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Research article

Assessing the efficacy of *Stemona collinsiae* roots extract against third-stage larvae of *Gnathostoma spinigerum* and its safety profiles

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ABSTRACT

Gnathostomiasis, caused by the advanced third-stage larvae of *Gnathostoma spinigerum*, demands novel treatment avenues. The ethanolic root extract of *Stemona collinsiae* has been postulated to have anthelminthic properties, suggesting its potential as an alternative remedy. In this study, *S. collinsiae* roots were collected, identified, and extracted with 95 % ethanol. The crude extracts were standardized using didehydrostemofoline as chemical marker. The efficacy of the *S. collinsiae* root extract against third-stage larvae of *G. spinigerum* and its toxicity to Wistar rats were evaluated. Both *in vitro* and *in vivo* tests were performed, where the *in vitro* tests assessed the anthelminthic potential of *S. collinsiae* extract against *G. spinigerum* larvae, while *in vivo* tests examined the extract's efficacy against *G. spinigerum* larvae in infected Wistar rats and the efficacy was compared with albendazole. Parallelly, Wistar rats underwent acute and sub-chronic toxicity tests to establish the safe dosage of the extract. The *in vitro* tests showcased significant anthelminthic activity, marked by discernible morphological alterations in the exposed larvae. Acute toxicity proved fatal at 2000 mg/kg body weight, while a dose of 300 mg/kg proved non-toxic. Using the Globally Harmonized Classification System, an LD50 of 500 mg/kg was determined. *In vivo* trials revealed a pronounced decline in *G. spinigerum* larvae among rats treated with the *S. collinsiae* extract. The larvae were also observed to be encysted post-treatment, while those treated with albendazole were not encysted. The *S. collinsiae* extract, with its noteworthy *in vitro* efficacy and favorable safety metrics in rodents, can be a potential anthelminthic agent. The diminished inflammatory response compared to albendazole hints at *S. collinsiae* being a safer gnathostomiasis treatment alternative. The promising results in these preliminary trials warrant a

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deeper investigation to determine the root extract's optimal dosing, suitable delivery methods, and its broader clinical implications.

1. Introduction

Gnathostoma spinigerum is the major species causing gnathostomiasis, a food-borne zoonosis [1]. The prevalence of gnathostomiasis varies widely depending on the geographic location and the population studied. While gnathostomiasis is endemic in some parts of Asia, particularly Thailand, Japan, and China, this disease has also been reported in other parts of the world, including Central and South America and the United States [2–4]. In Thailand, the reported incidence of gnathostomiasis ranged from 0.2 to 4.3 cases per 100,000 population, depending on the region studied. In Japan, the reported incidence was 0.08 cases per 100,000 population. Also, sporadic cases have been reported in many other parts of the world [5].

The life cycle of *G. spinigerum* begins with eggs being released via the feces of definitive hosts. Freshwater copepods serve as the first intermediate host, while the second intermediate hosts include fish or amphibians. Humans can become infected by consuming raw or undercooked meat containing the infective third-stage larvae [1]. As humans are accidental hosts of *G. spinigerum*, the larvae can be found in various tissues, causing intermittent cutaneous migratory swelling, allergic inflammation, and mechanical damage. Fatal eosinophilic meningoencephalitis or tumor formation/eosinophilic abscesses can result in severe cases [3].

In cutaneous gnathostomiasis, treatment is effective with oral albendazole, which binds to the colchicine-sensitive site of beta-tubulin, thus inhibiting their polymerization into microtubules and causing the parasite's death. This drug can reduce the absorptive function of the parasite's intestinal cells, deplete glycogen storage, and decrease ATP production [6,7]. Although oral albendazole is generally considered safe, some side effects are present [8]. These side effects include fever, headache, vomiting, nausea, abdominal pain, liver toxicity, and leukopenia. Additionally, its insolubility in water and poor absorption rate may lead to recurrent gnathostomiasis in some patients [9]. With albendazole's undesirable effects and reduced efficiency, further research is needed to develop alternative treatments for gnathostomiasis.

Stemona collinsiae Craib is an endemic medicinal plant primarily distributed in Southeast Asia, particularly Thailand and Cambodia. This plant is vernacularly named "Non-Tai Yak Lek" or the small roots type of *Stemona,* and is known for its anti-helminthic properties [10,11]. *Stemona collinsiae* also primarily contains the didehydrostemofoline (DSF) alkaloid [12]. Using the DSF compound isolated from *S. collinsiae*, our laboratory has previously discovered that it could kill the infective stage of *G. spinigerum* within 18 days of incubation [unpublished data]. Previous studies have also demonstrated the insecticidal, acetylcholinesterase inhibitory, acaricidal, multidrug resistance modulating, and acetylcholine system-affecting properties of *S. collinsiae* root extract [10].

As the effectiveness of *S. collinsiae* crude extracts on *G. spinigerum* and their toxicological safety, especially in animal models, remains unclear, we aimed to evaluate the efficacy of standardized *S. collinsiae* root extract in controlling the third-stage larvae of *G. spinigerum.* Toxicity tests (acute and sub-chronic) of *S. collinsiae* crude extracts on Wistar rats were performed, following the Organization for Economic Co-operation and Development (OECD) Good Laboratory Practices (GLP) protocols.

2. Materials and methods

2.1. Chemicals

High performance liquid chromatography (HPLC)-grade methanol was obtained from Labscan (Thailand). Deionized water was purified by the Ultra Clear™ system (Siemen Water Technologies Corp.). Ammonium acetate, hydrochloric acid (Merck), Penicillin/Streptomycin (Capricon Scientific Gmbh), RPMI Medium 1640 (Gibco), and all other reagents were of analytical grade if not stated otherwise.

2.2. Plant materials

S. collinsiae samples were collected from Chonburi, Nakhon Ratchasima, and Ubon Ratchathani provinces in Thailand. Identification of *S. collinsiae* was done based on their morphological characteristics [13,14]. The plant roots were thoroughly washed with tap water, chopped into small pieces, and dried at 55 °C for 72 h using a hot air oven. The dried plant roots were subsequently pulverized into a fine powder and stored in an airtight container at room temperature until used.

2.3. S. collinsiae crude extraction

The root powder of *S. collinsiae* was subjected to extraction with 95 % ethanol under sonication for 30 min at room temperature. The resulting supernatant was decanted, and extraction was repeated 3–5 times until exhaustion. The combined supernatant was filtered and subsequently concentrated under reduced pressure using a rotary evaporator (55 °C, 180 mbar). The resulting concentrated supernatant was dried in a boiling water bath, yielding a crude extract.

To formulate the *S. collinsiae* crude extract, *S. collinsiae* extract was dissolved with ethanol and mixed with corn starch at ratio (1:1–2, w/w). The resulting mixture was then heated at 55 °C in a vacuum oven until dry. The dried mixture was grounded and sieved to a fine powder extract. To keep the moisture content below 5 %, the fine powder extract was stored in a desiccator until further use.

Total aerobic microbial, yeast, and mold counts were also done. The DSF content formulated *S. collinsiae* extract was standardized to 2.5 ± 0.25 % w/w.

