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Antiallergic, anthelmintic and cytotoxic potentials of dried aerial parts of *Acanthus ilicifolius* L.

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Abstract

Background: *Acanthus ilicifolius* L. is admired for its traditional usage in the folk medicine for the treatment of numerous diseases including allergy and helminthiasis in various parts of the planet. The ethanolic extract of the aerial parts of this shrub (EEAI) was investigated in the present study for its major phytochemical groups, antiallergic activity, anthelmintic activity, cytotoxicity and for acute toxicity.

Methods: Antiallergic activity was carried out using Toluene 2, 4-diisocyanate (TDI)-induced allergic mice model by assessing various symptoms of allergic rhinitis like sneezing, scratching, swelling and watery rhinorrhea as well as counting the total and differential leukocytes profile of blood. The paralysis and death time of parasites, *Haemonchus contortus* (Nematoda) and *Paramphistomum cervi* (Trematoda) were used for anthelmintic activity test. Mortality of mice was counted to evaluate the acute toxicity whereas the mortality of brine shrimp was taken into account to assess cytotoxic potential of the extract.

Results: Phytochemical screening of the extract demonstrated the presence of alkaloids, phenolic compounds, tannins, flavonoids, glycosides, saponins, steroids and triterpenoids. Oral pretreatment of the extract significantly ameliorated the TDI-induced allergic symptoms like sneezing ($p < 0.05$), scratching ($p < 0.05$), swelling and watery rhinorrhea in experimental mice. The extract also reduced the differential count of leukocytes in the blood which was increased due to induction of allergic conditions through TDI sensitization. In anthelmintic activity test the extract revealed a dose dependent decrease in the relative index of paralysis and death for both *H. contortus* and *P. cervi* parasites and thus indicated the extract to be parasiticidal at higher concentrations. In brine shrimp lethality bioassay of toxicity assessment, the LC_{50} of the standard drug vincristine sulfate was 0.43 μg/mL whereas the extract showed the LC_{50} as 44.57 μg/mL indicating a promising cytotoxicity of the extract. In acute toxicity study the highest dose 3 g/kg failed to show any mortality in Swiss albino mice and thus confirmed the safety of the extract for in vivo administration.

Conclusions: The present study corroborated the traditional uses of the aerial parts of *Acanthus ilicifolius* L. in allergic diseases and in helminthiasis.

Keywords: *Acanthus ilicifolius*, Antiallergic, Anthelmintic, Cytotoxicity, Acute toxicity

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Background

Mankind, from antiquity, has continuously searched curing agents to various maladies and practiced the use of different medicinal plants as a remedy thereof. Conforming to some estimates, medicinal plants have served, directly or indirectly, as the source of about 80% of the present day medicines [1] and have been explored due to having strong pharmacological activity, less adverse pharmacological effects and due to lower costs [2]. Among the pharmacological activities, antiallergic activity is crucial as the prevalence of allergic diseases is significantly increasing around the planet [3]. Allergic rhinitis, affecting 5–22% of the global population, is one of the most common allergic diseases in the world [4]. Helminthic manifestation, due to high prevalence particularly in third-world countries, is a major health problem associated with several severe complications like anemia, malnutrition, dysentery etc. resulting from this infection [5]. Although the second-generation antihistamine drugs, used for the treatment of allergic rhinitis, itching, urticaria etc., are devoid of the major adverse effects of first-generation antihistamines, nevertheless those drugs have several adverse effects including somnolence, headache, appetite simulation, body-weight gain, cardiac arrhythmia etc. [6]. Again, most of the currently available anthelmintic drugs, though less frequently, exhibit various adverse effects including fatigue, drowsiness, insomnia, headache, dizziness, rashes, hallucinations, Stevens-Johnson syndrome, tinnitus etc. [7]. This situation has prompted researchers to investigate new antiallergic and anthelmintic drugs [8].

Acanthus ilicifolius Linn. (Family: Acanthaceae) is an around 2 m tall shrub, having somewhat elongated to oval shaped leaves [9], with about 4 cm long, light blue, sessile flowers and up to 3 cm long, brown-colored capsule fruits [10]. It is a true mangrove species abundantly found in estuaries, from Bangladesh, India to Polynesia of Asian tropics and northern Australia [11].

Various parts of *Acanthus ilicifolius* L. have been used in the treatment of a wide range of ailments. In ethnomedical practices, the leaves of the plant are used, in different parts of the world, for the treatment of asthma, rheumatism, paralysis and snakebite [12–14]. Its roots have been used traditionally as antidote, expectorant, nervine tonic, and in the treatment of general debility, neuralgia, leucorrhoea, asthma, paralysis, dropsy, strangury, rheumatism, and psoriasis [15–18]. Folklore claims for this plant in Indian and Chinese system of traditional medicine are usage as diuretic, blood purifier, aphrodisiac, in treating asthma, backache, diabetes, dyspepsia, excitation, gastralgia, hepatitis, leprosy, leucorrhoea, leukemia, malignant-tumors, neuralgia, paralysis, ringworm, rheumatism, allergic skin diseases, snakebite, stomach pain, and swelled spleen [15–17, 19, 20]. Moreover, the bark-extracted water is used as antiseptic and to treat cold allergy and dermatitis in Thailand [15, 20].

The plant has been reported to have antioxidant [21–23], anti-inflammatory [12, 24, 25], antinociceptive [26], anticancer [27, 28], antiasthmatic [29], antimicrobial [30, 31] antidiabetic [32], antiprotozoal [33, 34], cytotoxic (activity of flower extract) [22], gastroprotective [35], hepatoprotective [13, 36], hypercholesterolemic [37, 38], antihyperglycemic [39], osteoblastic activity [40] and activity against neurological disorders [41].

