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ANTIPROLIFERATIVE ACTIVITY OF PHYTOCHEMICALS PRESENT IN AERIAL PARTS AQUEOUS EXTRACT OF AMPELOCISSUS LATIFOLIA (ROXB.) PLANCH. ON APICAL MERISTEM CELLS

ANWESA CHAUDHURI AND SANJIB RAY*

Molecular Biology and Genetics Unit, Department of Zoology, The University of Burdwan, Golapbag, Burdwan-713104, West Bengal, India

ABSTRACT

This study aimed to explore antiproliferative activity of aerial parts aqueous extract of *Ampelocissus latifolia* (Roxb.) Planch. (AAEAL). Antiproliferative activity of AAEAL was tested in root and/or shoot apical meristems of green gram and onion by analysing growth retardation and /or by scoring mitotic index, mitotic cell phase frequency. Phytochemical contents of AAEAL were detected and the total phenolic, tannin and flavonoid contents were estimated using standard protocols. The results indicate AAEAL treatment could induce significant growth retardation in apical meristems. In onion root tip cells, 74 % mitotic index reduction was observed with 2 mg/ml of AAEAL treatment for 12 h. Tannins, terpenoids, saponins, flavonoids, carbohydrates and anthraquiones were relatively of higher quantities and a trace amount of alkaloids and glycosides were detected from AAEAL. Tannic acid equivalents were determined from AAEAL as 21.03±0.9, 9.54±0.7 and 12.1±0.9 % respectively for total phenols, non tannin phenols and tannins while 11.7±0.5 % flavonoids were estimated as quercetin equivalents in AAEAL. In summary, the AAEAL possesses the significant antiproliferative potentials which may be due to its bioactive polyphenolics.

KEYWORDS: Antiproliferation; *Allium cepa*; *Ampelocissus latifolia*; Phenolics

***Corresponding author**

SANJIB RAY
Molecular Biology and Genetics Unit, Department of Zoology, The University of Burdwan, Golapbag, Burdwan-713104, West Bengal, India.

INTRODUCTION

The genus *Ampelocissus* (Family: Vitaceae) has been cited in many indigenous systems of health care for the treatment of a variety of disorders. *Ampelocissus latifolia* is a native herb to Indian subcontinent and its root decoction is used to treat dental troubles, ulcers and dysentery [1, 2]. Fresh crushed tuber is boiled in *Ricinus communis* oil and then applied externally for the treatment of gout[3]. The tuber extract is also used to treat fractured bone, dyspepsia, indigestion and tuberculosis[3, 4].The root decoction is used as an antidote for snake bite, applied on wound, abscess and for an easy labor and delivery of a baby [5]. Fresh stem node paste is applied on the swelled joints of cattle[6]. Medicinal herbs are a good source of a wide variety of bioactive components[7]. Acetone extract of this plant possesses alkaloids, fixed oils and fats, flavonoids and saponins while chloroform and alcoholic extract possess alkaloids[8]. Acetone, chloroform and alcoholic extracts of this plant exhibit significant anti-inflammatory activity which was reported to be due to its inhibitory effect on histamine kinin and prostaglandin release[8]. Recently the antibacterial and antioxidant activities of this plant have been reported[9, 10]. In our previous study we have reported phytotoxic and cytogenotoxic potentials of aqueous extract of this plant [11]. Acetogenins like 22-epicalmistrin, uvaribonin and chalcone were isolated from the root of Phillippine *Ampelocissus* and showed their significant cell growth inhibitory activity against a panel of human cancer cell lines[12]. The antiproliferative pharmacological activities of plant derived secondary metabolites appear to elucidate the chemo-preventive or anticancer effects. The efficient anticancer chemotherapeutic agents, such as Paclitaxel (Taxol®) from *Taxus brevifolia* and vincristine and vinblastine, isolated from *Catharanthus roseus,* provide realistic clue that the natural products of plant origin are a potential source of novel cancer chemotherapeutic agents[7]. At present, the chemical composition of aerial parts aqueous extracts of *A. latifolia* and their antiproliferative activities are not well studied. Therefore, in the present study, antiproliferative activity of AAEAL was analysed on apical meristem cells. Furthermore, the secondary metabolites present in AAEAL were qualitatively detected and the total phenolic, tannin and flavonoid contents were measured.

