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RESEARCH ARTICLE

Anthelmintic Activity of Ellagic Acid, a Major Constituent of *Alternanthera sessilis* Against *Haemonchus contortus*

Himangsu Mondal[1], Hemayet Hossain[2], Khalijah Awang[3,4], Sanjib Saha[1], Sheikh Mamun-Ur-Rashid[1], Md Khirul Islam[1], Md. Sohanoor Rahman[1], Ismet Ara Jahan[2], Mohammad Mahfuzur Rahman[2] and Jamil A. Shilpi[1,4]*

[1]Pharmacy Discipline, Life Science School, Khulna University, Khulna 9208, Bangladesh; [2]BCSIR Laboratories& IFST, Bangladesh Council of Scientific and Industrial Research, Dhaka 1205, Bangladesh; [3]Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; [4]Centre for Natural Products and Drug Discovery, University of Malaya, Kuala Lumpur 50603, Malaysia
*Corresponding author: jamilshilpi@yahoo.com; jamilshilpi@um.edu.my

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ABSTRACT

Alternanthera sessilis,also known as 'sessile joyweed' or 'dwarf copperleaf', is a popular vegetable and used in traditional medicine in some Asian countries including Bangladesh for the treatment of various ailments. Anthelmintic activity of the ethanol extract of *A. sessilis* (ASE) and one of its major constituents ellagic acid (EA) was tested against cattle nematode *Haemonchus contortus* by adult motility test and egg hatch assay. In adult motility test, both ASE (1.56-50 mg/ml) and EA (0.09-3 mg/ml) showed a concentration dependent inhibitory effect on *H. contortus*. All test worms died 6 h post-exposure of 12.5 mg/ml of ASE treatment and 6 h post-exposure of 1.5 mg/ml of EA treatment. For the concentration of 1.5 mg/ml of the reference drug albendazole, all test worms died 2 h post-exposure of the treatment. In egg hatch assay, both ASE (0.0125-25 mg/ml) and EA (0.0125-25 µg/ml) showed a concentration dependent inhibition of the larval production from *H. controtus* eggs with the LC_{50} value of 150.00 and 3.097 µg/ml, respectively. The LC_{50} for albendazole (0.0125-25 µg/ml) was 0.163 µg/ml. In the HPLC analysis, EA, rutin, (+) catechin and quercetin (3007.26, 490.74, 117.72 and 13.85 mg/100 g extract, respectively) were detected. Phytochemical group test of ASE indicated the presence of reducing sugars, steroids, terpenoids, saponins, tannins and flavonoids. Thus, high level of EA in ASE, along with other phytochemical constituents might be responsible for the observed activity of the extract.

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INTRODUCTION

Helminthiasis in cattle is a serious problem in tropic and subtropical region resulting in the reduction of productivity, increased mortality, loss of weight, meat and wool caused by reduced appetite, retarded growth and impaired digestive efficiency (Raza *et al.*, 2010). Control of gastrointestinal nematodes is under threat due to increased incidences of drug resistance towards the commonly available anthelmintic drugs. Although search for synthetic anthelmintic drug continues, it might not help the farmers of third world countries due to the high cost and availability of new therapeutic agents (Alawa *et al.*, 2010; Sindhu *et al*., 2014; Fatima *et al*., 2014).

Medicinal plants have been of great interest in recent years for the management of helmithiasis due to the fact that plants often contain wide range of compounds with anthelmintic properties (Zahir *et al.*, 2012). Phytochemicals are also gaining interest for their ability to reduce parasitic burden and reducing the chance of developing drug resistance (Nunomura *et al.*, 2006; Masood *et al*., 2013; Abbas *et al*., 2014a & b; Hamad *et al*., 2014; Hamad, 2014).

