ISSN No: 0976-5921

Research Article

In-vitro antioxidant, anticancer, wound healing activity profile on aerial parts of *Phyllanthus reticulatus* poir.

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Received: 04-03-2025 Accepted: 26-09-2025 Published: 30-09-2025

Abstract

Background: Antioxidants and anticancer herbs play a major role in preventing and curing current life-threatening disorders due to their cell damage repair properties. Phyllanthus reticulatus Poir. is a shrub, growing commonly in tropical areas, aerial parts (PRAP) of which are used for malignancy, wound healing, and as a rejuvenator. Materials and Methods: PRAP was collected, aqueous extract of the Phyllanthus reticulatus Poir. leaves (PRL) was screened for antioxidant (free radical scavenging activity and reducing power assay), anticancer activity (MTT assay), and wound healing activity on HEK 293 cell line as per standard protocol. Results: Both free radical scavenging activity and reducing power assay have shown positive results, compared to Vitamin C, in higher concentrations. In the MTT assay, as the concentration of test drug extract increased from lower to higher (1 to 5000 µg/mL), % viability was gradually decreased up to 5.3 %, thus showing better cytotoxic activity. This study proved the efficacy of PRL as an antioxidant, anticancer, and wound healing activity agent. Conclusion: Drugs possessing antioxidant, wound healing, and anticancer activities is the need of the hour to prevent lethal diseases like malignancy. Aqueous extract of aerial parts P. reticulatus has shown promising results in the pre-clinical model, which can be taken for further study.

Keywords: Anticancer, Antioxidant, HEK 293, MTT assay, *Phyllanthus reticulatus*, SiHa

Access this article online

Website: https://ijam.co.in

DOI: https://doi.org/10.47552/ijam.v16i3.5910

Introduction

Phyllanthus reticulatus Pior. is an erect straggling shrub belonging to the family *Phyllanthaceae* (1). The plant is common on low moist ground along river banks, irrigation channels and waste places (2). Leaves are simple, elliptic with axillary flowers. Fruits are astringent globose berry with small seeds. Leaves of the plant are useful to treat bleeding gums, sores and wounds; these are also eaten in times of scarcity at a few places (3). Phytochemical constituents of leaves are β-Sitosterol, glochidonol, betulinic acid, friedelin, and its derivatives. Plant shown effective results in antidiabetic, anti-plasmodial, antimicrobial, anti-inflammatory pharmacological assays (4). In *Ayurveda*, it is used as *Vranaprakshalana dravya* (wound washing agent) and known by synonyms like *Kambhoji, Bhudhatri* (5). In traditional medicine, leaves decoction is used for wound healing (6). Matured leafy twigs along with fruits are claimed to be anticancer, antiseptic and rejuvenating. Dried leaf powder is used for wound cleaning and washing (7). Though used popularly in traditional medicine as a potent wound healing, rejuvenating drug, evident scientific research gaps are observed in this area. Millions of free radicals are produced each day which produces cell damage. The imbalance between the formation and neutralization of free radicals results in oxidative stress, which is the major cause of malignancy (8). Natural products with antioxidant properties have shown promising results in wound healing and as an anticancer agent (9).

Even though *P. reticulatus* is used as an efficient wound-healing agent in *Ayurveda*, in vitro study on its antioxidant and wound-healing potential are still lacking. In vitro studies provide a controlled platform to evaluate their pharmacological activities before moving to animal or human studies. Hence with this intention anti-oxidant activity, cytotoxic activity and wound healing activities are evaluated by using the dried powder of leaves of *P. reticulatus* (PRL) for documenting scientific evidence for the traditional claim.

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Hence an attempt was made through this research to evaluate the wound healing activity by in-vitro model on HEK 293 cell line along with in-vitro antioxidant and anticancer activity.

Materials and Methods

Plant materials

Matured aerial parts of *P. reticulatus* was collected from their local habitat along with fruits, authenticated, voucher specimen deposited at the Pharmacognosy department of SDM Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi, Karnataka (Voucher No. 1381/22061003). Around 1kg of wet sample collected, were shade dried which yielded 600 g powder used for preparation of aqueous extract used in the study (10).