MATERIALS AND METHODS

Chemicals

Glacial acetic acid, orcein and methanol were obtained from BDH chemicals Ltd., UK. Quercetin was obtained from Sigma-aldrich, USA. Folin-Ciocalteu's phenol reagent was obtained from MERCK Specialities Pvt. Ltd., Mumbai, India. Polyvinylpolypyrrolidone (K-30) was purchased from SRL Pvt. Ltd., Mumbai, India. Tannic acid powder was obtained from HIMEDIA Laboratories Pvt. Ltd., Mumbai, India. Other chemicals used in the study were of analytical grade and obtained from reputed manufacturers.

Plant products collection, storage and extract preparation

Fresh aerial parts of *A. latifolia* were collected from Burdwan University campus, West Bengal, India in August 2011. This plant species was taxonomically identified by Dr. Ambarish Mukherjee (Taxonomist), Professor, Department of Botany, the University of Burdwan. The voucher specimens (No.BUGBAC012) are maintained in the department for future reference. Collected plant materials were washed in tap water, shade dried, directly crushed into small pieces and followed to pulverize using an electric grinder (Philips Mixer Grinder HL1605). Ground powder was stored in air tight container for future use. Twenty grams of dried powdered plant material were extracted in 400 ml of distilled water for 6 h at slow heat (50 $^{\circ}$C) in water bath. At the end of 6 h extract was filtered through No. 1 Whatman® filter paper and stored at -20 $^{\circ}$C for further use. For determining the extract value (29.97±1.3 %) and concentration, few ml of extract was evaporated to dryness with hot air oven.

Experimental plants

Green gram (*Vigna radiata*) seedlings and onion (*Allium cepa*) roots were used as experimental plant models. Green gram seedlings were used for root and shoot growth

retardation assay. Onion roots were used for morphometric antiproliferation and cell cycle kinetics analyses.

AAEAL induced apical meristem growth retardation

Culture and treatment of green gram seedlings Green gram seeds were surface sterilized with 1 % sodium hypochlorite solution for 2 min and washed with distilled water vigorously for ten minutes and allowed for germination in dark at 25±2 ^{o}C on wet filter paper in glass Petri dishes, containing six different concentrations (0.25, 0.5, 1, 2, 4 and 5 mg/ml) of AAEAL, covered with another Petri dish. Root and shoot lengths were recorded at 96 h. Three replica of each with 10 seeds were arranged for each treatment and for untreated controls. Distilled water was used as culture medium for untreated control seedlings. *Culture and treatment of onion roots* similar sized onion bulbs were selected and allowed for root sprouting in test tubes containing four different concentrations (1, 3, 4 and 6 mg/ml) of AAEAL at 25±2 $^{\circ}$C in the culture room. For concentration dependent root growth retardation analysis, onion bulbs were continuously exposed with AAEAL from the beginning of root sprouting and simultaneously untreated bulbs were maintained with distilled water. The root length was recorded at 72 h and the growth retardation percentage was calculated in each case.

AAEAL *induced antiproliferation in apical meristem cells*

Treatment and preparation of mitotic phases from onion root meristem cells

The forty eight hours aged onion root meristem cells were exposed with two different concentrations (0.5 and 2 mg/ml) of AAEAL for 2-24 h. The untreated roots were maintained simultaneously in distilled water. Root tips were fixed in aceto-methanol (3 parts methanol: 1part glacial acetic acid) for 24 h and hydrolyzed for 10 min in 1 N HCl at 60^{o}C, stained with 2% aceto-orcein and squashed in 45 % acetic acid for each treatment[13]. Slides were randomly coded and for each set of experiment at least five slides were studied under bright field light microscope with 40x objective lens. The frequency of prophase-metaphase, anaphase-telophase, and mitotic index depressions in relation to the untreated controls were calculated [14].