2.4. LC-MS/MS characterization of major components in crude S. collinsiae extract and quantification of didehydrostemofoline in crude S. collinsiae extract

An UHPLC-DAD-Q-Orbitrap was performed on Vanquish UHPLC system (Thermo Fisher Scientific Inc.) equipped with Thermo Scientific Vanquish- Binary Pump F, Thermo Scientific Vanquish- Split Sampler FT, Thermo Scientific Vanquish- Column Compartment H, and Thermo Scientific Vanquish- Diode Array Detector FG, coupled with Thermo Scientific Orbitrap Exploris™ 120 mass spectrometer. The separation was done on a BDS Hypersil C18 column (50 × 2.1 mm i.d., 3 μm). The mobile phases were (A) 1 mM ammonium acetate in water and (B) methanol. A mobile phase time program was set up with 45 % B in A for 2 min, linear increasing from 45 % to 100 % B in A for 8 min, then 100 % B for 2 min. Before each injection, the column was equilibrated with 100 % A for 2 min. The column temperature was controlled at 25 °C with a constant flow rate of 1.0 mL/min. DAD detection was set at the wavelength of 290 nm. Injection volume setting was 2 μL for all samples.

For mass spectrometer, mass analysis was done in both positive and negative mode using internal mass calibration EASY-IC™. The ion source type was Heated-ESI. Spray voltage setting was static mode with positive ion 3500 V and negative ion 2500 V. Nitrogen gas mode was static with flow setting: sheath gas 50 Arb, Aux gas 10 Arb, and sweep gas 1 Arb. Ion transfer tube temperature was 325 °C. Vaporizer temperature was 350 °C. Full scan mode range was 200–1000 *m/z* with resolution of 60000 and RF Len 70 %. ddMS^2 mode was triggered with intensity threshold of 5.0 x 10^5. The MS^2 parameters were isolation window: 1.5 *m/z*, collision energy type: normalized, orbitrap resolution: 15000, and scan range mode: automatic.

HPLC quantitative analysis of DSF in crude *S. collinsiae* extract was done according to previous work [15] with slight modifications. HPLC was performed on an Agilent 1260 Series (Agilent Technologies) equipped with a 1260 Quat pump VL quaternary pump, 1260 ALS autosampler, 1260 TCC column thermostat, and 1260 DAD VL diode array detector. The separation was done on a Hypersil BDS C18 column (4.6 × 100 mm i.d., 3.5 μm) with a C18 guard column. The elution was performed on an isocratic solvent system using methanol: 1 mM ammonium acetate (50:50, v/v). The flow rate was set at 1.0 ml/min with a controlled temperature of 25 °C. DAD detector was set at the wavelength of 295 nm. The injection volume was 10 μl for every sample and standard.

2.5. In vitro test of S. collinsiae crude extract on G. spinigerum

2.5.1. Preparation of G. spinigerum third-stage larva

The third-stage larvae (L3) of *G. spinigerum* were harvested from naturally infected eel livers. The liver tissue was initially excised and homogenized in a 0.7 % acid-pepsin and 1 % hydrochloric acid solution using an electronic blender. The resulting homogenate was incubated for 1 h at 37 °C in a water bath with continuous stirring. After incubation, the homogenate was allowed to settle, and the supernatant was removed. The sediment was thoroughly washed several times with tap water to remove debris and distributed into multiple Petri dishes. The Petri dishes were then observed under an Olympus SZX7 stereomicroscope (Olympus Corporation, Tokyo, Japan) to detect and collect the L3 of *G. spinigerum*. The collected live L3 were washed thoroughly with 0.85 % normal saline solution (NSS).

2.5.2. In vitro test

The L3 of *G. spinigerum* was cultured in RPMI medium supplemented with 1 % penicillin-streptomycin to prevent bacterial and fungal contamination. The effectiveness of *S. collinsiae* root extract against *G. spinigerum* larvae was evaluated at five concentrations ranging from 10 to 50 mg/ml, while albendazole was also tested at five concentrations ranging from 1 to 10 mg/ml. Five replicates were conducted for each concentration. A control group (culture media without treatment with *S. collinsiae* or albendazole) containing the L3 larvae was also set up. The culture medium was replaced every two days to maintain a constant concentration and prevent the build-up of toxins secreted from the larvae. The LC_{50} values were then calculated for the concentration that effectively killed *G. spinigerum* larvae.

2.5.3. Scanning electron microscopy

Scanning electron microscopy was employed to examine the morphological features of the control and *S. collinsiae*-treated deceased larvae. The survival drug-treated immature stage (STIM) specimens were examined under a scanning electron microscope (SEM) (JEOL JSM-6610LV, Japan) with an acceleration voltage of 15 kV and were compared to untreated third-stage larvae used as controls. Five larvae undergoing treatment were fixed in a solution of 2.5 % glutaraldehyde in 0.1 M sucrose-phosphate buffer (SPB) for 1 h. The fixed larvae were washed in 0.1 M SPB and then treated with 1 % osmium tetroxide in 0.1 M SPB for 1 h. Following this, all larvae underwent dehydration using varying ethanol concentrations before drying in liquid CO_2 utilizing a critical point dryer machine (HITACHI HCP-2, Japan). Each larva was then mounted onto an aluminum stub with double-sided carbon tape and coated with a 20 nm gold film utilizing a sputter coater (EMITECH K550, UK).

2.6. Acute and sub-chronic toxicity study

2.6.1. Acute toxicity study

The acute oral toxicity study of *S. collinsiae* ethanolic extract followed the OECD guideline for testing chemicals (OECD 423: Acute

Oral Toxicity Acute Toxic Class Method) [16]. Female Wistar rats (8 weeks) weighing 200 ± 20 g were obtained from the National Animal Center, Salaya, Mahidol University. These rats were housed in a controlled environment with a temperature of 22 ± 3 °C and relative humidity of less than 70 %. The experimental rats underwent at least a 5-day acclimatization period under the same environmental conditions, and they fasted for 12 h before the start of the experiments. The experiments were conducted with a stepwise procedure with 3 animals per step. Briefly, a dose of 2000 mg/kg of *S. collinsiae* extract was first administered to 3 rats, followed by a 300 mg/kg dose with another 3 rats. Subsequently, another 3 rats were administered with the same dose of *S. collinsiae* extract. Standardized *S. collinsiae* extract containing DSF 6.25 % w/w was administered by No. 18 stomach tube gavage in a single dose.

After administration, the rats were closely observed for signs of behavioral alteration, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic, and central nervous systems, somatomotor activity, food and water intake, and changes in body weight. In the first 4 h, the rats were observed at 30-min intervals. Mortality was checked daily for a 14-day duration. The weight of each rat was recorded throughout, and the mean body weights for each group were calculated. At the end of 14 days, the animals fasted overnight, and a blood sample was collected and centrifuged at 3000 rpm for 10 min. Blood chemistry and hematology results were conducted at The National Laboratory Animal Center (Nakhon Pathom, Thailand). A gross necropsy was performed on all test animals. The lethal dose of 50 % (LD_{50}) cut-off value for the *S. collinsiae* extract was determined following the guideline principle (OECD 423) [16].