Scientific investigations claimed the plant to contain a wide range of compounds including, Alkaloids, e.g. Acanthicifoline [42], Trigonellin [43], Benzoxazin-3-one [44], Benzoxazinoid glucosides [45], 4-O-b-D-glucopyranosyl-benzoxazolin-2(3-H)-one [46]; Flavonoids, e.g., Quercetin, quercetin 3-O-β-D-glucopyranoside [44], Acacetin 7-O-α–L rhamnopyranosyl-(1‴6″)-O- β -D-glucopyranoside [47]; Aliphatic glycosides, e.g., Ilicifolioside B [48], Ilicifolioside C [49]; Lignan glycosides, e.g., (+)-Lyoniresinol 3a-[2-(3,5-dimethoxy-4-hydroxy)-benzoyl]-O-β-glucopyranoside, (8R,7'S,8'R)-5,5′-dimethoxylariciresinol4-O-β-D-glucopyranoside, Acanfolioside, Alangilignoside C [50], (+)-lyoniresinol 2a-O- α-D-galactopyranosyl-3a-O- β -D-glucopyranoside, (+)-lyoniresinol 3a-O-α-D-galactopyranosyl-(1–6)-β–D glucopyranoside [51]; Megastigmane and phenolic glycosides, e.g., (Z)-4-coumaric acid 4-O- β –D glucopyranoside [48]; (6R,7E,9R)-9-hydroxy-megastigman-4,7-dien-3-one-9-O- β –D glucopyranoside [47], 2,6-dimethoxy-p-hydroquinone 1-O- β -D-glucopyranoside, syringic acid O-β-D-glucopyranosyl ester [50], 5,11-epoxymegastigmane glucoside [52]; Phenylethanol glycosides, e.g., ilicifolioside A, ilicifolioside D [48, 53]; Triterpenoids, e.g., lupeol, oleanolic acid and ursolic acid [44, 54, 55]; Steroids e.g., stigmasterol, stigmast-7-en-3 β -ol, stigmasterylβ –D glucopyanoside [44]; Miscellaneous, 7-chloro-(2R)-2-O-β-D-glucopyranosyl-2H-1,4-benzoxazin-3(4H)-one [50] etc.

While *Acanthus ilicifolius* L. enjoys its traditional uses in curing skin allergies in Thailand in their traditional medicinal system, [15, 19, 20, 55] or in helminthiasis [12, 56], or in treatment of cancer and in many other diseases, no scientific evidence is so far available on the antiallergic, anthelmintic or cytotoxic activities of aerial parts of the plant. Moreover, phenolic antioxidant constituents being associated with antiallergic property [57], due to the presence of lots of phenolic antioxidant constituents in the plant, the present study was designed and conducted to assess the antiallergic, anthelmintic and cytotoxic activity of aerial parts of *Acanthus ilicifolius* L. In addition, an acute toxicity study was also carried out to assess the toxicity profile of *A. ilicifolius* L. aerial parts.

Materials and methods

Plant materials

The aerial parts (leaves and stems) of *Acanthus ilicifolius* were collected from the Sunderbans of Satkhira region

of Bangladesh, in May, 2015. The plant materials were taxonomically authenticated by experts at Bangladesh National herbarium, Mirpur, Dhaka, where a voucher specimen (Accession no. DACB 43824) was submitted as future reference. The collected plant part were separated from undesirable materials or plants or plant parts, were washed with water and shade-drying was carried out to remove moisture from those. The dried plant materials were then crushed into coarse powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powdered plant materials were then dried in an electrical drier for 2 h at 45 °C, kept in an airtight container to avoid fungal contamination and stored in a cool dark and dry place.

Preparation of extract
Powdered plant materials were macerated with 95% ethanol for fourteen days and periodic shaking was carried out to ensure optimum maceration. Cotton plug and Whatman filter paper was used as filtering media to obtain a clear solution through the removal of plant debris. The ethanolic crude extract of aerial parts of *A. ilicifolius* (EEAI), obtained following the evaporation of ethanol in a rotary evaporator at 50 °C, was stored at 4 °C in refrigerator (Yield: 9.6% of dried plant material). To ensure optimum release profile in experimental animals, the crude extract was brought to room temperature before further experimental use.

Chemicals and reagents
TDI and standard antihistamine drug cetirizine were obtained from Wako Chemical, Tokyo, Japan. Standard albendazole, Tween-80, vincristine sulphate and dimethyl sulfoxide (DMSO) were purchased respectively from Beximco Pharmaceuticals Ltd., Dhaka, Bangladesh, Loba Chemie, India; Cipla Pharmaceuticals India and Merck, Darmstadt, Germany. All other chemicals including sodium carbonate, ferric chloride, acetic acid, ethyl acetate etc. were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Experimental animals and parasites
Young Swiss-albino mice, aged 4–5 weeks, average weight 25–30 g., were used for the present experiment. They were kept in the animal laboratory of Pharmacy Discipline, Khulna University, Khulna- 9208, and a temperature of 23 ± 1 °C and humidity of 55 ± 5%, with 12-h light/12-h dark cycle was maintained for one week for adaptation after purchasing them from Jahangirnagar University, Bangladesh. The animals were fed standard laboratory food and tap water ad libitum. Live parasites *P. cervi* (Trematoda) and *H. contortus* (Nematode) were collected from freshly slaughtered cattle at local abattoirs. After cleaning normal saline (0.9% NaCl), parasites

were stored in 0.9% phosphate-buffered saline (PBS) of pH 7.4. All animal experiments were carried out following the guidelines of Animal Ethics Committee, Pharmacy Discipline, Life Science School, Khulna University, Bangladesh.

Preliminary phytochemical screening
Phytochemical group test of the extract was performed using standard testing methods [58] for the identification of carbohydrates/gums, reducing sugars, alkaloids, flavonoids, glycosides, steroids, triterpenoids, tannins, and saponins.

Acute toxicity test
Acute oral toxicity test was carried out according to the Organization for Economic Co-operation and Development (OECD) guidelines-425. Male Swiss albino mice were divided into seven groups containing six animals each. The control group received normal saline (2 ml/kg, orally p.o.). The other groups received 100, 200, 500, 1000, 2000 and 3000 mg/kg of test extract, respectively. Immediately after dosing, the animals were observed continuously for 24 h for any behavioral changes and for any sign of toxicity. Neither any sign of toxicity nor death was observed within 24 h. The animals were then observed for next 14 days with an interval of 24 h to find out the mortality [59, 60] or any other toxic responses.

Antiallergic activity test
Antigen for sensitization and provocation
Toluene 2, 4-diisocyanate (TDI) was used as antigen for sensitization and provocation of allergy and was prepared at a concentration of 5%, in ethyl acetate solvent.