Phytochemical detection

The AAEAL was tested to detect alkaloids, terpenoids, triterpenoids, anthraquinones, flavonoids, phlobatannins, tannins, steroids, saponins, glycosides and carbohydrates following the standard procedures[15-17, 20].

Estimation of phytochemicals

The total phenolic and tannin contents were estimated following the procedure as described by Makkar et al.[18] with slight modification, briefly, 20 µl of AAEAL was taken in a test tube and the volume was made up to 2 ml adding double distilled water. Then, 1 ml Folin-Ciocalteu's phenol reagent (1N) was added and mixed thoroughly. Then 5 ml 20 % Sodium carbonate solution (50 g of Na_2CO_3, 10 H_2O was dissolved in 250 ml double distilled water) was added and mixed properly and it was kept for 40 minutes at room temperature (25±2 ºC). Optical density was recorded using spectrophotometer, UV-1800 Series, Shimadzu, Japan, at 725 nm and the concentration was determined using tannic acid standard curve. Total phenolic was estimated as tannic acid equivalent and expressed on dried extract matter basis. For non-tannin phenol estimation, tannins were precipitated with polyvinylpolypyrrolidone (PVPP). In a test tube 200 mg PVPP, 2 ml distilled water and 2 ml AAEAL were added, mixed well by vortexing, and kept in refrigerator at 4 ºC for 15 minutes. The mixture was vortexed again and filtered through filter paper. Now the filtrate was used for estimation of non tannin phenols. In a test tube, 20 µl filtrate was taken and the volume was made up to 2.0 ml by adding double distilled water and then it was processed like that of total phenol estimation. Non tannin phenol content (%) was calculated from the tannic acid standard curve and it was expressed on the dried extract basis. Total tannin content was calculated by subtracting non tannin phenol from total phenol. Total flavonoid was estimated spectrophotometrically[19] with slight modifications, briefly, 0.5 ml of AAEAL was added to 1 ml distilled water and to this 1.5 ml 5 % $NaNO_2$ was added. Five minutes later,

0.15 ml of 10% $AlCl_3$ was added and after 6 min, 1 ml of 1 M NaOH was added and the total volume was adjusted to 5 ml with distilled water and finally the absorbance was measured at 510 nm with UV-VIS spectrophotometer (UV-1800 Series, Shimadzu, Japan). Calibration curve was constructed using quercetin standard. Data were reported as mean ± SEM for three replicate measurements.

Scoring and Statistical analysis

Green gram seedlings and onion roots growth were recorded and the growth retardation percentages were calculated. The difference between the untreated and treated groups for the root length was analysed with the Student's t test. The mitotic index and cell phase frequencies were calculated from onion root tip cells. Cell division phase frequencies were scored on the basis of the nucleus and chromosomal characteristics. The effects on cell cycle kinetics were determined by scoring mitotic index (MI), prophase-metaphase and anaphase-telophase cumulative index. MI was calculated as No. of cells in dividing phase / Total No. of cells scored X 100. Prophase-metaphase and anaphase-telophase cumulative indices were calculated as No. of cells in that particular dividing phases / Total No. of dividing cells X 100. The statistical significance of the difference between the control and treated groups for MI and cell phase frequency were analysed using 2X2 contingency $\chi 2$-test.

RESULTS

Morphometric assays for antiproliferation

Green gram root and shoot growth retardation assay

Data clearly indicate that AAEAL could induce dose dependent growth retardation on green-gram roots and shoots. In the present study, the maximum root length (5.6±0.54 cm) was recorded from the untreated roots and the minimum length (1.7±0.40 cm) was recorded from the highest concentration (5 mg/ml) of AAEAL at 96 h. The root growth inhibitions were calculated as 25.2, 29.3, 34.6, 39.5, 52.9 and 69.5% respectively for the concentrations 0.25, 0.5, 1, 2, 4 and 5 mg/ml of AAEAL at 96 h. Like root growth retardation, the shoot growth retardation was also dose dependent. At 96 h, AAEAL (5 mg/ml) could reduce 54.6 % shoot length in green gram seedlings (Table1).