Alternanthera sessilis (L.) RBr ex DC (Family: Amaranthaceae) is commonly known as "Sachi-shak", "Haicha", "Chanchi" in Bangladesh, and is distributed almost in all parts of the country, particularly in marshy areas. It grows widely in other Asian countries including Nepal, China, India, and Taiwan. It is a perennial herb,

usually rooting near the nodes and sometimes ascending. It has white flowers which usually appear between December to March. Although it is considered as an obnoxious weed in some parts of the world, the plant is used as a popular vegetable in the rural areas of Asia. The plant is used in the management of diarrhoea, helminthiasis, malaria, night blindness, post-natal complaints and dysentery. It can eliminate laziness, tiredness and sleep after eating. It produces anthelmintic action when the plant juice is taken in empty stomach with two spoons of warm water (Ghani, 1998). The plant is reported to possess antimicrobial, wound healing (Jalalpure *et al.*, 2008), cytotoxic (Balasuriya and Dharmaratne, 2007), antioxidant (Borah *et al.*, 2011), antipyretic (Nayak *et al.*, 2010), hematinic (Arollado and Osi, 2010), hepatoprotective (Lin *et al.*, 1994) and anti-inflammatory (Sahithi *et al.*, 2011) properties. Previous phytochemical investigations revealed that the plant contains lupeol, stigmasterol, *β*-sitosterol, handianol, campesterol, *α* and *β*-spinasterol, 24-methylene-cycloartanol, cycloeucalenol, 5*α*-stigmasta-7-enol (Sinha *et al.*, 1984).

As a part of our research with the aim of identifying locally available medicinal plants with anthelmintic property, we describe herein the anthelmintic property of *A. sessilis* and that of ellagic acid (EA), one of the major phenolic constituents of this plant.

MATERIALS AND METHODS

Plant material and extraction: Several whole plants of *Alternanthera sessilis* were collected from Gopalgonj, Bangladesh, during October, 2011. A voucher specimen (DACB 36542) of the plant was lodged in the Bangladesh National Herbarium, Dhaka, Bangladesh, for authentication. The powdered plant material (270 g) was macerated in ethanol for a week with occasional stirring. Upon filtration, the solvent was dried under reduced pressure in rotary vacuum evaporator to get the crude ethanol extract (ASE) (yield: 5.3% of dried plant material).

In vitro anthelmintic assay

Adult motility test: Live adult *H. contortus* were collected from freshly slaughtered cattle at local abattoirs of Gallamari, Khulna and kept in 0.9% phosphate-buffered saline (PBS) of pH 7.4 at the temperature of 37±1ºC.The nematodes, divided into groups (*n*=10) were treated with ASE (50-1.56 mg/ml) and albendazole (1.5 mg/ml). Control group received 1.0% tween-80 in PBS (Badar *et al.*, 2011). Ellagic acid (Sigma-Aldrich) was tested within the concentration range of 3-0.09 mg/ml. To assist EA to go into solution, it was first dissolved in 40 µl of 0.1 N KOH with further equilibration with phosphate buffer saline. The worms were checked every 2 h for a period of 8 h. Finally, the treated and control worms were placed in lukewarm PBS to assess the restoration of motility.

Egg hatch assay: Eggs harvested from female worms of *H. contortus* were resuspended in deionized water and the number of eggs estimated in a 50 µl sample was adjusted to 100-150 eggs/ml. Test wells containing 1 ml of the egg suspension were exposed to serially diluted different concentrations of ASE (25-0.0125 mg/ml) and EA (25-0.0125 µg/ml), while the positive control and control group contained albendazole (25-0.0125 µg/ml) and 1.0% tween-80 in distilled deionized water, respectively. After an incubation period of 48 h, Lugol's iodine solution was added. Number of unhatched eggs and first stage larvae were counted in each well to determine the effect of the treatments. The LC_{50} was determined using LdP line probit analysis software (USA) (Badar *et al.*, 2011).

Phytochemical investigation

Group test: Phytochemical group test was performed to detect the presence of reducing sugars, alkaloids, steroids, tannins, glycosides, gums/carbohydrates, flavonoids and saponins (Habib, 1980; Harborne, 1998).

HPLC analysis for phenolic constituents: HPLC analysis was carried on a Dionex Ultimate 3000 Rapid Separation LC (RSLC) system (Thermo Fisher Scientific Inc., MA, USA), equipped with quaternary rapid separation pump (LPG-3400RS), Acclaim® C_{18} (4.6×250 mm; 5 µm) column (Dionex, USA) and rapid separation photodiode array detector (DAD-3000RS). For the preparation of calibration curve, a standard stock solution was prepared in methanol containing gallic acid, vanillic acid, (+)-catechin, (-)-epicatechin, *p*-coumaric acid, rutin, ellagic acid (20 µg/ml each), caffeic acid (8 µg/ml) and quercetin (6 µg/ml). The standards were purchased from Sigma-Aldrich. For the extract, a solution of ASE was prepared in methanol having the concentration of 5 mg/ml (Sakakibara *et al.*, 2003; Islam *et al.*, 2014).