TDI sensitization and provocation of animals
Antiallergic activity of aerial parts extract of *A. ilicifolius* L. was assessed on adult male Swiss albino mice according to procedures of Dev et al. with minor modifications [61–63]. After acclimatization with the standard housing conditions of the animal house, fifty adult Swiss albino male mice were randomly selected and divided into five groups (10 in each group) denoted as group-I, group-II, group-III, group-IV and group-V. Group-I (Control) received ethyl acetate (10 μL) bilaterally in the nasal vestibules and 2% aqueous solution of tween-80 water orally. Group-II served as TDI control and received TDI (10 μL of 5% TDI in ethyl acetate) bilaterally in the nasal vestibules and 2% tween-80 water equivalent to the vehicle given with the extract orally. Group-III served as standard group and was given TDI (10 μL of 5% TDI solution in ethyl acetate) bilaterally in the nasal vestibules and an antihistamine drug, cetirizine, at a dose of 20 mg/kg body weight orally. Group-IV and group-V received ethanolic extract of *A. ilicifolius* (300 mg/kg and 500 mg/kg body weight respectively) orally and TDI (10 μL of 5%

TDI solution in ethyl acetate) bilaterally in the nasal vestibules.

All the mice (except Group-I) were sensitized, with 10 μL of 5% TDI in ethyl acetate, bilaterally on the nasal vestibule of each mice, once a day for five consecutive days [63]. After an interval of two days, the same sensitization procedure was then repeated. Following nine days of the second sensitization (on 21st day of the experimental protocol), for provoking nasal allergy-like symptoms, 10 μL of 5% TDI solution was again applied to the nasal vestibule of the experimental animals (except Group-I). The control group (Group-I) was sensitized and provoked with 10 μL of ethyl acetate only, according to the same procedure. Group-IV and group-V received ethanolic extract of *A. ilicifolius,* at the dose of 300 mg/kg and 500 mg/kg respectively, once a day throughout the entire experiment, and was continued even during the interval period. The extracts were administered 1 h prior to TDI application. Group-III received cetirizine at a dose of 20 mg/kg body weight, only on the 21st day just 1 h before TDI provocation (Fig. 1).

Assessment of allergy-like symptoms
Nasal allergy-like symptoms including the number of sneezes, number of scratches (rubbings), the nasal scores, the extent of watery rhinorrhea were measured following the methods described previously [63–65]. Immediately after TDI provocation, the number of sneezes, number of scratching and severity of watery rhinorrhea were carefully assessed for each group for 10 min, by placing the animals in various cages (ten animals per cage). The extent of watery rhinorrhea was measured on a grading scale ranging from 0 to 3 (Table 1), where 0 is lowest and 3 is highest.

Blood collection and blood cell count
On 21st day of that experimental period, the nasal allergy-like symptoms were assessed and 24 h following provocation with 5% TDI, animals of each group were anesthetized by injecting sodium pentobarbital and blood sample was collected from the cervical vein as methods described by Mahajan et al., 2007 and Smita et al., 2014 [62, 66]. The collected blood was then placed in a heparinized tube and used for total and differential leukocyte count. Total leukocytes were counted after the blood was diluted at 1:10 ratio with 1% acetic acid to lyse red blood cells. Total blood cell counts were then carried out on automated cell counter (DS-500i, 5 part automated hematology analyzer; Edan Instruments Inc., Shenzhen, China). For determining differential counts of blood cells, slides were prepared and were then stained with Leishman stain. Following drying of the slides, 300 cells/slide were counted on a compound microscope oil immersion power at 400 × magnification. In order to carry out differentials, using standard morphological determinants, cells were identified as eosinophils, lymphocytes, monocytes, neutrophils or basophils.

Anthelmintic activity test
Anthelmintic activity of the extract was assessed on live parasites *P. cervi* and *H. contortus* of cattle [5, 67, 68]. First off, the collected parasites were divided into six groups consisting of six parasites in each group. The extract was prepared at concentrations of 25, 50, 100, and 200 mg/mL of 10 mL in phosphate buffered saline (PBS). Standard albendazole was prepared at the concentration of 15 mg/mL (10 mL) in PBS and was used as reference drug. Tween-80 of 0.1% concentration in PBS was used to treat control group. All the extracts, reference drug and control was then transferred to Petri dishes. Six
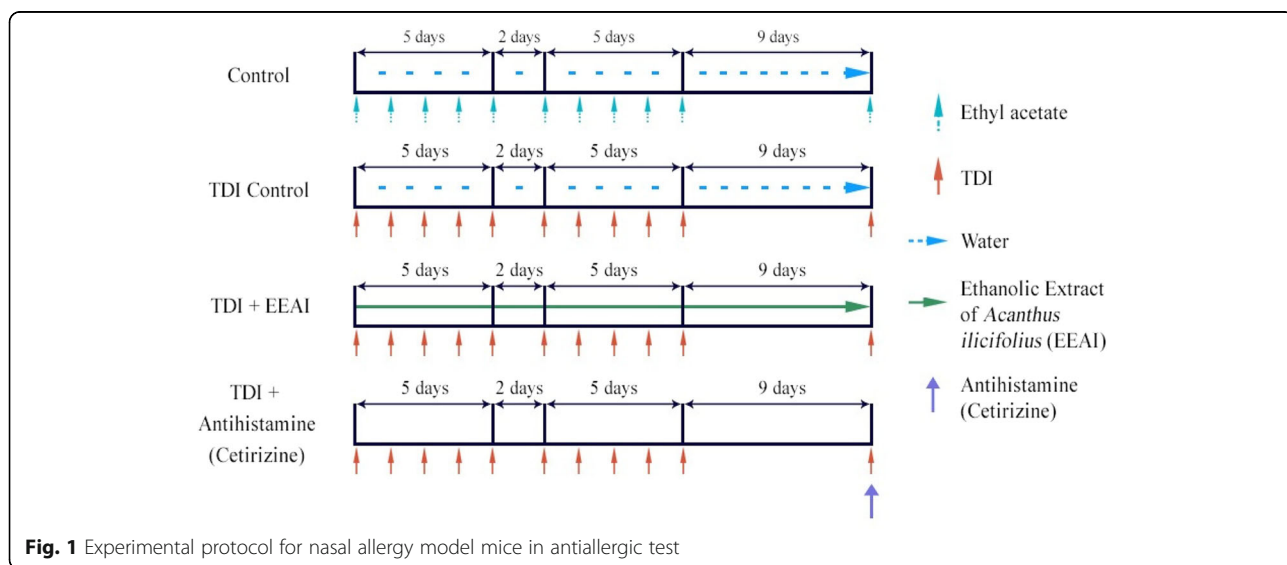