2.6.2. Sub-chronic toxicity study

The sub-chronic toxicity study followed the OECD guideline for testing chemicals (OECD TG 408: Repeated Dose 90-day Oral Toxicity Study in Rodents) [17]. Male Wistar rats (250 ± 25 g body weight) and female Wistar rats (200 ± 20 g body weight) were obtained from the National Animal Center, Salaya, Mahidol University. The experiment was carried out with doses of 125, 250, and 500 mg/kg body weight of *S. collinsiae* root extract, where all rats were fed daily for 90 days. A satellite group with a 500 mg/kg dose was observed to detect the recovery from toxic effects. The animal model and the number of animals required for the study are presented in Table 1. Throughout the 90-day study, all animals were monitored daily for signs of toxicity, changes in behavior, food and water intake, and body weight. The body weight of Wistar rats in the sub-chronic toxicity study was measured each week and demonstrated. Following the final dose of standardized *S. collinsiae* crude extract, animals fasted overnight, and blood samples were collected 24 h later. Surviving animals were then subjected to necropsy, and their organs were collected for pathological study. The sub-chronic toxicity test was conducted at the National Laboratory Animal Center (Nakhon Pathom, Thailand), and the rat organs (liver, spleen, kidney, heart, brain, testis, and ovary) were stored in 10 % formaldehyde and sent to the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University. Tissue samples from each organ were cut into small pieces, fixed with formalin, and subjected to routine histology techniques, including trimming, embedding in paraffin, sectioning into 5 μm thickness, mounting on glass slides, and staining with hematoxylin and eosin (H&E). The histopathology slides were examined under a light microscope, and the histopathological service unit performed the process at the Phramongkutklao Hospital in Bangkok, Thailand. Blood chemistry parameters include Glucose (GLU), blood urea nitrogen (BUN), creatinine (CREA), cholesterol (CHOL), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), while red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), white blood cell (WBC) were the hematological parameters.

2.7. In vivo efficacy test of S. collinsiae crude extract in wistar rat

2.7.1. Animals

Male Wistar rats (250 ± 50 g body weight) were housed in controlled environmental conditions (temperature, 22 ± 3 °C; relative humidity less than 70). Before the experiments, the experimental rats were housed under the above conditions for 5 days of climatization and fasted for 12 h.

2.7.2. Efficacy study

All Wistar rats were orally infected with 20 L3 of *G. spinigerum,* where the larvae were collected and prepared as previously

Table 1
Animal model and the number of animals required.

Group	Substance	Wistar rats		Dose (mg/kg)	Duration of treatment
		Males	Females		
High dose	*S. collinsiae* crude extract 500 mg/kg containing DSF 31.25 mg/kg	10	10	500	90 days daily
Medium dose	*S. collinsiae* crude extract 250 mg/kg containing DSF 15.625 mg/kg	10	10	250	90 days daily
Low dose	*S. collinsiae* crude extract 125 mg/kg containing DSF 7.8125 mg/kg	10	10	125	90 days daily
Control	Distilled water	10	10	1 mL	90 days daily
Satellite	*S. collinsiae* crude extract 500 mg/kg containing DSF 31.25 mg/kg	5	5	500	90 days daily and then kept without treatment for 14 days

mentioned. Five days after infection with L3 of *G. spinigerum*, Wistar rats (three rats per group) were treated three times daily with crude extract of *S. collinsiae* at 500 mg/kg, or treated with 20 mg/kg albendazole once daily or treated three times with corn starch (control group). At the end of 21 days, the Wistar rats were euthanized, and the L3 of *G. spinigerum* was observed in the tissues of the rats through the tissue compression technique under a light microscope. The larvae recovered were counted, and percent reduction was calculated– [(worms found in treated rats/worms found in untreated rats) x 100] [18].

2.7.3. Histopathological study

The L3 of *G. spinigerum* found in the Wistar rat tissues was prepared for pathological study to evaluate host–parasite interaction regarding their general microscopic appearances. Fixed tissues were subjected to standard tissue processing, which included dehydration with graded ethanol, infiltration with graded paraffin, embedding with absolute paraffin, sectioning into 5 μm thickness, and staining with hematoxylin and eosin (H&E). The sections were examined under a light microscope focusing on histopathological changes around parasite interfacing area, particularly fibrosis and white blood cell accumulation (including macrophage, giant cell, eosinophil, lymphocyte, and neutrophil). These changes were scored using an H-score (0–300; multiplication of a severity score [0–3;

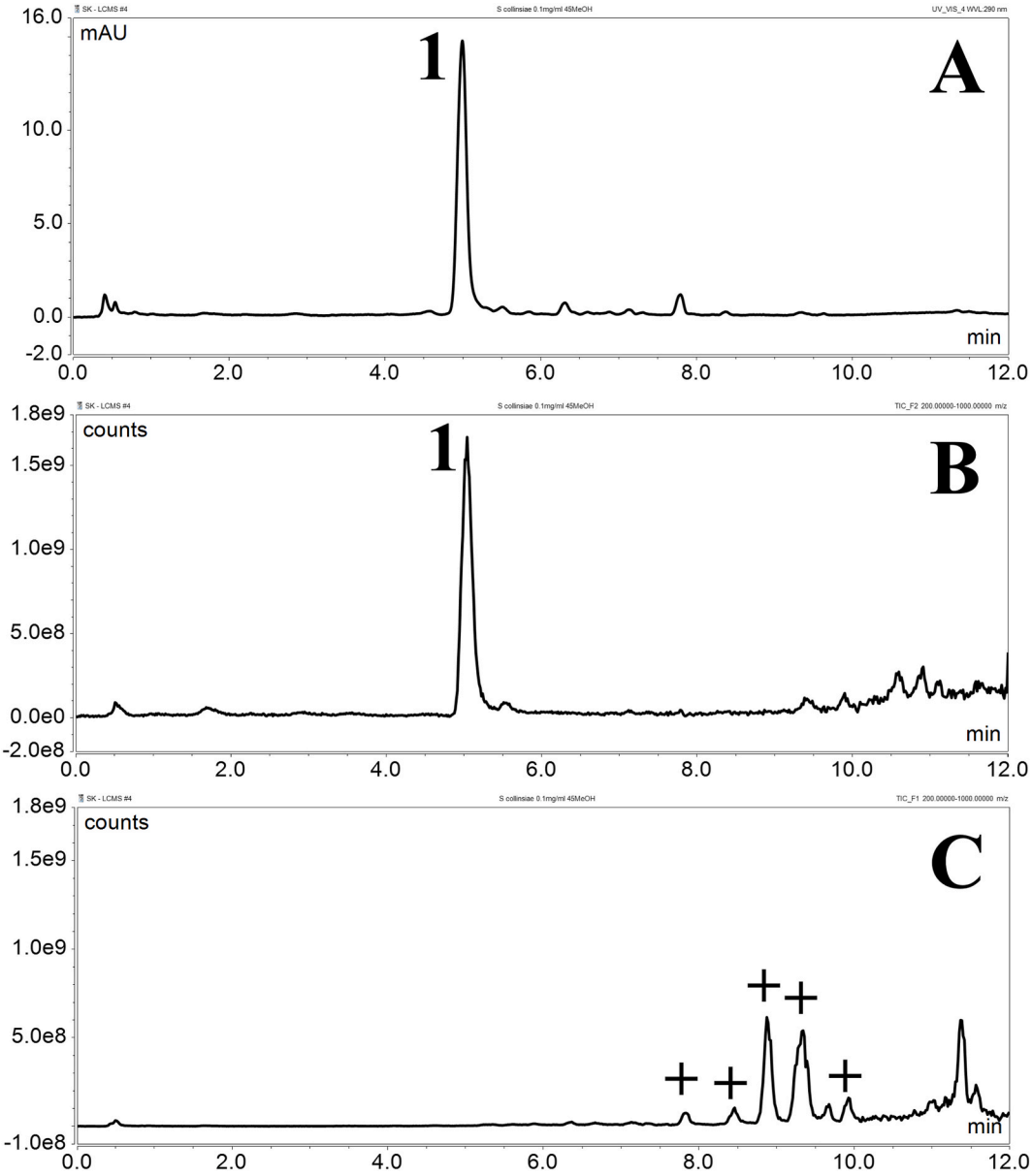

Fig. 1. LC-DAD-MS chromatograms of *S. collinsiae* extract detected at UV 290 nm (A) full scan in positive mode (B) and full scan in negative mode (C). Peak identification: (1) didehydrostemofoline. Peak indicated with + showing the presence of stilbenoids.

