) extracted from different, but mostly plant sources are active against parasitic nematodes such as naringenin, quercetin, and luteolin (Mordvinov *et al.*, 2021; Olmedo-Juárez *et al.*, 2022).

Our study demonstrated a significant dose-dependent decrease in the head-trashing activity of the worms when exposed to increasing concentrations of both extracts of *A. paniculata*, *A. indica*, and *L. elliptica*. Our finding is supported by other studies by Qiao *et al.* (2014), Zenebe *et al.* (2017), and Widaad *et al.* (2022), which showed the efficacy of the plant extracts against different nematode species. The egg-laying activity in this study was also significantly reduced after exposure to both extracts of *A. paniculata*, *A. indica*, and *L. elliptica*. Similar research findings concordantly showed the inhibition of egg-laying and -hatching post-treatment with extracts of the

studied plants in different nematode species (Costa *et al*., 2008; Nawaz *et al*., 2014; Banerjee *et al.*, 2019). Additionally, the current study demonstrated that both extracts *A. indica* and *L. elliptica* significantly increased the percentage of worm mortality in a dose-dependent manner, which generated LC_{50} values of 10.71 mg/mL and 11.53 mg/mL for *A. indica* and *L. elliptica* water extracts, and 23.74 mg/mL and 1.01 mg/mL for *A. indica* and *L. elliptica* ethanol extracts, respectively. Ethanol extract of *A. paniculata* only demonstrated significant mortality at the highest concentration with an LC_{50} of 66.86 mg/mL. Nonetheless, our results show that all the plants had anthelmintic activity. Our results are in line with the findings of Zenebe *et al.* (2017), Mumed *et al.* (2022), and Widaad *et al.* (2022), in which the mortality effect of botanicals was indicated to be dosage (concentration) dependent.

Overall, our data demonstrates exposure to both extracts of *A. paniculata*, *A. indica*, and *L. elliptica*. could induce anthelmintic action by altering the reproduction and locomotion behavior and reducing the nematodes' survival rate. These phytochemical substances may be responsible for the observed anthelmintic activity of plant extracts in the present study. *L. elliptica* exhibits the most potent anthelmintic activity among others, mainly due to the presence of more secondary metabolites in this plant as compared to other plants, as shown in the results above (Table 2 and Table 3). However, further studies are required on the isolation and characterization of its active constituents to validate its anthelmintic action and determine the mechanism of action of the active compound(s) in inducing such an effect.

Conclusion

In conclusion, this study demonstrates the potential of both water and ethanol extracts of *Andrographis paniculata*, *Azadirachta indica,* and *Litsea elliptica* as alternative anthelmintics in treating helminth parasite infection. *Litsea elliptica* shows the most potent anthelmintic activity among other plant species being studied. Phytochemical screening performed confirms the presence of active compounds that might contribute to their anthelmintic action. More research is required to determine the active compound(s) responsible for such an effect. Isolation of the respective compound(s) should be done to evaluate further these plants' anthelmintic efficacy involving the nematodes' various developmental stages. Additionally, determining the mechanisms of action of the plant extracts for its anthelmintic action is crucial to draw a complete picture of the active compound(s) that is/are responsible for such effect.

Acknowledgments

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