Fig. 1 Experimental protocol for nasal allergy model mice in antiallergic test

Table 1 Criteria for grading the severity of TDI-induced nasal responses in mice

Nasal Response	Score			
	0	1	2	3
Watery rhinorrhea	–	At the nostril	Between 1 and 3	Drops of discharges
Swelling and redness	–	Slightly swollen	Between 1 and 3	Strong swelling with redness

parasites were placed in each Petri dish and observed for mobility or death. When no movement of the parasites was observed unless shaken vigorously, that time was recorded as time of paralysis. Again, when the parasites did not move as shaken vigorously, dipped in warm water of 50 °C, or subjected to external stimuli, that time was taken to note as death time. The time required for paralysis and death of parasites when compared to control was used to express anthelmintic activity of the extract. The experiment was triplicated to avoid statistical error.

Cytotoxic activity test
The general cytotoxicity of the ethanolic extract of the plant was tested using brine shrimp lethality bioassay [69]. Simulated sea water (3.8% NaCl in water) was prepared, dissolving 38 g NaCl in 1 L of double-distilled water. Brine shrimp (*Artemia salina*) eggs were hatched using a conical shaped vessel containing previously prepared 1 L artificial seawater at 25–30 °C temperature with a constant oxygen supply. The *Artemia salina* eggs took about 24–48 h to be hatched. The larvae (nauplii) were collected using a pipette. Dimethyl sulfoxide (DMSO, 1%) was used to dissolve the sample extract and the extract was then transferred into seven test tubes following the method of serial dilution in such way that each test tube contained 10 mL of artificial sea water having seven different sample concentrations viz. 400, 200, 100, 50, 25, 12.5 and 6.25 μg/mL. Ten nauplii were placed in each test tube. DMSO (1%) was added to seven test tubes and Vincristine sulphate, an anticancer drug, was used as the positive control following the method of serial dilution in such way that each test tube contained 10 mL of artificial sea water having seven different vincristine sulphate concentrations at 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 μg/mL. Again, ten nauplii were placed in each test tube of positive control group. A negative control group was also tested with 10 nauplii in 10 mL sea water containing DMSO (1%). Following the period of 24 h at room temperature, the number of viable nauplii were counted. The experiment was triplicated to avoid statistical error.

Statistical analysis
Results were expressed as mean ± S.D. (Standard Deviation). All statistical analysis were carried out using Student's t-test. Statistical analysis and graphics generation were accomplished using GraphPad Prism® 5.03 (GraphPad software

Inc., San Diego, CA). Results were considered as significant when $p < 0.05$. The LC_{50} value in brine shrimp lethality bioassay was determined using Ldp line probit analysis software, USA.

Results
Preliminary phytochemical screening
Various phytochemicals tests conducted on the extract indicated the presence of alkaloids, steroids, terpenoids, saponins, tannins, phenolic compounds, glycosides and flavonoids and the absence of reducing sugars and carbohydrates (Table 2).

Acute toxicity test
Acute toxicity study revealed the non-toxic nature of the extracts. There was no lethality or any toxic reactions found at any of the doses even at 3000 mg/kg until the end of the study period, suggesting the LD_{50} of the extract of the plant is above 3000 mg/kg.

Antiallergic activity test
Effect on TDI-induced nasal allergy-like symptoms
In the mice of TDI-control group (Group II), the total number of sneezes (Fig. 2b), scratching (rubbing) (Fig. 2b) and nasal score (Fig. 2c) were 30.1 ± 15.35, 195.63 ± 50.95 and 3.00 ± 0 respectively. Whereas the

Table 2 Results of phytochemical group test of the extract

Tests for phytochemical groups	Reagents/Tests	Results[a]
Reducing sugar	Fehling's test	–
	Benedict's test	–
Gum/Carbohydrate	Molish's test	–
Alkaloids	Dragendorff's test	+
Steroids and terpenoids	Salkowski's test	+
	Libermann-Burchard reagent	+
Saponins	Frothing test	+
Flavonoids	Shinoda test	+
	Alkaline reagent test	+
Tannins	Ferric chloride test	+
Glycosides	Borntrager's test (Anthraquinone glycosides)	+
	Keller Killiani test (cardiac glycoside)	+

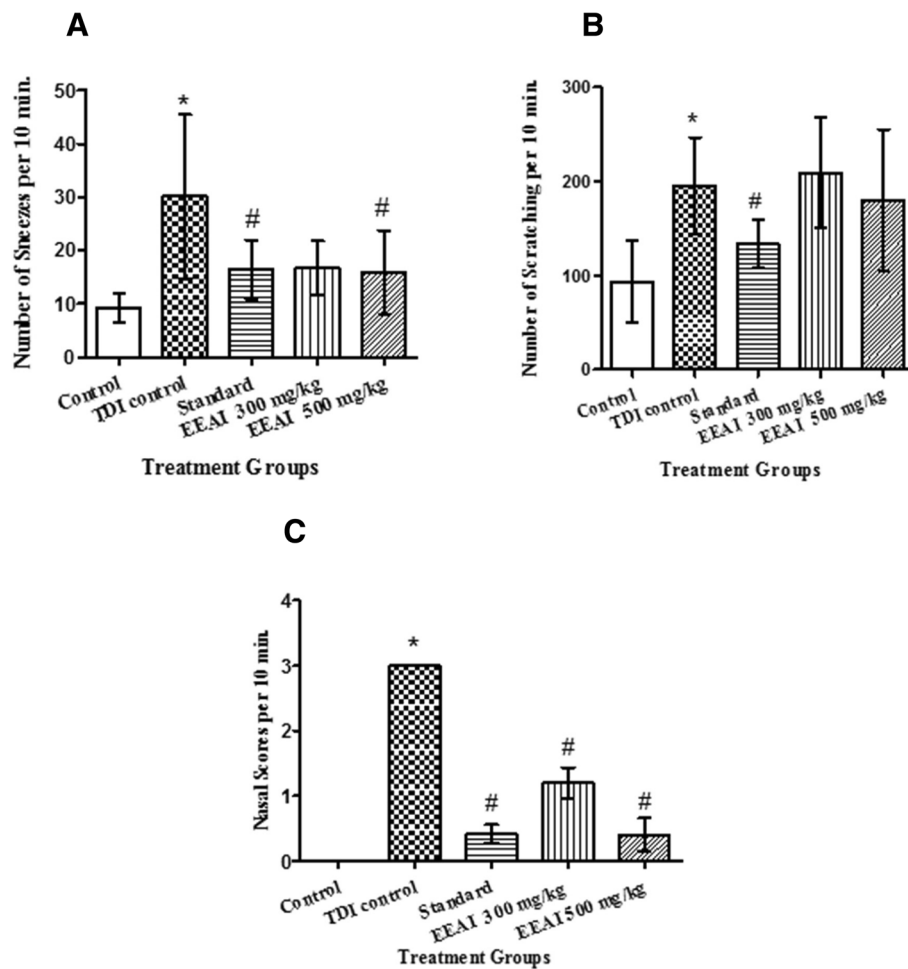[a] + indicates presence and – indicates absence