Statistical analysis: The statistical analysis of the results of adult motility test was performed by student's t-test while that of HPLC was performed using one way analysis of variance (ANOVA) followed by Dunnett's test using SPSS 11.5.

RESULTS

Adult motility test: The extract showed a concentration dependent anthelmintic activity when tested on live nematodes of *H. contortus* and the results were statistically significant ($P<0.05$). The highest activity was observed at 4 h for the concentration of 50 mg/ml. All the test worms died within the observation period of 8 h for the concentration of 12.5 mg/ml and above. For EA, 100% death occurred for 1.5 and 3 mg/ml. Albendazole, reference drug used in this assay, caused 100% death of the test worms at 2 h for the concentration of 1.5 mg/ml while no death was recorded in control group (Table 1).

Egg hatch assay: In egg hatch assay, both ASE and EA showed a concentration dependant ovicidal activity on the eggs of *H. contortus*. The LC_{50} of ASE and EA observed at 150.00 and 3.097 µg/ml, respectively, while that of albendazole, used as the reference standard was 0.163 µg/ml.

Phytochemical group test: The phytochemical group test indicated the presence of reducing sugars, steroids, terpenoids, saponins, tannins and flavonoids (Table 2).

Table 1: Effect of *A. sessilis* extract and ellagic acid on survival of *H. contortus*

Treatment	Conc. (mg/ml)	Mean no of dead worms at different hour of experiment				
		0 h	2 h	4 h	6 h	8 h
Control	-	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
A. sessilis	50	0.00±0.00	8.33±0.41[a]	10.00±0.00[a]	10.00±0.00[a]	10.00±0.00[a]
	25	0.00±0.00	6.67±0.41[a]	9.00±0.71[a]	10.00±0.00[a]	10.00±0.00[a]
	12.5	0.00±0.00	5.00±0.71[a]	7.33±0.41[a]	9.33±0.41[a]	10.00±0.00[a]
	6.25	0.00±0.00	4.00±0.71[b]	5.33±0.41[a]	7.33±0.41[a]	9.33±0.41[a]
	3.13	0.00±0.00	1.67±0.41[b]	4.00±0.71[b]	5.67±0.41[a]	7.67±0.41[a]
	1.56	0.00±0.00	0.00±0.00	1.67±0.41[b]	3.33±0.41[a]	5.33±0.41[a]
Ellagic acid	3	0.00±0.00	5.33±0.41[a]	7.00±0.71[a]	10.00±0.41[a]	10.00±0.00[a]
	1.5	0.00±0.00	2.33±0.41[a]	5.00±0.71[a]	7.33±0.82[a]	9.67±0.41[a]
	0.75	0.00±0.00	0.33±0.41	1.33±0.41[c]	4.67±0.41[a]	7 .00±0.00[a]
	0.38	0.00±0.00	0.00±0.00	0.00±0.00	1.33±0.41[b]	3.67±0.82[b]
	0.19	0.00±0.00	0.00±0.00	0.00±0.00	1.66±0.82	3.33±0.82[b]
	0.09	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.67±0.41
Albendazole	1.5	0.00±0.00	10.00±0.00[a]	10.00±0.00[a]	10.00±0.00[a]	10.00±0.00

Results expressed as mean±SE (All experiments done in triplicate with *n*=10, SE: standard error); Student's *t*-test, [a]P<0.001, [b]P<0.01, [c]P<0.05 vs. control.

Table 2: Effect of *A. sessilis* extract and ellagic acid on egg hatch assay of *H. contortus*

Treatment (*n*=3)	LC_{50} (µg/ml)	Regression values and correlation of regression
A. sessilis	150.00	$y = 8.8846x - 23.5$, $R^2 = 0.9482$
Ellagic acid	3.097	$y = 8.4336x - 14.848$, $R^2 = 0.9257$
Albendazole	0.163	$y = 9.542x - 26.439$, $R^2 = 0.9419$

Using simple linear regression equation, $y=mx+c$, where y: y points, x: x points, *m*: gradient, c: vertical intercept, and R^2: Pearson correlation coefficient.