Chemicals used for Antioxidant study

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) (Sigma Aldrich), vitamin C (ascorbic acid), methanol, potassium ferricyanide 1%, trichloroacetic acid 10 %, ferric chloride 0.1%, isopropanol, fetal bovine serum (FBS), MTT (Sigma Aldrich), tryphan blue, antibiotic (100 X), minimum essential medium (MEM), phosphate buffered saline (PBS), trypsin, hemocytometer, 60 mm petri dish, sterile needle, 4% formaldehyde and 0.05% gentian violet.

Cell lines for MTT assay, Wound healing assay

SiHa and HEK 293 cell lines were obtained from NCCS Pune and sub cultured at SDM Center for Research in Ayurveda and Allied Sciences, Udupi as per standard protocol were used (11).

Methodology

In-vitro Antioxidant Study

DPPH assay (Radical scavenging assay)

DPPH is a stable free radical with purple colour. It degenerates yellow colour when free radicals have been scavenged. The change in colour from purple to yellow which is measured at 517nm in a spectrophotometer. Test drug was taken in varying concentration (1- 1000µg/mL) for the present study and Vitamin C used as standard drug (Table 1) (12). Percentage inhibition of the discoloration of DPPH by the extract was expressed as follows:

DPPH scavenging activity (%) = [(OD of Blank ─ OD of Sample) / OD of Blank] × 100

Reducing power assay

Varying doses of test drug extract(1- 1000µg/mL) was mixed with 0.75ml of potassium ferricyanide(1%v/v) and incubated at 50° C for 20 min. Reaction was stopped by adding 0.75ml of 10% trichloroacetic acid and centrifuged at 800rpm for 10min. 1.5ml of supernatant was mixed with 1.5ml distilled water and 0.1 ml ferric chloride (0.1%). Incubated at room temperature for 10min and the absorption at 700nm was measured with double beam UV visible spectrophotometer. Higher absorption of reaction mixture indicates the greater reducing power (13).

MTT assay using SiHa cell line

Cell line study of aqueous extract of test drug was carried out on the SiHa cell line, a human cell line designated as cancerous tissues of the cervix (14). SiHa cell line was procured from NCCS Pune, sub-cultured as per standard procedure and MTT assay was carried out.

A confluent cell line flask was taken to trypsinize the cells. The cells were washed twice with Phosphate buffer saline (PBS) centrifuged and re-suspended the pellet in a suitable medium (medium with 10 % fetal bovine serum). Later the cells were counted using hemocytometer. The cells were platted (10,000 cells /well) to 96 well plates. Thereafter it was incubated at 37° C in a CO^2 incubator for 24 h. After 24 h, old medium was carefully discarded from 96 well plates, dissolved the different concentrations of the drug in a suitable serum free medium, after that it was added to the different test groups and incubated for 24 h at 37° C in CO^2 incubator. After completion of incubation time, 20 µL of MTT dye (5 mg/mL in PBS) was added to all wells and covered the plate with aluminum foil and incubated in a CO^2 incubator for 4 h.

After 4 h 100 µL of acidified isopropanol (0.4 N HCl and Isopropanol (1:24) 100 µl /well) was added to all the wells and mixed by careful shaking. Using multi well plate reader absorbance at 540 nm was taken. The percentage of viable cells was calculated using the formula. Cell line with media was treated as the control, whereas cisplatin was used as the positive control.