Fig. 2 a, **b**, **c** Suppressive effect of *A. ilicifolius* extract on Sneezing (**a**), Nasal Scratching (**b**) and Nasal Score (**c**). Values are expressed as Mean ± S.D., n = 10; * *p* < 0.05 vs. Control; #*p* < 0.05 vs. TDI Control

number of sneezes, scratching and nasal score, for standard cetirizine were 16.37 ± 5.57, 133.3 ± 25.51 and 0.4 ± 0.28; for 300 mg/kg dose of the extract those values were 16.67 ± 5.04, 209.0 ± 59.07 and 1.2 ± 0.23; and for 500 mg/kg dose of the extract those values were 15.87 ± 7.82, 180.3 ± 75.21 and 0.4 ± 0.25. As such, oral pre-treatment of ethanolic extract of *A. ilicifolius* for 3 weeks, significantly decreased the number of sneezes, and nasal score and the efficacy of extract was comparable with the standard antihistamine drug, cetirizine, in case of sneezes and nasal score at the dose of 500 mg/kg (Fig. 2a, b, c).
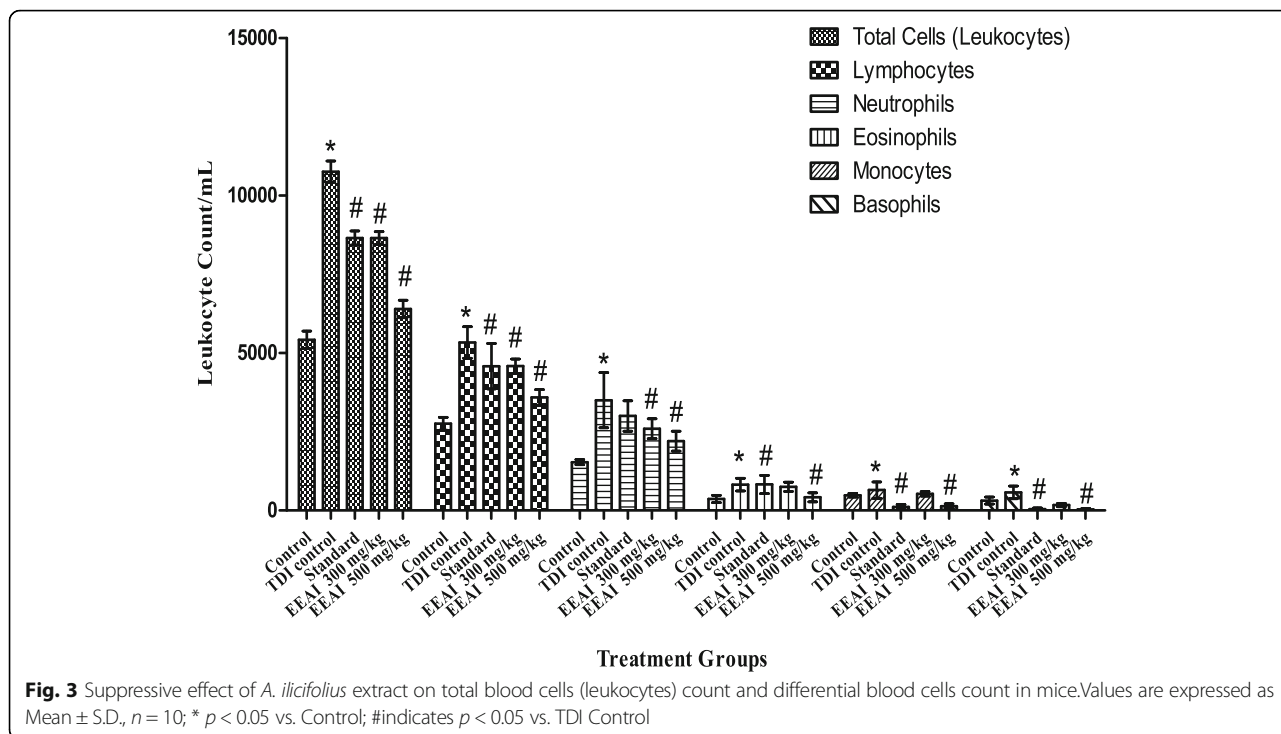
Effect on circulating total blood cell (leukocyte) count and differential blood cell count

In the blood samples of the mice of TDI-control group, the total numbers of circulating leukocytes, eosinophils, lymphocytes, neutrophils, monocytes and basophils were markedly increased (*p* < 0.05) in comparison with that of the mice of Control group. In contrast, the numbers of total leukocytes, eosinophils lymphocytes, neutrophils, monocytes and basophils were decreased in the blood samples of mice treated with ethanolic extract of *A. ilicifolius* L. and in mice treated with standard cetirizine, as compared to those levels in TDI-control group mice. The efficacy of the extract at the dose of 500 mg/kg was comparable with standard cetirizine (20 mg/kg) (Fig. 3).