Table 3: Group test for *A. sessilis* extract

Test for phytochemical group	Reagent	Results
Reducing sugar	Fehling's test	+
	Benedict's test	+
Alkaloid	Mayer's test	-
	Dragendorff's test	+
Steroid and terpenoid	Salkowski's test	+
	Libermann-Burchard reagent	+
Tannin	Ferric chloride test	+
Glycoside	Keller Killiani test (cardiac glycoside)	-
	Borntrager's test (anthraquinone glycosides)	-
Gum/Carbohydrate	Molish's test	-
Flavonoid	Shinoda test	+
	Alkaline reagent test	+
Saponin	Frothing test	+

+ Indicates presence and – indicates absence

Table 4: Contents of polyphenolic compounds in *A. sessilis* extract (*n*=5)

Polyphenolic compound	Content(mg/100 g extract)	% RSD
(+)-Catechin	117.72	1.04
Rutin	490.74	1.91
Ellagic acid	3007.26	3.89
Quercetin	13.85	0.63

RSD: Relative standard deviation

Alkaloid was considered absent based on negative Mayer's test and false positive result with Dragendorff's reagent.

HPLC analysis: The extract showed high level of EA and rutin (3007.26 and 490.74 mg/100 g of extract). (+)-Catechin and quercetin were also detected but with lower concentrations (117.72 and 13.85 mg/100 g of extract) (Table 3**)**. The HPLC chromatogram also displayed peaks in regions that represent simple polyphenols, catechins, anthocyanins, flavonoid aglycones and flavonoid glycosides (Fig. 1).

DISCUSSION

In the present study, ASE caused the death of live adult nematodes and inhibited larval production from the eggs of *H. contortus*. The activity of the extract was comparable to the positive control, albendazole, which acts through binding with β-tubulin, preventing the formation of microtubules (Martin, 1997). In the HPLC analysis, EA was found to be a major constituent of ASE. Ellagic acid was further subjected to the adult motility and egg hatch assay to investigate its role in the observed anthelmintic activity of the extract, in which EA showed a strong anthelmintic activity against *H. contortus*.

Phytochemical investigation of *A. sessilis* indicated the presence of terpenes, saponins and tannins which might have played a role in the observed anthelmintic activity (Katiki *et al.*, 2013). Lupeol, a lupane type triterpene previously reported from this plant showed anthelmintic activity against *Caenorhabditis elegans* (Shai *et al.*, 2009). However, the activity was of moderate level and less than that of the crude extract indicating that some other compounds present in the extract also contributed towards the observed activity. Suggested mechanisms involved in the anthelmintic activity of saponins include disrupting cell membrane permeability through pore formation, disintegration of integuments at specific site, inhibition of cAMP phosphodiesterase and Na^+/K^+ ATPase (Wang *et al.*, 2010). Tannins are known to produce anthelmintic activity through a number of mechanisms which include uncoupling oxidative phosphorylation, antioxidant activity and their ability to bind with metals and proteins (Katiki *et al.*, 2013). Various simple phenolics including gallic acid, caffeic acid, flavonoids, which give rise to tannins, are also known to exert anthelmintic action.

Ellagic acid (Fig. 2**)**, one of the major constituents of ASE, caused the death of adult nematodes, as well as inhibited the larval production from the eggs of *H. contortus*. In a recent study, EA has been found to inhibit the parasite *C. elegans* (Ndjonka *et al.*, 2013a). However in another study, EA found to be inactive against *C. elegans* (Thomsen *et al.*, 2012). In the first case, EA was dissolved in 0.3M KOH and further equilibration with phosphate buffer solution, while in the later, it was dissolved in DMSO for the bioassay. It is possible that solubility might have played a role in the observed results,