% of viable cells = [(Test sample-blank) / (Control-blank)] x 100

In Vitro Wound healing assay using HEK cell line

The Human Embryonic Kidney (HEK293) cell line was secured from NCCS Pune and sub-cultured at SDM Centre for Research in Ayurveda and Allied Science, Udupi. Around 70-80 % confluent HEK cell line flask was selected and the previous medium was removed and washed twice with phosphate buffered saline. Cell line was treated with 100 µl of 0.25 % trypsin and incubated at 37° C incubator for about 5 minutes. Once the cells detached from the flask the trypsin was carefully removed using the pipette and floating cells were transferred to a new fresh 15 ml centrifuge tube with medium and centrifuged for 5 minutes at 800 rpm. After centrifugation, the old medium was removed, and cells were re-established in a fresh MEM medium with 10% fetal bovine serum. Next cells were counted using a hemocytometer and around 50,000 cells were transferred to a 60 mm Petri dish with MEM medium and incubated at a 37° C incubator. After incubation time, a sterile needle was used to create a wound on the cell line. Next cells were washed with PBS and re-suspended through different concentrations of serially diluted (10, 20, 40, 80 100, and 500 µg/mL) aqueous extract of PRL to wounded cells and kept for incubation at 37° C for about 72 h. MEM medium was added to unwounded control group cells. After incubation time, the earlier medium was removed and cells were cleaned twice with phosphate buffered saline. Cell fixation was carried out with 4% formaldehyde and these were stained with 0.05% gentian violet solution and incubated for 20 minutes at room temperature (15). Later, the excess stain was removed and these cells were carefully washed with phosphate-buffered saline and dried at room temperature and stained Petri dishes were observed under an inverted microscope to check the wound healing activity (16).

Results

Table 1: DPPH assay of aqueous extract of aerial parts of *P. reticulatus*

Conc. (µg / mL)	Vitamin C	P. reticulatus (Mean ± SE)
1	60.678	39.354 ± 0.289
2	75.607	45.830 ± 0.595
4	79.994	71.236 ± 0.321
8	80.620	71.589 ± 0.032
10	82.693	74.787 ± 0.209
20	83.272	78.033 ± 0.177

40	83.352	83.175 ± 0.112
80	83.481	83.609 ± 0.161
100	83.593	84.911 ± 0.080
200	83.802	86.418 ± 0.032
400	84.027	88.559 ± 0.129
800	84.123	89.796 ± 0.241
1000	84.381	90.503 ± 0.048

Table 2: Reducing Power assay of aqueous extract of aerial parts of P. reticulatus

Conc. (µg / mL)	Vitamin C	P. reticulatus (Mean ± SE)
1	0.01863	0.00927 ± 0.000
2	0.02702	0.01698 ± 0.000
4	0.03607	0.02747 ± 0.009
8	0.06697	0.04098 ± 0.021
10	0.07277	0.05517 ± 0.016
20	0.12873	0.09117 ± 0.028
40	0.21483	0.14983 ± 0.047
80	0.24782	0.23822 ± 0.004
100	0.27198	0.25297 ± 0.010
200	0.27997	0.27902 ± 0.018
400	0.28453	0.28537 ± 0.023
800	0.28563	0.28657 ± 0.023
1000	0.29353	0.31093 ± 0.036

Table 3: MTT assay using SiHa cell line with aqueous extract of aerial parts of P. reticulatus

Conc. (µg / mL)	% Viability (Mean ± SE)
1	62.922 ± 2.539
2	59.483 ± 1.234
4	45.918 ± 1.499
8	42.587 ± 1.151
10	40.109 ± 0.917
20	36.218 ± 0.034
40	33.972 ± 0.857
80	31.646 ± 0.141
100	29.852 ± 0.200
200	26.326 ± 0.594
400	18.082 ± 1.802
800	15.061 ± 0.240
1000	13.520 ± 0.125
2000	10.675 ± 2.186
4000	6.644 ± 2.383
5000	5.309 ± 2.630
Cisplatin 500	1.786 ± 0.011
Cisplatin 1000	1.137 ± 0.007

Figure 1: DPPH assay of aqueous extract of aerial parts of P. reticulatus

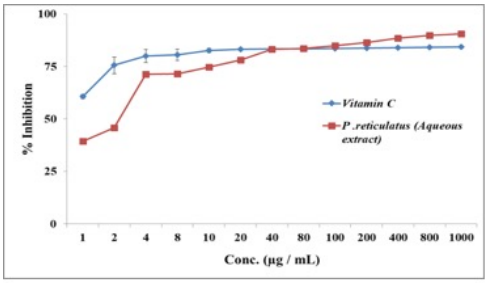


Figure 2. Reducing power assay of aqueous extract of aerial parts of P. reticulatus

