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Research Article

Evaluation of *In-vitro* Anti-oxidant and Cytotoxicity activity of Aqueous extract of *Pergularia daemia*

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Abstract: The plant *Pergularia daemia* (Asclepiadaceae) is traditionally used as a medicinal agent and they are widely distributed to tropical and subtropical region of India. Therefore, the present work has been designed to evaluate antioxidant and cytotoxicity potential of *Pergularia daemia so* as to claim folklore uses of *P. daemia.* The *in vitro* anti-oxidant activity was studied using DPPH Scavenging, Nitric Oxide Scavenging and β-carotene-linoleate scavenging model and cytotoxic activity of AEPD was studied by the MTT assay using using A-549 (Human, Lung Carcinoma) HT- 29 (Colon Carcinoma) cell lines. AEPD was studied at different concentrations, in which it showed a moderate to potent activity which confirms that it is having cytotoxic activity. AEPD was also studied for its *in vitro* antioxidant property by different methods. AEPD has a hydrogen donating property, which may be attribute to the good antioxidant activity. The *in vitro* data suggest that the extract is having both the *in vitro* antioxidant and cytotoxicity activity.
Keywords: *Pergularia daemia*, Anti-oxidant, cytotoxicity, Aquous extract, Cell lines

INTRODUCTION

Cancer is the second leading cause of death worldwide. A number of undesired side effects sometimes occur during chemotherapy. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects. Currently, a few plant products are being used to treat cancer. However, a myriad of many plant products exist that have shown very promising anticancer properties *in vitro*, but have yet to be evaluated in humans [1].

Herbs have been used as food and for medicinal purposes for centuries. Research interest has focused on various herbs that possess hypolipidemic, antiplatelet, antitumor, or immune-stimulating properties that may be useful adjuncts in helping reduce the risk of cardiovascular disease and cancer [2]. Cancer can be frightening. Most cancer treatments are accompanied by a degree of herbal supplements. There are beneficial effects of medicinal plants on cancer. Around the world, there are countless herbs, trees, and fruits that possess anticancer properties [3].

The plant *P.daemia* of family Asclepiadaceae is known as “Veliparuthi.” in Tamil, “Uttaravaruni” in Sanskrit and “Utranajutuka” in Hindi. [4]. Traditionally the plant *P.daemia* is used as anthelmintic, laxative, antipyretic and expectorant, and is also used to treat infantile diarrhoea and malarial intermittent fevers [5-6]. Latex of this plant is used for toothache [7]. Stem bark of this plant is remedy for cold [8] and fever [9]. Aerial parts of this plant are reported to have various pharmacological activities like hepatoprotective [10], antifertility [11], anti-diabetic [12] analgesic, antipyretic and anti-inflammatory [13-14]. Phytochemically the plant has been investigated for cardenolides, alkaloid and saponins [14] and it has been found that contains various triterpenes and steroidal compounds [15].

Therefore, the present work has been designed to evaluate antioxidant and cytotoxicity potential of *Pergularia daemia* with a view to contributing to the search for beneficial uses of this invasive plant which is a menace to farmers.

MATERIAL AND METHODS

Collection, authentication of plant

The plant *Pergularia daemia* was collected locally from the surrounding places of Hyderabad and authenticated by Botanical Survey of India (BSI), Hyderabad, India.

Preparation of Aquous extract

Fresh aerial parts of *Pergularia daemia* was collected and washed with water to remove the dust and soil. Then the fresh plant part was filled into a round bottomed flask and sufficient quantity of water is added to it. Boiled for 3hrs and filtered with muslin cloth. Same process is repeated with the marc until to get the extract. All the filtrates are pooled together and evaporated at 45-50^{o}C, until the filtrate convert into a semi solid. Then dried in a desiccator over calcium chloride and stored. This aqueous extract named as AEPD and utilized in our present study.

***In-vitro* antioxidant study**

Diphenyl picryl hydrazyl(DPPH) radical scavenging assay

The ability of the plant extract to scavenge 1,1-diphenyl-2-picryhydrazyl (DPPH) free radicals was assessed by the standard method [16]. 0.3mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 1ml of various concentrations of sample and the reference compound (10, 20, 30, 40 and 50 µg/ml), were shaken vigorously and left to stand in the dark at room temperature for 30 min and then absorbance was measured at 517 nm. A control reaction was carried out without the test sample. All the tests were performed in triplicate in order to get the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Antiradical activity was expressed as inhibition percentage (I %) and calculated using the following equation:

Inhibition percentage (I %) = (Abs $_{control}$- Abs $_{sample}$/Abs $_{control}$) X 100