Onion root growth retardation assay

The AAEAL could induce dose dependent growth retardation on onion roots. In the present study, the maximum root length, 2.85±0.10 cm, was recorded from the untreated groups of onion, while the minimum length, 0.42±0.10 cm, was recorded from the highest concentration, 6 mg/ml, of AAEAL at 72 h. The AAEAL, 1 mg/ml, also showed the significant ($p<0.001$) growth retardation, 71%, on onion roots (Table 2).

Mitotic index depression bio assays with onion root tip cells

Data indicate the trends of mito depression in AAEAL treated onion root tip cells as compared to untreated control roots. Dose dependent mitotic index reduction was observed in AAEAL treated samples. The significant ($p<0.001$) differences in mitotic index were seen between treated and untreated root tip cells. The maximum reduction in mitotic index percentage (74 %) was calculated in AAEAL (2 mg/ml) treated samples at 12 h. Prophase-metaphase and anaphase-telophase cumulative index ratios were 55/45, 73/27 and 85/15 respectively, for the concentrations 0, 0.5 and 2 mg/ml of AAEAL treated for 12 h. More or less the similar patterns of increased prophase-metaphase cumulative index and decreased anaphase-telophase cumulative index were also observed at 2-24 h of AAEAL treatments (Table 3).

Table1

Pooled data showing root and shoot growth retardation effects of different concentrations of AAEAL on green gram seedlings

Conc. (mg/ml)	Root length			Shoot length		
	Range(cm)	(Mean±SEM)	Inhibition %	Range(cm)	(Mean±SEM)	Inhibition %
0.00	3.2-9.5	5.60±0.54	--	0.5-14	7.70±1.04	--
0.25	0.1-11.7	4.19±1.16	25.2	1.0-9.2	5.03±0.90	34.7
0.50	0.1-8.5	3.96±0.83	29.3	0.6-10	5.16±0.91	33.0
1.00	0.5-7.5	3.66±0.77*	34.6	0.5-11	4.53±0.94*	41.2
2.00	0.1-5.8	3.39±0.42**	39.5	0.5-9.5	4.25±0.64**	44.8
4.00	0.1-6.5	2.64±0.59**	52.9	0.1-8.5	3.46±0.77**	55.1
5.00	0.1-4.5	1.71±0.40***	69.5	0.3-8.3	3.50±0.75**	54.6

******Significant at p < 0.001, **at p < 0.01 *at p < 0.05 with Student's t- test. SEM; Standard error of mean. Conc.; Concentration. Data taken from 30 seedlings, triplicate set of ten seedlings, at 96 hrs after extract application. AAEAL was applied to seeds at the beginning of culture set.***

Table 2

Pooled data showing growth retardation effects of AAEAL on onion roots

Conc.(mg/ml)	Time (H)	Root length (cm)		
		Range	(Mean±SEM)	Growth Inhibition %
00	72	1.5-3.0	2.85±0.10	0
01		0.2-1.6	0.82±0.16***	71
03		0.3-1.0	0.62±0.08***	78
04		0.2-1.0	0.45±0.08***	84
06		0.2-1.1	0.42±0.10***	85

******Significant at p < 0.001 with Student's t- test. Each data point is the mean root length of 15 roots, five roots considered from each set of three onion bulbs.***

Table 3

Pooled data showing the influence of AAEAL on mitotic index, prophase-metaphase and anaphase-telophase cumulative frequency of root apical meristem cells

H	Conc. (mg/ml)	No. of cells Scored			MI Mean±SEM (Reduction %)	Cells Percentage	
		TC	IP	MP		PR +ME	AN+TE
2	0.0	07343	06977	366	5.22±0.7	56	44
	0.5	06102	05902	200	2.79±0.9*** (47)	70	30
4	0.0	09712	09274	438	4.62±0.5	52	48
	0.5	10790	10570	220	2.08±0.4*** (55)	71	29
	2.0	15866	15612	254	1.78±0.6*** (62)	77	23
6	0.0	10615	10130	485	4.65±0.8	56	44
	2.0	12945	12673	272	2.33±0.7*** (50)	79	21
12	0.0	04934	04716	218	4.62±0.7	55	45
	0.5	4591	4648	123	2.98±0.6*** (39)	73	27
	2.0	03522	03519	046	1.38 ±0.4*** (74)	85	15
24	0.0	08356	07917	439	5.45±0.8	53	47
	0.5	07214	07071	143	2.00±0.4*** (62)	80	20