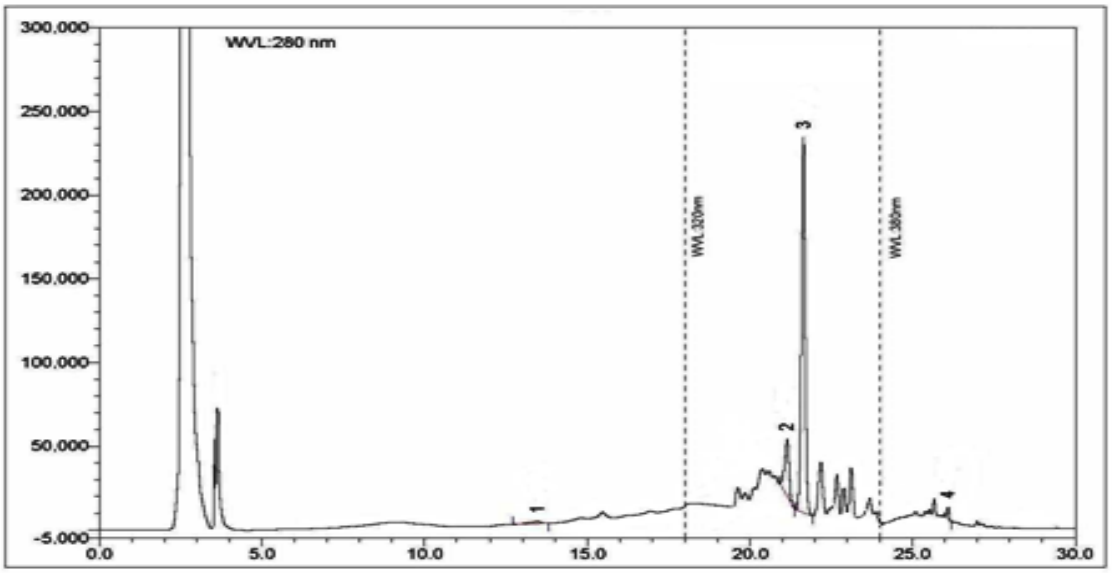

Fig. 1: HPLC chromatogram of *A. sessilis* extract (Peaks 1: (+)-catechin, 2: rutin, 3: ellagic acid, 4: quercetin).

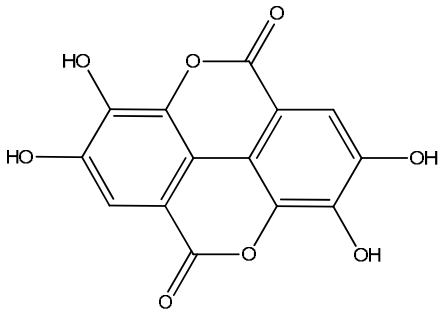

Fig. 2: Structure of ellagic acid

since solubility of EA in DMSO is only ~0.14 mg/ml, while in alkaline solution, it is as high as 10 mg/ml. It can be assumed that maximum amounts of ingested EA will remain in the solution in the alkaline milieu of the intestine to give anthelmintic action. In another report, EA inhibited wild type as well as levamisole and albendazole resistant strains of *C. elegans* indicating that the mechanism of action of EA is different from that of aforementioned anthelmintic drugs (Ndjonka *et al.*, 2013b). Ellagic acid and related antioxidants are excellent acceptor of free radicals. Thus, they can also quench electrons from various biological systems including that of electron transport system (ETS). Disruption of electron flow in ETS results in the inhibition of oxidative phophorylation (Vattem and Shetty, 2005). Depletion of ATP in nematodes might be one of the mechanisms through which EA and other phenolics exert anthelmintic action. The content of EA in ASE was found to be much higher (1.59 g/kg dried plant material) than many other plants including that of chestnut bark (1.17 g/kg dried plant material), one of the commercial sources of ellagic acid (Vekiari *et al.*, 2008). Phytochemical group test indicated the presence of tannins in ASE. Thus, it is possible that the ellagitannis present in ASE are converted to ellagic acid through hydrolysis, thus further increasing the concentration of ellagic acid in the intestine (Vekiari *et al.*, 2008; Arapitsas, 2012).

Conclusion: Present investigation revealed that EA possesses strong anthelmintic activity and was the major responsible constituent for the observed activity of ASE. Thus, EA rich food can be a good choice for control and prevention of helminthiasis. Synergism often plays an important role for enhanced bioactivity of plant extracts. Thus, plant products containing a range of phytochemicals with anthelmintic action can often be a good choice to control helminthiasis. Present investigation also supported the ethnobotanical use of *A. sessilis* in helminthiasis. Moreover, *A. sessilis* which is considered as an obnoxious weed can be an economical source of ellagic acid.

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Authors' contribution: HM, SS, KI, SMR and MSR carried out the experiments on nematodes; HH and MMR carried out the HPLC analysis under the guidance of IAJ;JAS designed the work and prepared the manuscript under the supervision of KA.

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