0–absent, 1–mild, 2–moderate, and 3–severe] and an extent of distribution [0%–100 %]).

2.7.4. Immunohistochemical study

To characterize host–parasite interaction in terms of specific immunological responses, an immunohistochemical study was conducted. Anti-inflammatory cytokines (transforming growth factor [TGF]-β, interleukins [IL]-4, and IL-10) and pro-inflammatory cytokines (IL-1β, tumor necrotic factor [TNF]-α, interferon [INF]-γ, and neutrophil elastase) were used as primary antibodies (MyBioSource, USA). Sections were subjected to deparaffinization with xylene and hydration with ethanol, followed by microwave-induced antigen retrieval in citrate buffer (pH 6). They were further treated with 1 % v/v of hydrogen peroxide in methanol and 2 % v/v of bovine serum albumin (BSA; [EMS, USA]), respectively. Each primary antibody, polymer HRP anti-mouse/rabbit labeling (DAKO, Denmark), and diaminobenzidine visualization (DAKO, Denmark) were applied to the sections and then counter-stained with hematoxylin. The immunolocalization was examined under a light microscope.

Each cytokine's expression level was measured using the H-score (percentage area of expression × intensity score) [19]. ImageJ software was used to quantify the immuno-distribution area in terms of percentage. Color images (4 images/group) of tissue interfacing areas were captured at 400× magnification. A threshold mode measured the immunolabeled area to obtain the percentage of

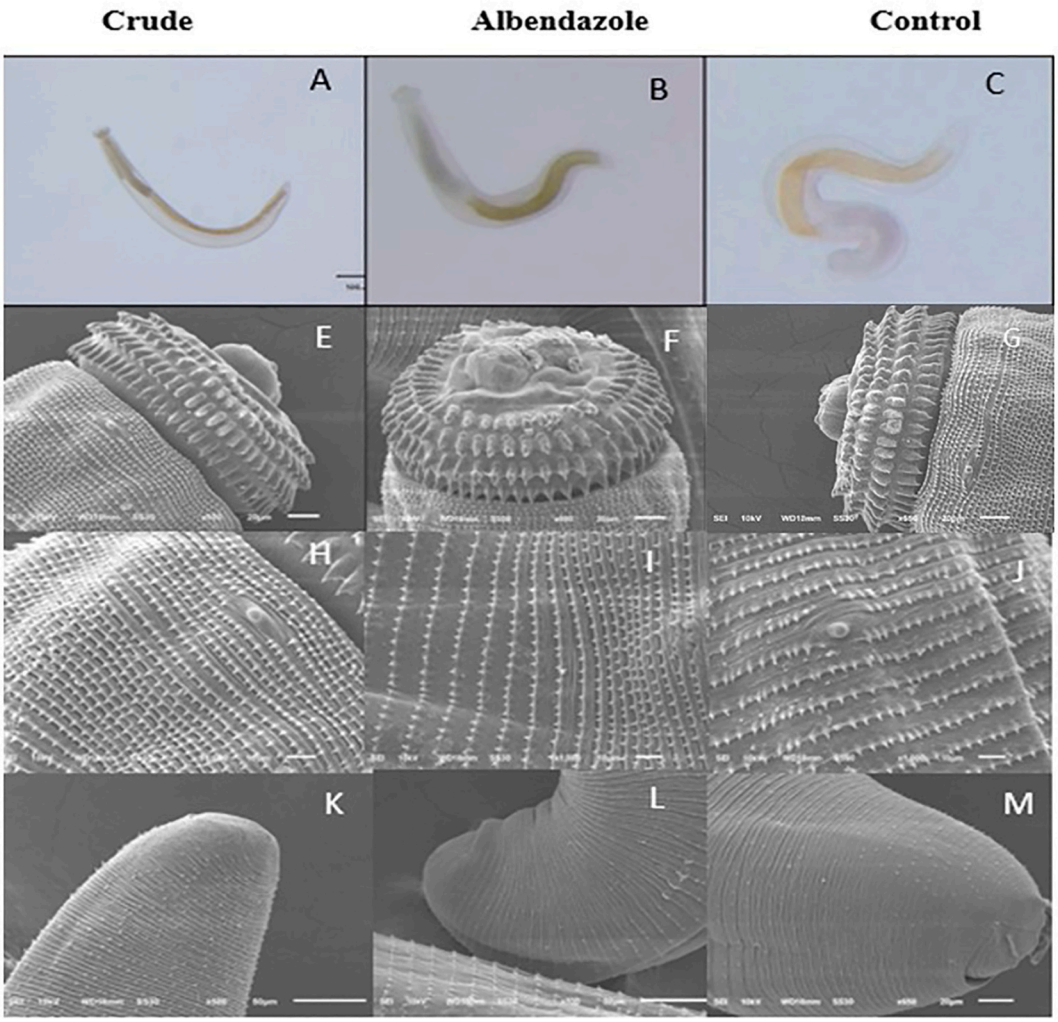

Fig. 2. Morphological changes observed in L3 *G. spinigerum*. Light microscopy photograph of the third stage larvae of *G. spinigerum*: (A) worm treated with *S. collinsiae* crude extract; (B) worm treated with albendazole; (C) Untreated worm. Scanning electron micrograph of the third stage larvae of *G. spinigerum*: (E) cephalic bulb of the third stage larvae of *G. spinigerum* treated with *S. collinsiae* crude extract; (F) cephalic bulb of the third stage larvae of *G. spinigerum* treated with albendazole; (G) cephalic bulb of untreated third stage larvae of *G. spinigerum*; (H) spines in the middle region of the body of the third stage larvae of *G. spinigerum* treated with *S. collinsiae* crude extract; (I) spines in the middle region of the body of the third stage larvae of *G. spinigerum* treated with albendazole; (J) spines in the middle region of the body of untreated worm; (K) posterior part of the third stage larvae of *G. spinigerum* treated with *S. collinsiae* crude extract; (L) posterior part of the third stage larvae of *G. spinigerum* treated with albendazole; (M) posterior part of untreated worm.

positive pixels after converting images to grayscale. The intensity was scored from 0 to 3 and was classified into four grading scales: 0–negative staining, 1–low-intensity staining, 2–moderate-intensity staining, and 3–high-intensity staining.

2.8. Statistical analysis

The results are presented as mean ± standard deviation (SD), and the student's t-test was performed to compare the means of each independent group, with statistical significance defined as *P*-value <0.05.

Ethical approval

The animal research protocols, including the acute toxicity test (Protocol No. RA 2017-15) and sub-chronic toxicity test (Protocol No. RA 2017-47), were approved by the National Laboratory Animal Center Animal Care and Use Committee (NLAC-ACUC) at Mahidol University. The efficacy study (Protocol no. 028–2020) was approved by the Faculty of Tropical Medicine Institute Animal Care and Use Committee (FTM-IACUC) at Mahidol University.