******Significant at p < 0.001 as compared to their respective control with 2x2 contingency χ2 -test (d.f. = 1). H, Treatment hours; Conc., Concentration; TC, total cells; IP, Interphase; MP, Mitotic phase; MI, Mitotic index; PR, Prophase; ME, Metaphase; AN, Anaphase; TE, Telophase.***

Phytochemical detection

The preliminary chemical analyses indicate the presence of relatively higher quantities of tannins, saponins, terpenoids, flavonoids, carbohydrates and anthraquinones and a trace amount of alkaloids and glycosides in AAEAL (Table 4).

Table 4
Phytochemicals detected in AAEAL with the standard procedures

S. No.	Phytochemicals	Tests performed	Results
1	Tannins	$FeCl_3$ test	++++
		Alkaline reagent test	+++
2	Saponins	Froth test	+++
3	Steroids	Kantamreddi et al.[20]	_
4	Terpenoids	Kantamreddi et al. [20]	+++++
5	Triterpenoids	Kantamreddi et al. [20]	-
6	Alkaloids	Mayer's test	+
		Wagner's test	+
		Hager's test	-
		Tannic acid test	+
7	Flavonoids	Shinoda test	+++
		Alkaline solution test	+++
8	Anthraquinones	Borntrager's test	+++
9	Phlobatannins	HCl test	_
10	Glycosides	Fehling's test	+
		Alkaline reagent test	+
11	Carbohydrates	Benedict's test	+++
		Fehling's test	+++

Symbols "+"and "-" indicate presence and absence of corresponding phytochemicals respectively and repetition of symbols indicates relative abundance.

Phytochemical estimation

Tannic acid equivalents were determined from AAEAL, following the procedure as described by Makkar et al.[18] as 21.03±0.9 % total phenols, 9.54±0.7 % non tannin phenols and 12.1±0.9 % tannins. Flavonoids were estimated from AAEAL as quercetin equivalents with aluminum chloride colorimetric method as 11.7±0.5 % (Figure 1).

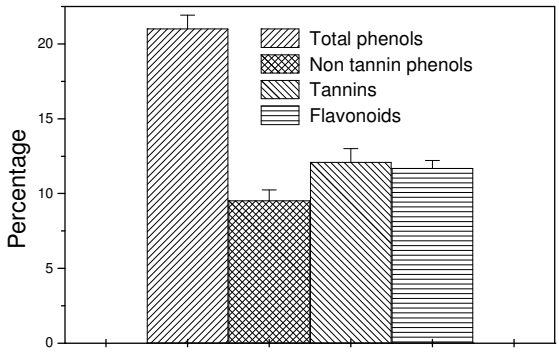

Figure 1
Showing total phenolics, non tannin phenols, tannins and flavonoids contents in dried AAEAL.

UV-VIS Spectrum

UV-VIS spectral analysis of AAEAL showed characteristic absorption spectra with two peaks and three valleys where maximum absorption peak (λ max) at 272 nm indicating relatively higher abundance of phenolics (Figure 2).