Nitric Oxide Scavenging Activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [17] Different concentrations (500 to 2500 µg/ml) of extracts and as well as ascorbic acid (standard) were prepared in methanol and mixed with phosphate buffer (2.5ml) and Sodium nitroprusside (2.5ml) and incubated at 25^{o}c for 30 minutes. A portion of Griess reagent (1.5ml) was added to 1.5 ml of the reaction mixture. The absorbance was measured at 546nm. Increased absorbance of the reaction mixture indicated the increased reducing power. The % scavenging activity was calculated by using the formula:

% scavenging = (Abs $_{control}$- Abs $_{sample}$/Abs $_{control}$) X 100

β-Carotene Linoleate Model

Antioxidant activity is evaluated by the β-carotene-linoleate model system [18]. A solution of β-carotene is prepared by dissolving 2mg of β-carotene in the 10ml of chloroform. This solution is pipette (2ml) into a 100ml round bottomed flask. After removal of chloroform under vacuum, 40mg of purified linoleic acid, 400mg of Tween 40 emulsifier and 100ml of aerated distilled water was added to the flask with vigorous shaking. Aliquot (4.8ml) of this emulsion was transferred into test tubes containing different concentrations of the extracts (0.2ml). As soon as the emulsion was added to each tube, the zero time absorbance is measured at the 470nm using a spectrophotometer. The tubes were placed at 50^{o}c in a water bath, and measurement of absorbance is recorded after 2hrs. Ablank devoid of β-carotene, is prepared for background substraction. The same procedure was separated with the ascorbic acid, as a positive control. Antioxidant activity was calculated using the following equation.

Antioxidant activity = <u>β-carotene content after 2hr of assay×100</u>

Initial β carotene content

***In-vitro* Cytotoxic activity**

The in-vitro cytotoxicity activity was done using A-549 (Human, Lung Carcinoma) HT- 29 (Colon Carcinoma) cell lines (National Centre for Cell Sciences (NCCS), Pune). Cells were grown in Minimal essential medium supplemented with 2 mM L- glutamine, 10% Fetal Bovine Serum, Penicillin (100 µg/ml), Streptomycin (100 µg/ml) and Amphoterecin B (5 µg/ml) and The cells were maintained at 37˚C in a humidified atmosphere with 5% CO_2 and subculture twice a week.

Determination of Mitochondrial Synthesis by Micro culture Tetrazolium (MTT) Assay [18]

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based.

The principle involved is the cleavage of tetrazolium salt 3-(4, 5 Dimethyl thiazole-2 yl) - 2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

Procedure:

The monolayer cell culture was trypsinized using TPVG and the cell count was adjusted to 1.0x10^{5} cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100 µl of (1000 to 15.6 µg/ml) drug concentrations were added to the cells in microtitre plates. The plates were then incubated at 37^{0}C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, the drug solutions in the wells were discarded and 50µl of MTT (MTT:

prepared in Hank's Balanced Salt Solution without phenol red [(HBSS-PR), 2 mg/ml, Sigma Chemicals)] was added to each well. The plates were gently shaken and incubated for 3 hours at $37^{o}C$ in 5% CO_2 atmosphere. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a Microplate reader (ELISA Reader, Bio- rad) at a wavelength of 540nm. The percentage growth inhibition was calculated using the formula below-

$$\text{Growth Inhibition (\%)} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

RESULT AND DISCUSSION

Result of DPPH scavenging activity

AEPD significantly scavenged the DPPH radical and the result is given in Table-1. The 1, 1-diphenyl -2-picryl hydroxyl (DPPH) radical was widely used as the model system to investigate the scavenging activities of several natural compounds such as extract of plants in a relatively short time. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of the extract and the standard quercetin as a reference compound. The radical scavenging activity of AEPD was evident at all the concentrations but only at moderate level not as significant as that of standard quercetin. The scavenging activity of the AEPD was increased with increase in concentration of extract and that of the standard. AEPD showed the % scavenging activity by 72.43 at 400 µg/ml when compared to quercetin showed 88.44 at 400 µg/ml.

Table-1: Result of DPPH scavenging activity

S. No	Concentration(µg/ml) Std (quercetin)	% scavenging activity	Concentration(µg/ml) AEPD	% scavenging activity
1.	50	73..26± 0.015	50	45.68± 0.035
2.	100	76.25± 0.022	100	54.99± 0.760
3.	200	80.46± 0.01	200	62.30± 0.315
4.	300	83.64± 0.02	300	66.53± 0.051
5.	400	88.44± 0.024	400	72.43± 0.380

Result of Nitric oxide scavenging activity

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities. In scavenging of Nitric oxide radical, AEPD non significantly scavenged the nitric oxide radical when compared with the Ascorbic acid scavenging effect (Table-2). % scavenging activity of AEPD was found to be 35.71at 2500 µg/ml when compared with Ascorbic acid by 78.57at 2500 µg/ml