3. Results

3.1. Preparation and standardization of S. collinsiae crude extract

S. collinsiae root samples were collected, identified, and used to prepare crude ethanolic extract. The variation of didehydrostemofoline in each production batch ranged from 5 to 7 %. To ensure consistency, the *S. collinsiae* extract was standardized with a didehydrostemofoline content of 6.25 % w/w. LC-DAD-MS characterization [Fig. 1(A–C)] of the *S. collinsiae* extract showed didehydrostemofoline as the major component, along with trace amounts of stilbenoids. Furthermore, assessments of total aerobic microbial count and total yeast and mold count revealed no microbial growth, affirming the sterility of the products.

3.2. In vitro effect of S. collinsiae crude extract and albendazole against G. spinigerum

The crude ethanolic extract of *S. collinsiae* root containing DSF 6.5 % w/w was compared with albendazole and tested for its effectiveness against advanced L3 of *G. spinigerum in vitro*. Upon exposure to the extract, morphological changes were observed, including body and gastrointestinal tract shrinkage. When the larvae died, their bodies appeared stretched [Fig. 2(A–M)]. The LC_{50} value, determined by a logarithmic regression equation based on the concentration of the *S. collinsiae* extract and the 5-day mortality rate of *G. spinigerum*, was found to be 15.77 mg/mL. In comparison, the L3 of *G. spinigerum* was not affected by albendazole (Table 2).

3.3. Acute toxicity study

The acute toxicity test was conducted as described in the OECD 423 guideline. Using the initial dose (2000 mg/kg body weight), the three rats convulsed 30 min after administration and died within an hour. In contrast, no mortality or observable toxicity signs were seen when the dose was reduced to 300 mg/kg body weight. There was also no change in coat skin/fur, eyes, pupil size, lacrimation, respiration, mucous membrane, lymph node, social interaction, posture mobility, and activity (Table 3). Additionally, the gross necropsy of all animals showed no toxic signs. Based on the acute toxicity tests, the LD_{50} cut-off value of *S. collinsiae* crude extract in Wistar rats after a single oral treatment was determined to be 500 mg/kg body weight, which fell into category 4 of the Globally Harmonized Classification System (GHS).

3.4. Sub-chronic toxicity study

The sub-chronic toxicity study was carried out with doses of 125, 250, and 500 mg/kg body weight of *S. collinsiae* root extract. No deaths and observable signs of toxicity were recorded, indicating that the rats tolerated the 90-day oral administration of *S. collinsiae* extract well across the three doses. Moreover, no differences in food intake and body weight were observed between male and female rats challenged with *S. collinsiae* extract and the control group.

Comparing the blood chemistry and hematological parameters among the dose-treatment and control groups (Tables 4), no toxicity signs were observed when compared with the control group except for glucose and cholesterol levels. For glucose, a significant dose-related effect was observed, with increased treatment dose associated with increased glucose levels. Similarly, a dose-related significant increase in cholesterol levels was observed in both male and female rats. However, we observed reduced glucose and cholesterol

Table 2
Effect of *S. collinsiae* crude extract and albendazole against *G. spinigerum* (n = 5).

Substance	LC_{50}	Duration (Days)	Result
S. collinsiae crude extract	15.77 mg/mL	5	Death
Albendazole	3 mg/mL	21	Active larvae
Control	–	30	Active larvae

Table 3
Signs, symptoms, and mortality of the Wistar rats for the acute toxicity test.

Group	Dose (mg/kg)	No. of rats used	No. of death rat	Sign and Symptom
1	2000	3	3	Convulse, Coat skin/fur
2	300	3	0	No toxicity signs
3	300	3	0	No toxicity signs

levels in the satellite and control groups, respectively, indicating that the rats can recover from the treatment.Table 5 and 6

Examination of internal organs across the groups revealed No Observed Adverse Effect Concentration (NOAEC) for the sub-chronic toxicity of the formulated standardized extract (500 mg/kg body weight). Although there was an increase in the weight of the liver in both male and female rats, a recovery of increased liver weight was also observed in the satellite group. Furthermore, no gross abnormalities were observed in the internal organ's color, texture, size, or shape.Table 7

3.5. *In vivo efficacy test of S. collinsiae crude extract in wistar rat*

In vivo tests revealed that the advanced L3 of *G. spinigerum* was found in the adipose and subcutaneous tissue, forelegs, and hind legs of Wistar rats. The percentage of larvae recovered was 34.44 ± 5.09, 6.67 ± 2.89, and 67.92 ± 2.5 from treatment with *S. collinsiae* root extract, treatment with albendazole, and the control group, respectively (Table 8). Intriguingly, larvae encystment was detected in rats treated with *S. collinsiae* root extract and control, whereas larvae without encystation were detected in rats treated with albendazole. The treatment group has shown a significantly lower infection rate compared to the control group, approximately 60–80 % of individuals in the control group being infected with *G. spinigerum*.

3.6. *General histopathological changes in larval migration tissues*

Histopathological alterations demonstrated that granulomatous lesion was induced by gnathostomiasis larval migration, as shown in Fig. 3. It was noted that granuloma with fibrotic formation was observed only in the group treated with *S. collinsiae* root extract (Fig. 3A–i; *) and control (Fig. 3A–ii; *), while the group treated with albendazole presented non-fibrotic granuloma (Fig. 3A–iii; #). White blood cell accumulation was predominately found in the parasite and host tissue interfacing area when fibrotic formation was observed. Pathological H-score indicated that all groups had identical alteration on 3 days post-treatment, while the group treated with *S. collinsiae* extract had a significantly higher score than the control group on 10 days post-treatment [Fig. 3B(i-ii)].

3.7. *Pro- and anti-inflammatory responses in larval migration tissue*

Fig. 4(A–B) presents the immunohistochemical results to demonstrate host and parasite interaction on cytokine induction. In 3 days, post-treatment, the expressions of IL-1β, INF-γ, IL-10, and TGF-β were significantly increased in the control rats compared to those treated with *S. collinsiae* extract and albendazole. IL-4 expression was significantly reduced in rats treated *S. collinsiae* than in the control group. For neutrophil elastase, the rats treated with *S. collinsiae* and the control group had significantly higher expression levels than the albendazole-treated group. However, all groups had identical TNF-α expression levels.

Interestingly, pro- and anti-inflammatory cytokine ratios demonstrated that rats treated with *S. collinsiae* extract (4.37) had the highest ratio compared with control rats (1.29) and rats treated with albendazole (1.09) on 3 days post-treatment (Fig. 4C). At 10 days post-treatment, only IL-1β expression levels were significantly higher in the treatment group than in the control group. The rest of the cytokines were at similar levels.

4. Discussion

This study delved into the potential of the crude extract from *S. collinsiae* as a therapeutic alternative against gnathostomiasis while

Table 4
Blood chemistry of male-Wistar rats in sub-chronic toxicity study of *S. collinsiae* root extract.