Anthelmintic activity test

In a concentration-dependent manner, the aerial part extract exhibited significant anthelmintic activity which was comparable with standard drug albendazole. The results are presented in Table 3. In *P. cervi*, both paralysis and death occurred faster at higher concentrations of the extract (i.e. 5.50 ± 2.42 min and 10.17 ± 2.71 at 200 mg/mL). In *H. contortus*, a similar tendency was found (i.e. 9.33 ± 3.82 min and 18.83 ± 3.54 at 200 mg/mL). Again, the relative index values of paralysis obtained were 2.93, 1.84, 1.15 and 0.55 for *P. cervi* and 2.03, 1.30, 0.65 and 0.48 for *H. contortus* at concentrations of 25, 50, 100 and 200 mg/mL of the extract, respectively, vs.

Fig. 3 Suppressive effect of *A. ilicifolius* extract on total blood cells (leukocytes) count and differential blood cells count in mice. Values are expressed as Mean ± S.D., *n* = 10; * *p* < 0.05 vs. Control; #indicates *p* < 0.05 vs. TDI Control

albendazole (15 mg/mL). By contrast, the relative index values for death in *P. cervi* were 2.03, 1.30, 0.65, and 0.48 and that in *H. contortus* were 1.69, 1.10, 0.69 and 0.60 at concentrations of 25, 50, 100 and 200 mg/mL of the extract, respectively, vs. albendazole (15 mg/mL). These data suggest that the anthelmintic activity of the extract at high concentration is comparable with albendazole.

Cytotoxic activity test

The extract exhibited lethality on brine shrimp in a dose dependent manner. The LC_{50} for ethanolic extract of *A. ilicifolius* was found to be 44.57 μg/mL whereas that of standard anticancer drug vincristine sulphate was 0.42 μg/mL (Fig. 4a, b). Data set have been presented in Table 4.

Discussion

Evaluating the anti-allergic potential of ethanolic extract of aerial parts of *A. ilicifolius* L. on TDI-induced allergic rhinitis in Swiss albino mice was the objective of this study. The investigation was carried out on an established animal model in which mice were sensitized and provoked with TDI for inducing the onset of allergy-like symptoms, e.g., sneezing, rhinorrhea, nasal blockage, redness, swelling, scratching (rubbing), differential cell infiltration into tissues of lungs and airways, eosinophilia and release of biochemical substances like interleukins (IL)-4, IL-6 etc. [62, 63]. In this study, we showed that ethanolic extract of aerial parts of *A. ilicifolius* L. exhibits symptomatic relief of allergic rhinitis in mice. *A.*

Table 3 Anthelmintic activity of different doses of *Acanthus ilicifolius* L. aerial parts against *P. cervi* and *H. contortus*

Groups	Concentrations (mg/mL in PBS)	Test Parasites							
		Paramphistomum cervi				*Haemonchus contortus*			
		Paralysis		Death		Paralysis		Death	
		Time taken (min)	Relative index [a] (P)	Time taken (min)	Relative index (D)	Time taken (min)	Relative index (P)	Time taken (min)	Relative index (D)
Control	0	–	–	–	–	–	–	–	–
EEAI 25	25	28.83 ± 1.72	2.93	34.83 ± 3.18	2.24	39.00 ± 5.09	2.03	53.17 ± 7.02	1.69
EEAI 50	50	18.16 ± 1.94	1.84	23.00 ± 2.60	1.48	25.00 ± 4.93	1.30	34.67 ± 4.96	1.10
EEAI 100	100	11.33 ± 1.86	1.15	13.16 ± 2.04	0.84	12.50 ± 4.46	0.65	21.67 ± 5.50	0.69
EEAI 200	200	5.50 ± 2.42	0.55	10.17 ± 2.71	0.65	9.33 ± 3.82	0.48	18.83 ± 3.54	0.60
Albendazole	15	9.83 ± 1.47	1.00	15.50 ± 2.66	1.00	19.17 ± 4.02	1.00	31.33 ± 6.62	1.00