Table-2: Result of Nitric oxide scavenging activity

S.No	Concentration (µg/ml)	absorbance	% scavenging activity
Control (blank)		0.014	-
Ascorbic acid (STD)			
1.	500	0.007	50.00
2.	1000	0.005	64.28
3.	1500	0.005	64.28
4.	2000	0.005	64.28
5.	2500	0.003	78.57
AEPD			
1.	500	0.007	50.00
2.	1000	0.007	50.00
3.	1500	0.009	35.71
4.	2000	0.019	-35.71
5.	2500	0.019	-35.71

Result of Scavenging of linoleic acid radical by β-Carotene Linoleate Model

The linoleic acid free radical attacks the highly unsaturated b-carotene models. The presence of different antioxidants can hinder the extent of b-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system. AEPD significantly scavenged the β-Carotene Linoleate radical when compared to the Ascorbic acid showed the antioxidant potential of AEPD (Table-3). AEPD showed the % scavenging activity by 96.29 at 2000 µg/ml when compared to ascorbic acid showed 59.15 only at 2000 µg/ml

Table-3: Result of Scavenging of linoleic acid radical by β-Carotene Linoleate Model

S.No	Concentration(µg/ml) Ascorbic acid (STD)	Initial Absorbance	Absorbance after 2hrs	% scavenging activity
1.	500	0.063	0.038	63.33
2.	1000	0.070	0.045	64.28
3.	1500	0.074	0.042	56.75
4.	2000	0.071	0.042	59.15
5.	2500	0.071	0.049	69.01
AEPD				
1.	500	0.029	0.026	89.65
2.	1000	0.028	0.026	92.85
3.	1500	0.028	0.027	96.42
4.	2000	0.027	0.026	96.29
5.	2500	0.043	0.024	55.81

Result of *In vitro* cytotoxicity activity

In this phase of study the aqoues extract of *Pergularia daemia* was further evaluated for the cytotoxic activity. The cytotoxic test was carried out by using MTT method, by using different cell lines like HT-29 (colon cancer cell lines) and A-549(Human lung carcinoma cell line). In this study different concentration of the AEPD was treated with known quantity of cells and the % cytotoxicity in each dose level was measured by using MTT (Micro culture Tetrazolium) method. The extract shown significant % cytotoxicity in cell lines. The results are given in Table-4, and Table-5 and plotted in Fig-1 and Fig-2.

Table-4: Result of *In vitro* cytotoxicity activity of AEPD on HT-29 cell line

Conc. (µg/ml)	% cytotoxicity
1000	94.79
500	85.62
250	73.23
125	20.92
62.5	12.38
31.25	07.93
15.60	00.00
CTC 50(µg/ml)	195

Table-5: Result of *In vitro* cytotoxicity activity of AEPD on A-549 cell line

Conc. (µg/ml)	%
1000	100.00
500	95.85
250	78.72
125	66.87
62.5	36.73
31.25	24.28
15.60	17.91
CTC 50(µg/ml)	90

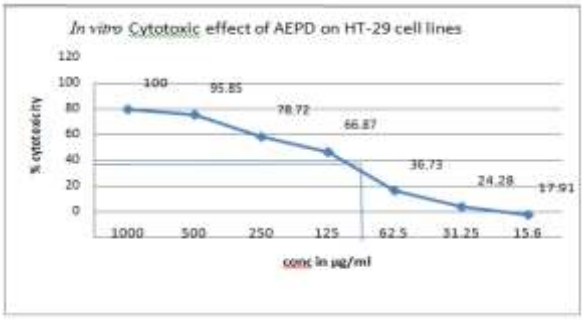

Fig-1: Result of *In vitro* cytotoxicity activity of AEPD on HT-29 cell line

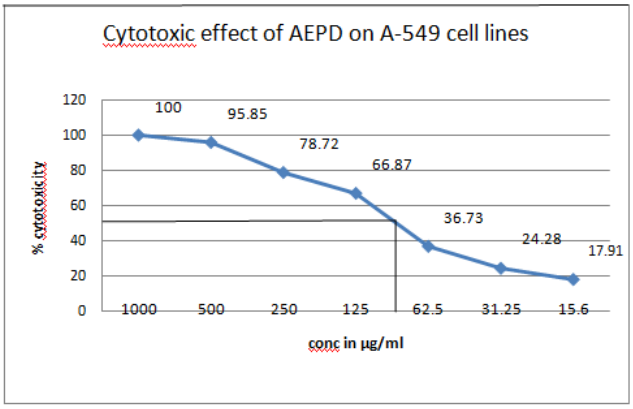

Fig-2: Result of *In vitro* cytotoxicity activity of AEPD on A-549 cell line

CONCLUSION

From the above finding it can be conclude that the plant *Pergularia daemia* (Forsk) posses anti-oxidant and cytotoxicity activity. So, it can be used in different cancer theraphy like colorectal cancer. Before, its clinical usage, thorough toxicological profile has to be determined to confirm the safety of the drug.

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