Parameters	Unit	Reference Value	Distilled water	125 mg/kg	250 mg/kg	500 mg/kg	Satellite
GLU	**(mg/dl)**	70–208	330.3 ± 53.3	345.7 ± 55.7	354.8 ± 42.9	328.4 ± 24.9	347.1 ± 36.4
BUN	**(mg/dl)**	12.30–20.10	16.8 ± 1.6	19.0 ± 1.6*	16.8 ± 2.7	19.5 ± 2.8*	15.5 ± 1.2
CREA	**(mg/dl)**	0.2–0.5	0.3 ± 0.02	0.3 ± 0.04	0.3 ± 0.03	0.3 ± 0.02	0.3 ± 0.02
CHOL	**(mg/dl)**	37–85	91.4 ± 15.8	102.3 ± 18.1	119.4 ± 16.5*	168.5 ± 23.6*	98.2 ± 15.1
TG	**(mg/dl)**	20–114	118.2 ± 25.9	117.5 ± 29.5	129.3 ± 49.9	151.1 ± 38.9 *	123.6 ± 33.4
AST	**(U/l)**	74–143	108.7 ± 33.3	125.4 ± 45.3	97.3 ± 29.7	80.0 ± 17.2*	103.2 ± 49.3
ALT	**(U/l)**	18–45	58.0 ± 24.0	85.1 ± 31.2*	75.6 ± 29.3	68.8 ± 21.6	75.8 ± 28.4
ALP	**(U/l)**	62–230	67.9 ± 10.0	70.7 ± 13.9	70.1 ± 11.5	66.7 ± 7.5	57.4 ± 9.8 *

Significant differences were compared with the control group, *P < 0.05. GLU: Glucose; BUN: Blood Urea Nitrogen; CREA: Creatinine; CHOL: Cholesterol; TG: Triglycerides; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

Table 5
Blood chemistry of female- Wistar rats in sub-chronic toxicity study of *S. collinsiae* root extract.

Parameters	Unit	Reference Value	Distilled water	125 mg/kg	250 mg/kg	500 mg/kg	Satellite
GLU	**(mg/dl)**	76–175	111.2 ± 39.3	176.2 ± 26.2*	193.7 ± 57.2 *	237.9 ± 27.9 *	209.2 ± 41.9 *
BUN	**(mg/dl)**	13.5–24.5	16.8 ± 2.6	17.8 ± 2	18.4 ± 2.3	18.4 ± 3.4	15.8 ± 1.7
CREA	**(mg/dl)**	0.2–0.6	0.4 ± 0.03	0.4 ± 0.03	0.3 ± 0.04 *	0.3 ± 0.04 *	0.4 ± 0.04
CHOL	**(mg/dl)**	24–73	114 ± 22	131 ± 21.1	151.5 ± 22.3 *	177.6 ± 24.7 *	118.3 ± 11.6
TG	**(mg/dl)**	14–46	72.6 ± 14.2	75.9 ± 16.6	84.8 ± 18.2	101.7 ± 14.5 *	73.7 ± 13.3
AST	**(U/l)**	65–203	84.9 ± 9.9	79.6 ± 8.7	78.5 ± 8	85.1 ± 18.2	72.1 ± 3 *
ALT	**(U/l)**	16–48	41.3 ± 6.3	42.4 ± 12.1	49.8 ± 10.2*	58.7 ± 15.2 *	35.6 ± 5.4 *
ALP	**(U/l)**	26–147	34.2 ± 5.7	43.3 ± 7.2 *	36.8 ± 5.2	37.8 ± 4	33.6 ± 10.3

Significant differences were compared with the control group, *P < 0.05. GLU: Glucose; BUN: Blood Urea Nitrogen; CREA: Creatinine; CHOL: Cholesterol; TG: Triglycerides; AST: Aspartate Aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

Table 6
Hematological parameter of male-Wistar rats in sub-chronic toxicity study of *S. collinsiae* root extract.

Parameters	Unit	Reference Value	Distilled water	125 mg/kg	250 mg/kg	500 mg/kg	Satellite
RBC	(10^6/μl)	7.27–9.65	9.57 ± 0.34	9.8 ± 0.2	9.54 ± 0.38	9.68 ± 0.32	9.33 ± 0.22
HGB	(g/dl)	13.7–17.6	17.02 ± 0.37	16.35 ± 0.44*	16.10 ± 0.53*	16.06 ± 0.37*	16.04 ± 0.37*
HCT	(%)	39.6–52.5	52.52 ± 1.87	51.76 ± 1.31	50.16 ± 1.81*	51.49 ± 1.35	48.12 ± 1.32*
MCV	(fl)	48.9–57.9	54.93 ± 1.62	52.83 ± 1.26*	52.59 ± 0.97*	53.21 ± 1.03*	51.66 ± 1.62*
MCH	(pg)	17.1–20.4	17.81 ± 0.59	16.68 ± 0.34*	16.87 ± 0.19*	16.59 ± 0.42*	17.18 ± 0.48*
MCHC	(g/dl)	32.9–37.5	32.44 ± 0.7	31.59 ± 0.27*	32.10 ± 0.41	31.20 ± 0.33*	33.34 ± 0.17 *
RDW	%	11.1–15.2	23.07 ± 0.84	24.18 ± 0.85	24.01 ± 0.78	24.18 ± 0.45	24.38 ± 0.9
PLT	(10^3/μl)	638–1177	933.6 ± 56.16	1005.3 ± 61.7*	1106.90 ± 106.75*	1188.20 ± 83.72*	1112.80 ± 127.37*
MPV	(fl)	6.2–9.4	7.16 ± 0.24	7.09 ± 0.21	7.27 ± 0.24	7.17 ± 0.27	6.90 ± 0.16 *
WBC	(10^3/μl)	1.96–8.25	6.73 ± 0.82	10.00 ± 1.71*	7.88 ± 1.34 *	8.76 ± 1.4 *	8.00 ± 2.08

Significant differences were compared with the control group, *P < 0.05. RBC: Red blood cells; HGB: Hemoglobin; HCT; Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red cell distribution width; PLT: Platelets; MPV: Mean platelet volume; WBC: White blood cells.

Table 7
Hematological parameter of female-Wistar rats in sub-chronic toxicity study of *S. collinsiae* root extract.

Parameters	Unit	Reference Value	Distilled water	125 mg/kg	250 mg/kg	500 mg/kg	Satellite
RBC	(10^6/μl)	7.07–9.03	9.35 ± 0.68	9.27 ± 0.39	8.81 ± 0.3*	8.80 ± 0.33 *	8.54 ± 0.33 *
HGB	(g/dl)	13.7–16.8	17.48 ± 1.14	17.17 ± 0.69	16.49 ± 0.38 *	16.11 ± 0.54 *	15.94 ± 0.63 *
HCT	(%)	37.9–49.9	55.23 ± 3.75	53.36 ± 2.56	51.42 ± 1.19 *	50.93 ± 1.73 *	48.10 ± 1.95*
MCV	(fl)	49.9–58.3	59.1 ± 0.98	57.60 ± 1.47*	58.4 ± 0.82	57.90 ± 0.87 *	56.36 ± 1.19 *
MCH	(pg)	17.8–20.9	18.71 ± 0.38	18.55 ± 0.47	18.72 ± 0.43	18.31 ± 0.3 *	18.7 ± 0.46
MCHC	(g/dl)	33.2–37.9	31.66 ± 0.24	32.20 ± 0.35 *	32.07 ± 0.41 *	31.63 ± 0.13	33.16 ± 0.38 *
RDW	%	10.5–14.9	19.84 ± 1.24	20.17 ± 0.68	19.23 ± 1.22 *	20.21 ± 0.74	20 ± 0.86*
PLT	(10^3/μl)	680–1200	918.9 ± 74.25	934.8 ± 66.9	969.5 ± 47.86	1022.10 ± 44.02 *	990.4 ± 131.75
MPV	(fl)	6.2–9.8	7.05 ± 0.21	6.9 ± 0.14	7.11 ± 0.26	6.86 ± 0.16*	7.04 ± 0.21
WBC	(10^3/μl)	1.13–7.49	6.64 ± 1.02	7.31 ± 1.13	7.46 ± 1.47	6.8 ± 1.02	4.96 ± 0.69 *

Significant differences were compared with the control group, *P < 0.05. RBC: Red blood cells; HGB: Hemoglobin; HCT; Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red cell distribution width; PLT: Platelets; MPV: Mean platelet volume; WBC: White blood cells.