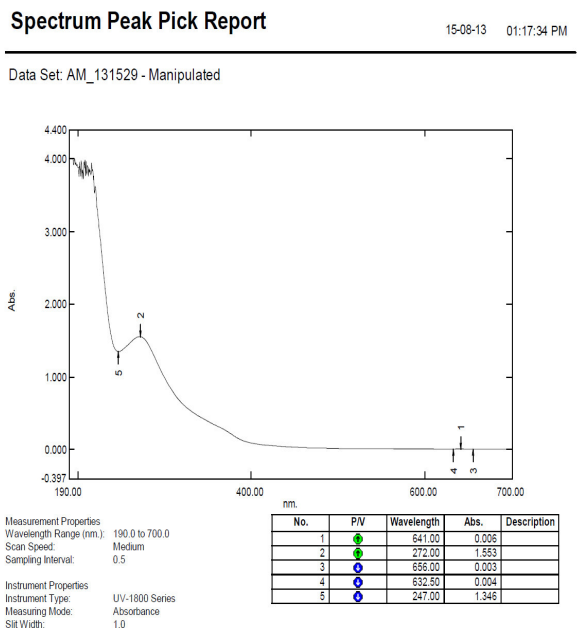

Figure 2

Showing UV-VIS absorption spectrum of AAEAL (concentration, 125 µg/ml, absorbance 1.553, two peaks and three valleys; λ max 272 nm; at which belongs to the UV VIS spectral bands of phenolic compounds) and absorbance was scanned with medium speed, 0.5 sampling interval, slit width 1.0 and the wavelength ranged from 190 to 700 nm.

DISCUSSION

Ampelocissus latifolia has been cited for the treatments of a variety of disorders [21-25]. The work presented herein focuses on the antiproliferative effects of AAEAL on apical meristem cells. In the initial experiments, a wide range (0.25–6 mg/ml) of AAEAL concentrations were used for morphometric bioassays on green gram seedlings and onion roots, and finally two concentrations 0.5 and 2 mg/ml were selected for microscopic analysis of antiproliferative activity in onion root tip cells. The AAEAL treatment could significantly reduce the root and shoot lengths of green gram seedlings (Table 1). Dose dependent growth retardation effects of AAEAL were also observed in onion roots ($p<0.001$). A number of earlier studies have also suggested that the level of growth inhibition increases with increasing extract concentrations[13,14,26]. Secondary metabolites like alkaloids, phenolics, saponins and terpenoids are recognized as allelochemicals. Phenolics are the most abundant substances that affect seedling growth and cell division [27]. We have shown earlier antiproliferative effects of leaf aqueous extracts of *Clerodendrum viscosum* and *Synedrella nodiflora* on root apical meristem cells[13-14]. Mitotic index depression bioassay on onion root apical meristem cells revealed that the AAEAL treatment could significantly ($p<0.001$) reduce the mitotic index. Such a dose dependent reduction in mitotic index percentage suggested that the exposure of AAEAL to root apical meristem cells led to cytotoxic stress, reduction in cell numbers entering into mitotic cycle and all together increased interphase cell frequency. Moreover, increased cumulative frequency of prophase-metaphase and decreased cumulative frequency of anaphase-telophase (Table 3) indicate AAEAL induced delayed cell cycle kinetics in onion root apical meristem cells. There are similar types of reports on mitotic index depressions[13, 14, 21, 28-31]. The present study indicates that AAEAL may contain bio-active compound(s) that might have interacted with the mitotic apparatus. Preliminary phytochemical analysis of AAEAL revealed the presence of tannins, saponins, terpenoids, flavonoids, anthraquinones, carbohydrates and trace amounts of alkaloids and glycosides. The UV-VIS spectrum indicates two distinct peaks and three valleys with the maximum absorption (λ max) at 272 nm which is in the absorption range for phenolics and that may be correlated to our

chemical analysis and quantitative analysis (Table 4, Figure 1 & 2).The therapeutic potentials of medicinal plants lie in their phytochemical ingredients [32]. Phenolics are the most abundant substances that affect seedling growth and cell division[33]. Terpenoids and tannins have been shown to suppress the growth of a variety of cancer cells[34-36]. Some of the efficient anticancer and anti-neoplastic agents exert their effect through the cell cycle progression machinery[37]. Moreover, dose-dependent increased interphase frequency and reduced prophase frequency, indicate AAEAL-induced cell cycle delay, and thus it may correlate with antiproliferative activity. Therefore, the novel findings of the present study are the exploration of antiproliferative potentials of AAEAL. In conclusion, the AAEAL possesses significant antiproliferative potentials in root apical meristem cells, which may be due to the presence of higher quantities of phenolics. Investigations are in progress to isolate the active principle(s) and to determine their influence on the cell cycle kinetics.

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