Values are expressed as Mean ± S.D., n = number of parasites (6), [a]Relative index denotes the time taken for paralysis (P) or death (D) to occur for EEAI/the time taken for paralysis or death to occur for the standard albendazole
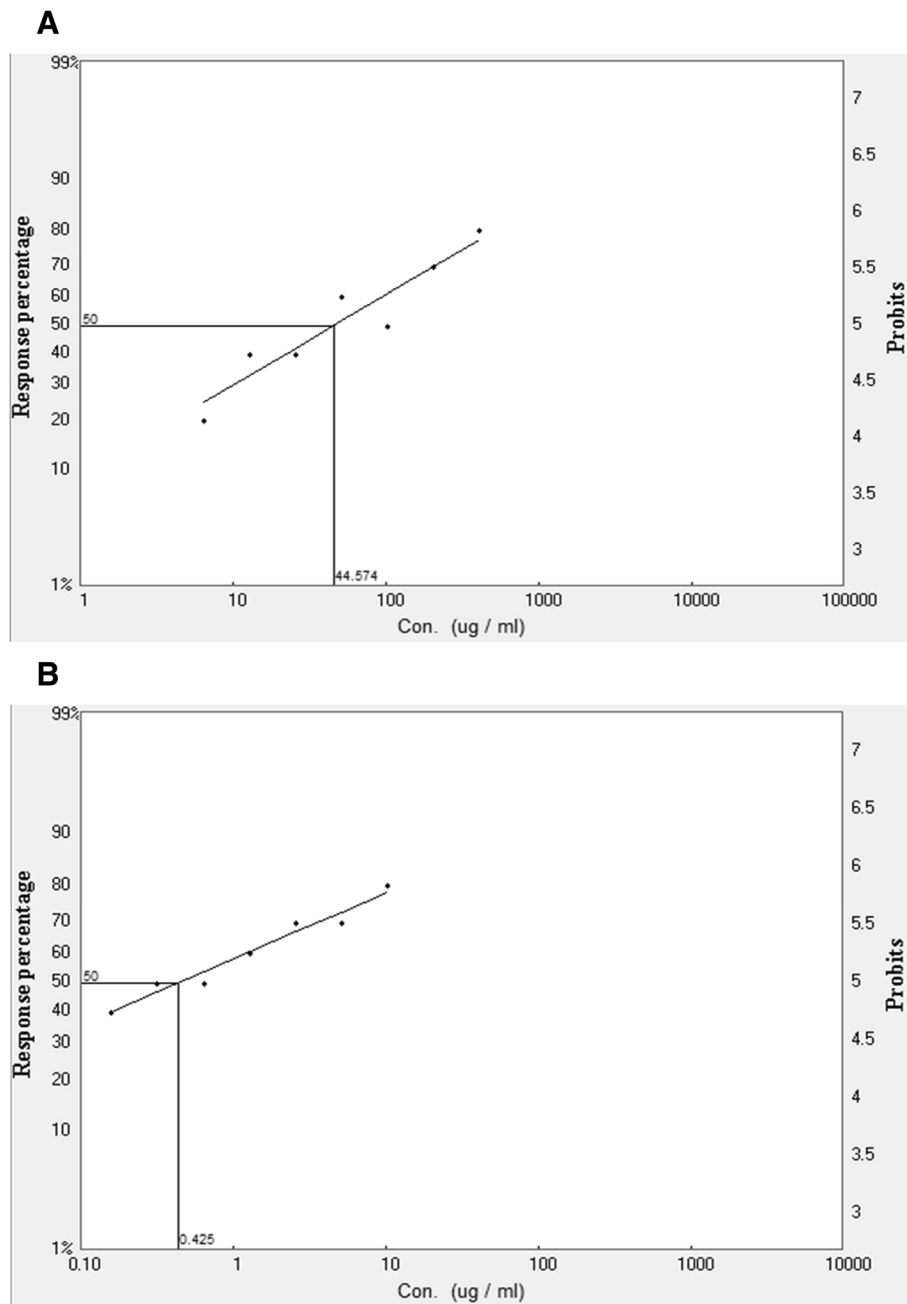
Fig. 4 a, **b** Determination of LC_{50} for the ethanolic extract of *A. ilicifolius* L. **a** and vincristine sulphate (**b**) using Ldp line software

Table 4 LC_{50} for EEAI and standard with lower and upper limit calculated by Ldp line software

	LC_{50} (µg/mL)	Lower limit (µg/mL)	Upper limit (µg/mL)
EEAI	44.574	23.338	81.640
Standard	0.424	0.234	0.645

ilicifolius L. extract significantly suppressed sneezing and nasal score as shown in Fig. 2a, b, c. It also decreased the nasal rubbing (scratching), but that was not significant. Suppression of these allergy-like symptoms (Fig. 2a, b, c) prompted us to study whether the extract has any effect on cells associated with inflammation and on the total and differential blood cell count in TDI-induced allergy model mice [8, 63]. In addition, in our study we have found increase in levels of total leukocytes, eosinophils, lymphocytes neutrophils, monocytes and basophils in blood of TDI-control mice (Fig. 3), and treatment with the ethanolic extract at an oral dose of 300 mg/kg and 500 mg/kg and cetirizine (20 mg/kg) reduced the count of these inflammatory cells as compared to TDI-control.

Mention-worthy, increased eosinophils in blood represent an overall state of allergic condition. Moreover, allergy/asthma patients show increased eosinophilia and leukocytosis and this increased eosinophilia and leukocytosis may work as a good cellular biomarker for allergic symptoms [70, 71]. Therefore, our experimental results (Fig. 3) are consistent with hypothesis and findings presented previously by several researchers [72–74]. In addition, the significant increases in the number of total and differential leukocytes, particularly, increase in the number of eosinophils, neutrophils, lymphocytes, monocytes and basophils, in the blood of the mice in TDI Control group, found in our study, is consistent with previous data reported by researchers who induced asthma using TDI in guinea pig model [61] and in rat model [62].

Preliminary phytochemical screening exhibited that *A. ilicifolius* aerial part extracts contains alkaloids, flavonoids, glycosides, steroids, tannins, triterpenoids and saponins (Table 1). It has been reported previously that the antiallergic activity of *Gymnema sylvestre* R Br is assumed to be due to the presence of tannins, total phenols, and flavonoids [74]. Histamine signaling, which is the major cause of allergic symptoms, is suppressed by several phytochemicals compounds e.g. tannins, including epigallocatechin-3-O-gallate isolated from green tea [75]. It has been reported that polyphenolic compounds e.g., gallic acid (3, 4, 5-trihydroxy benzoic acid) suppress various hypersensitivity reactions in mice and inhibit release of histamine and helper T cell cytokines, IL-4, IL-5 and IL-2 form mast cells in various experimental animal model of allergic diseases [76–78]. As *A. ilicifolius* extract has higher phenolic content, those could be the compounds responsible for the antiallergic effects of ethanolic extract of aerial parts of *A. ilicifolius* in a possibly similar mechanism. Moreover, as gallic acid is a tannin compound in chemical nature, and as *A. ilicifolius* extract exhibited presence of tannin in phytochemical screening, antiallergic activity of the extract could be due to the presence tannin contents in it.

Antioxidants reduce intracellular reactive oxygen species production and thus modulate both the function of mast cell as well as gene expression of IL-4 [79]. It has been reported that the antiallergic activity of the ethanolic extract of *Sanseveiria trifasciata* leaves is due to the presence of steroidal saponins, triterpenoids, flavonoids etc. which are known as potent antioxidants [72]. As the extract of *A. ilicifolius* L., in preliminary phytochemical analysis, exhibited presence of all of these compound including flavonoids, and as the extract exhibited free radical scavenging activity and possesses potent antioxidant property [12, 13, 22, 23], it is possible that these phytoconstituents either alone or combinedly, may have elicited the antiallergic activity observed in our investigation.

According to a previous scientific report, saponins inhibit degranulation of mast cells and thus inhibit histamine signaling and alleviate allergic symptoms [80]. Thus, presence of saponins in the extract exhibited in preliminary phytochemical analysis, could be responsible for the anti-allergic effect of the extract. In addition, Smita and other researchers have showed that a steroid, stigmasterol (stigmast-5, 22-dien-3β-ol), in combination with dexamethasone (an antiallergic drug), imparts antiallergic activity which is more potent than the activity of dexamethasone alone. Thus, as *A. ilicifolius* L. contains stigmasterol [44] as well, the ethanolic extract of *A. ilicifolius* may impart its antiallergic activity through the similar mechanism [66].