Table 8
Mean percentage of worm found, worm reduction, and location in Wistar rats.

Group	% Worm found	Location	feature	% Worm reduction
Control	67.92 ± 2.5	Body part, forelegs, and hind legs especially in adipose tissue	Encystment	–
S. collinsiae	34.44 ± 5.09	Subcutaneous tissue in Body part, forelegs, and hind legs	Encystment	51.50 %
Albendazole	6.67 ± 2.89	Muscle and adipose tissue	No encystment and movement	85.17 %

evaluating its safety using acute and sub-chronic toxicity assays. *In vitro* evaluations revealed that the ethanolic root extract of *S. collinsiae* exhibited potent anthelminthic capabilities against the advanced third-stage larvae of *G. spinigerum*. Notable morphological alterations in treated larvae, such as the body and gastrointestinal tract shrinkage, indicate potential disruptions to their standard physiological processes. Furthermore, the accompanying larval death, typified by body stretching, significantly impacts their nervous and muscular systems, potentially leading to paralysis and eventual fatality. These findings underscore the crude ethanolic

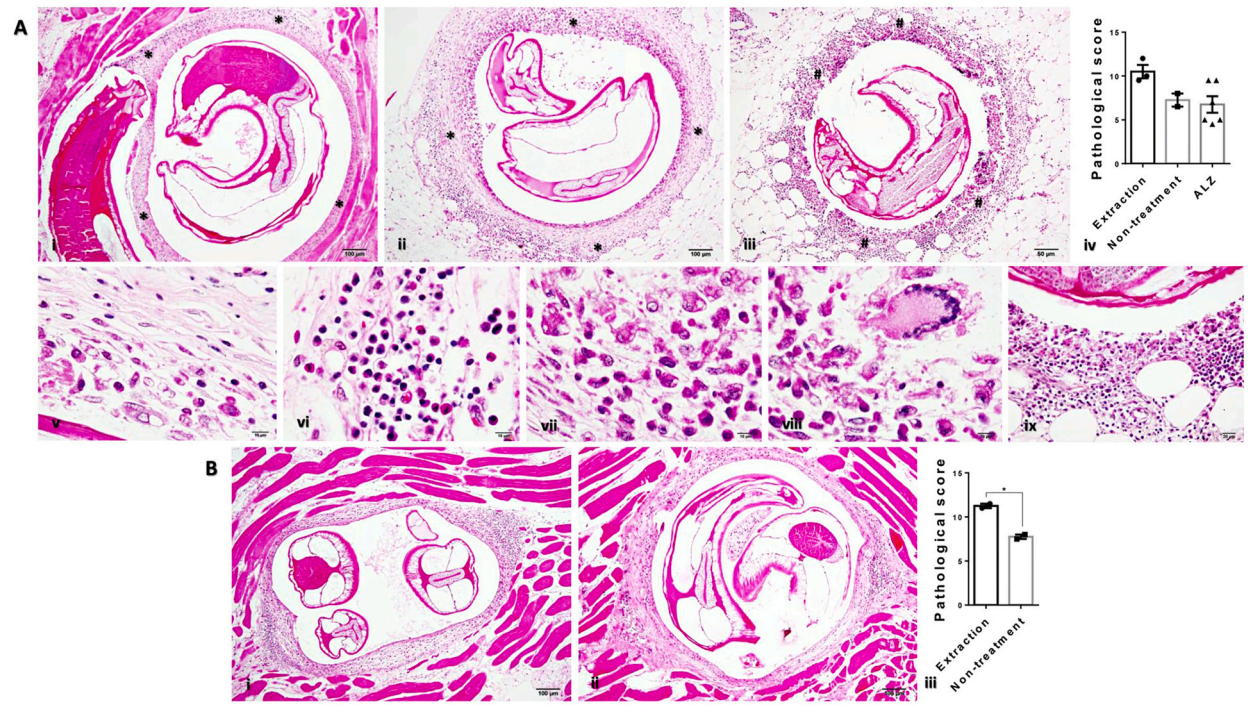

Fig. 3. Histopathological appearances and scores in L3 gnathostomiasis rats with any treatment: (**A**) 3 days post treatment; fibrotic granuloma in rats-treated with extraction (**i**) and non-treated rats (**ii**) exhibited the tissue interfacing area between host and parasite with thickening fibrosis formation (**v**) and the accumulation of several types of white blood cells majority with macrophage, neutrophil, and lymphocyte (**vi-vii**) and minority with eosinophil (**vi**) and giant cell (**vii**). Non-fibrotic granuloma in rats-treated with albendazole (**iii**) demonstrated only white cells infiltration on tissue interfacing area (**ix**). Bar graph of pathological score at day 3 post treatment (**iv**). (**B**) 10 days post treatment; fibrotic granuloma in rats-treated with extraction (**i**) and non-treated rats (**ii**) characterized the tissue interfacing area deposition with white cells majority with eosinophil, macrophage and giant cell. Bar graph of pathological score at day 10 post treatment (**iii**). * p-value <0.05.

extract's potential as a novel gnathostomiasis treatment.

S. collinsiae samples were collected, and an ethanolic root extract was prepared and analyzed for its DSF content, serving as a quality marker [15]. Stilbenoids were detected in trace quantities using negative mode in mass spectrometer with characteristic UV spectrum. However, their identification via mass spectra was hindered by the presence of similar compounds with the same molecular weight. Nonetheless, they are recognized as phytoalexins with pronounced antimicrobial properties [20]. To ensure consistency, the *S. collinsiae* extract was standardized to contain 6.25 % w/w didehydrostemofoline to reduce discrepancies in DSF content among different production batches.

Safety assessment, a critical facet of our investigation, employed acute and sub-chronic toxicity trials on Wistar rats. Initial acute toxicity tests illuminated potential adverse reactions at the 2000 mg/kg body weight dosage. An intriguing observation was the neurostimulant effect of the *S. collinsiae* extract, causing heightened alertness in the rats - an avenue warranting further exploration. Meanwhile, a markedly lower 300 mg/kg dose exhibited no toxic effects. Utilizing the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), an LD_{50} threshold of 500 mg/kg body weight was obtained, placing the *S. collinsiae* extract under category 4 [21]. It's crucial to note that this value serves mainly as a classification threshold rather than a precise lethal dose derived from our trials.

Subsequent sub-chronic toxicity evaluations at the 500 mg/kg concentration disclosed no aberrant findings in the subjects, where negligible treatment-related behavioral changes were observed. Although all treated rats maintained typical body weight, a standout observation was the significant weight reduction in male rats subjected to the 500 mg/kg dose. This anomaly may allude to potential metabolic shifts triggered by the extract, such as reduced food absorption or increased energy expenditure. However, clinical toxicity remained absent, bolstering the extract's safety profile at this dosage and enhancing its potential against *G. spinigerum* infections. We also analyzed various biochemical and histological markers to understand the extract's impact on internal organ functions. Hematological parameters, critical indicators of potential toxic effects, revealed no significant divergence from the control group.

In vivo studies demonstrated that a 500 mg/kg dose of *S. collinsiae* extract effectively reduced *G. spinigerum* larvae in infected rats compared to controls. While albendazole proved more potent in decreasing worm numbers than *S. collinsiae* extract, histopathological analyses highlighted a safer therapeutic profile. The safer therapeutic profile is attributed to the host's immune response towards encysted worms, which aids in further elimination of the parasite. In contrast, low immune response was exhibited in albendazole-treated rats, this bringing forth potential risks that may be associated with non-encysted worms migrating to vital organs or re-emerging after treatment with albendazole.

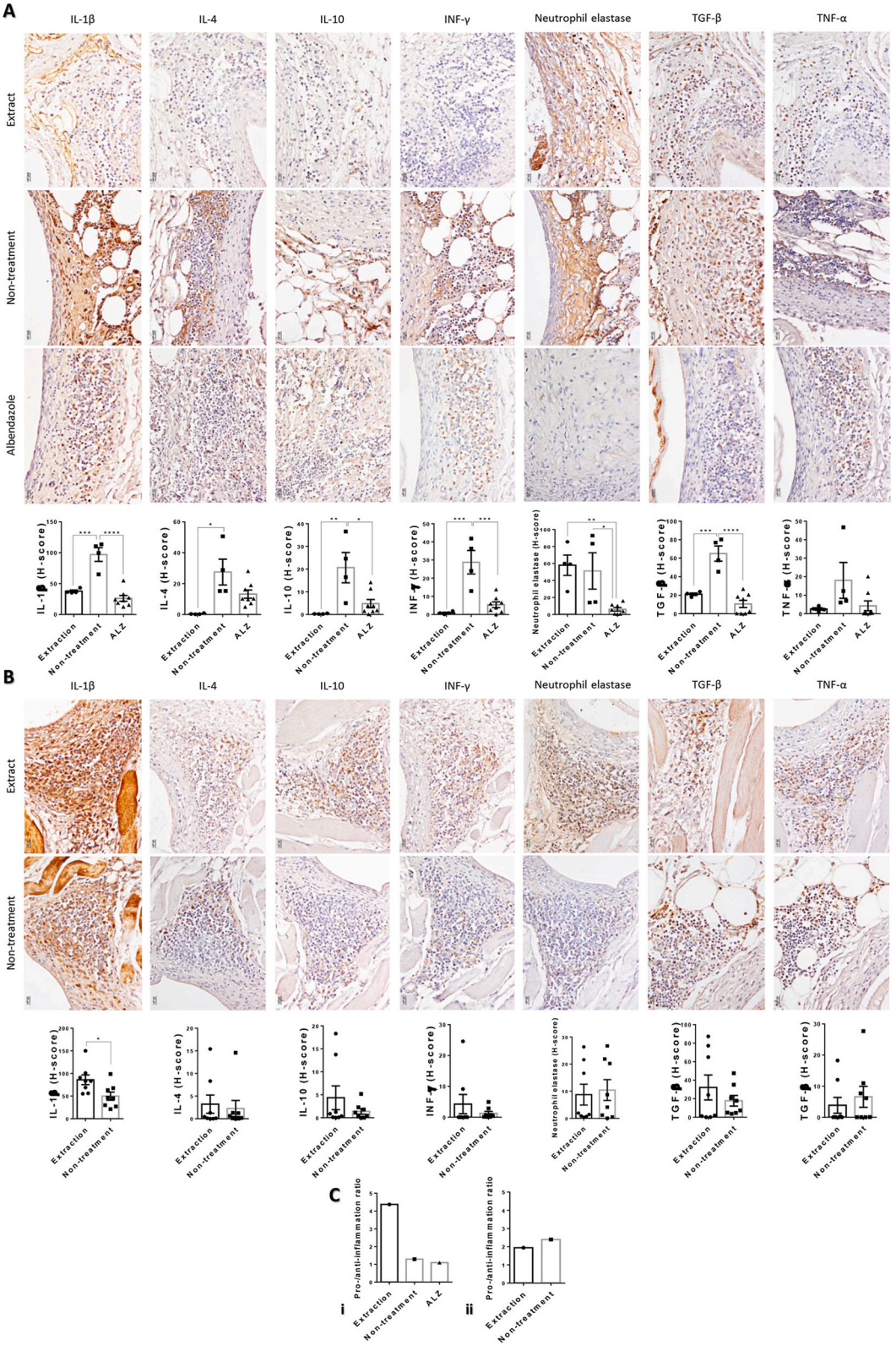

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Fig. 4. Pro- and anti-inflammatory cytokine levels in L3 gnathostomiasis rats with any treatment: Immunological expression of IL-1β, IL-4, IL-10, INF-γ, neutrophil elastase, TGF-β, and TNF-α in L3 migratory tissue on 3 (**A**) and 10 (**B**) days post treatment with or without extraction and albendazole. Bar graph represented the cytokine expression score with * p-value <0.05, ** p-value <0.01, and *** p-value <0.001. (**C**) Pro- and anti-inflammatory cytokine ratio in any treatment on both 3- and 10-days post treatment.

The promise of our *S. collinsiae* extract against *G. spinigerum* larvae, especially with its paralyzing prowess, postures it as a potential treatment contender for human gnathostomiasis. In juxtaposing our findings with prior studies, our understanding of *S. collinsiae* extract's potential is fortified. Our research casts *S. collinsiae* extract in a promising light against gnathostomiasis; it also underlines the need for future rigorous and expansive follow-ups. Our research has limitations on its applicability to humans despite its favorable *in vitro* anthelminthic activity in Wistar rats. Future endeavors should prioritize efficacy evaluations in diverse animal models and, eventually, human clinical trials. Additionally, distilling the crude extract to isolate its primary bioactive components will not only streamline its therapeutic effects but could also minimize potential side effects [22]. Adjusting dosage and pinpointing the best administration method will also be instrumental in optimizing these preliminary findings for clinical applications.

5. Conclusions

Our study demonstrates the therapeutic promise of *S. collinsiae* crude extract for human gnathostomiasis. We determined a NOEAC dose of 500 mg/kg for the *S. collinsiae* extract enriched with 6.25 % DSF through rodent toxicity assessments. Extended oral administration of this extract did not induce any discernible behavioral alterations or fatalities in the test animals. As we progress, addressing the study's constraints is pivotal, scrutinizing the extract's safety and effectiveness in clinical scenarios and pinpointing the active molecules for crafting new, potent, and safer anthelminthic agents against gnathostomiasis and similar infections. A deeper dive into potential interactions between *S. collinsiae* extract and current treatments like albendazole could illuminate any complementary or counteractive effects. Our findings thus serve as a foundation and a clarion call for further exploration in this domain.

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Data availability statement

The data included in the article and its supplementary materials.

CRediT authorship contribution statement

Norinee Arlee: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Sumate Ampawong:** Writing – review & editing, Visualization, Supervision, Investigation, Conceptualization. **Sumet Kongkiatpaiboon:** Writing – review & editing, Writing – original draft, Supervision, Investigation. **Yanin Limpanont:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Kuntarat Arunrungvichian:** Writing – review & editing, Supervision, Conceptualization. **Apanchanid Thepouyporn:** Supervision, Methodology, Investigation. **Wallop Pakdee:** Supervision, Methodology. **Urusa Thaenkham:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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