Accumulating all these data, in conclusion, our experimental findings provide adequate scientific basis for traditional use of *A. ilicifolius* L. in the treatment of allergic rhinitis. Thus, according to our experimental data, we may hypothesize that prolonged oral administration of the extract might play an important role in the management of TDI-induced allergic rhinitis.

In in vivo studies of anthelmintic activity of plant materials, loss of movement or paralysis and complete destruction or death of live parasites are usually utilized as the parameters [81–83]. In vivo anthelmintic activity of *A. ilicifolius* has previously been reported on adult earth worm model and worm parasite of chicken [56]. In the present work, we established significant anthelmintic activity of aerial parts of the extract of *A. ilicifolius* in a concentration dependent manner on freshly collected live parasites (Trematoda and Nematode) from cattle which is consistent with the earlier report by Husori and colleagues. Anthelmintic activity of secondary metabolites obtained from plants have been attributed to the presence of alkaloids, flavonoids, steroids, tannins and saponins [56]. This is consistent with our data (Table 1) for the phytochemical analysis of the plant, as the preliminary phytochemical screening exhibited that *A. ilicifolius* extract contains alkaloids, flavonoids, steroids, tannins and saponins.

Though, too little is known regarding the precise mechanism involved with the obtained anthelmintic activity of

the extract, a role may have been played in it by the poly-phenolics or other biochemical compounds present in the extract. It has been reported, for instance, that by uncoupling oxidative phosphorylation or binding to the glycoprotein on the cuticle of parasites, tannins are capable of interfering with energy generation in helminthes parasites and thus to cause death of the parasites [84, 85]. Capability to bind with the free protein in the host animal's digestive tract has been reported as another mechanism of tannins anthelmintic activity. According to some reports, tannins contained in the plant are capable of enhancing the absorption of protein which is obtained through the formation of protein complexes in the rumen, which at the low pH in the small intestine break down later [86].

Moreover, flavonoids and saponins have recently been reported to have the capability of exerting anthelmintic activity. Saponins have been described to increase the membrane permeability and pores formation, both of which actions are similar to the mechanism of established anthelmintic praziquantel and toltrazuril [87, 88]. Saponins and tannins may cause damage to the mucopolysaccharide membrane of worm which results in paralysis and death of those [89].

Furthermore, the alkaloids and steroids in the extracts may play key role in providing the suppressive effect of sucrose transfer to the small intestine which eventually can reduce glucose support for the worm parasites. These effects provided by alkaloid and steroids together with the antioxidant effects exerted by flavonoids can reduce the production of nitrate to be used in synthesis of protein [90]. Paralysis and death of parasites can possibly result from the action of alkaloid on CNS of parasites [91]. In addition, all these phytochemicals and their interaction may possibly have synergistically enhanced therapeutic efficacy of anthelmintic activity of *A. ilicifolius* aerial parts extract.

The brine shrimp lethality bioassay is an established, easy, safe, economic and practical method for predicting important pharmacological activities such as enzyme inhibition, ion channel interference, cytotoxic activity and several other bioactivities of synthetic compounds and of plant products [69, 92–94]. The significant correlation between the brine shrimp assay and human solid tumor cell lines in vitro growth inhibition demonstrated by the National Cancer Institute (NCI, USA) has shown the value of this bioassay as a pre-screening tool for antitumor drug research [95, 96]. According to Meyer et al., natural products' extracts exhibiting $LC_{50} \leq 1000\,\mu g/mL$ in brine shrimp bioassay have been claimed to possess bioactive constituents [69]. Several criteria for toxicity in brine shrimp lethality bioassay for synthetic compounds or natural products have been established where the LC_{50} values above $1000\,\mu g/mL$ are non-toxic, between 500 and $1000\,\mu g/mL$ are weak toxic and below $500\,\mu g/$ mL are toxic [97]. In the present study, both the extract and vincristine sulphate showed a gradual increase in mortality rate with the increase of concentration. The LC_{50} value for the ethanolic extract of *A. ilicifolius* was found to be very low ($44.57\,\mu g/mL$), signifying that the extract may contain potent pharmacologically active compound(s). The toxicity of plants is mainly attributed to the presence of alkaloids, phenolic compounds, glycosides, steroids, tannins, phlobatannins, terpenoids, flavonoids and saponins [98–101]. This is consistent with our observation as well, as the phytochemical group analysis of the extract exhibited the presence of alkaloid, tannins, flavonoid, steroids and saponins (Table 1). Thus, the principal advantage of the extract with regard to its cytotoxic effect could be exploitation of the cytotoxic potentials of *A. ilicifolius* for the development of natural product derived better medication for cancer.

Conclusions

The present study was carried out to evaluate the antiallergic, anthelmintic, cytotoxicity and acute toxicity study of ethanolic extract of aerial parts of *A. ilicifolius* L. Significant inhibitory activity for allergic symptoms, differential leukocytes counts in blood, and for helminthic infestation were observed. The extract was also found to be toxic for brine shrimp nauplii but safe in acute toxicity test for in vivo administration. The outcome of the present study corroborated the traditional use of *A. ilicifolius* in allergic disease and in helminthic infestation. Further investigation would be required to establish the safety parameters through biochemical and histopathological studies, and to discover novel phytochemicals responsible for the antiallergic and anthelmintic activity of the aerial parts of *Acanthus ilicifolius* Linn.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

Authors' contributions
The submitted research work was conducted in collaboration among the authors. The project was designed by SD, AKD and NNB. SD and PKS conducted the detail literature review and performed the phytochemical screening. PKS, SY, SP and MAAB have performed the extraction, antiallergic, anthelmintic, cytotoxic activities and acute toxicity tests. SD carried out the statistical analysis. Article was written by PKS, NNB and SD. Critical revision of the article was done by NNB and AKD. All authors read and approved the final manuscript.

Consent for publication
All co-authors have consented for the publication of this manuscript.

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