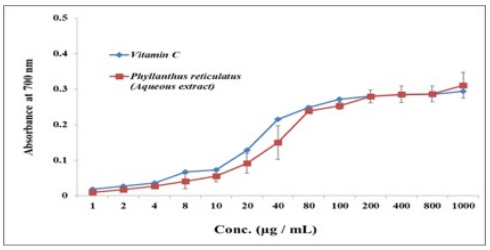


Figure 3: MTT assay using SiHa cell line with aqueous extract of aerial parts of P. reticulatus

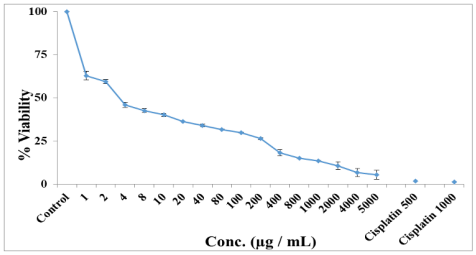
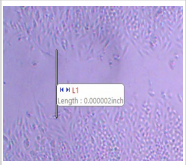
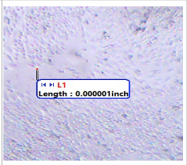
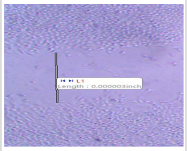
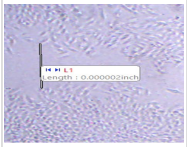
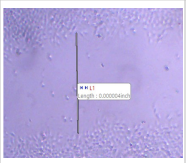
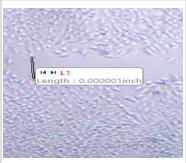
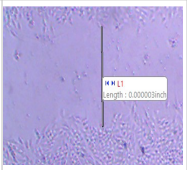
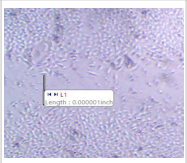
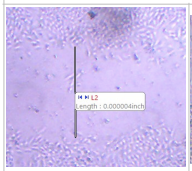
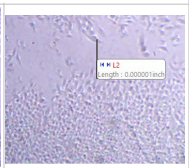
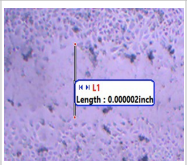
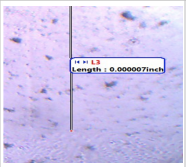
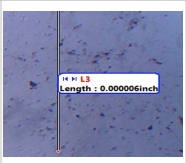
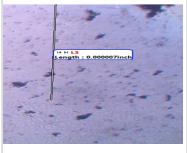
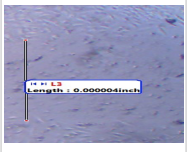


Figure 4: In Vitro Wound healing assay using HEK cell line with aqueous extract of aerial parts of P. reticulatus

Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
1 µg/ ml (control)		10 µg/ ml		20 µg/ ml		40 µg/ ml	
							
80 µg/ ml		100 µg/ ml		200 µg/ ml		500 µg/ ml	
							

Discussion

Focus on herbal drugs based on the traditional systems of medicine and remedies, built on folk knowledge, has been boosted due to their easy access, low cost, and faith of people (17). There is a prevalence of using plants and plant-based products in various contemporary and traditional systems of medicine, with little written documentation. It is essential to document with scientific evidence of all these activities for systematic regulation and widespread application (18). Natural products have been considered as precise sources of treatment used in traditional medicine for centuries to treat a variety of diseases related to cell injury and malignant diseases. Oxidative stress results from the imbalance between the formation and neutralization of free radicals. In this modern world, stress, poor nutrition, environmental pollution and lifestyle are the leading causes of oxidative stress. Oxidative stress can trigger the tumorigenesis. There is a much need for drugs that have anti-oxidant properties to nullify the ill-effect (19). Anti-cancer activity of the *P.reticulatus* Poir leaves methanolic extract was reported for colon cancer by previous researchers (20). *P.reticulatus* Poir leaves is being used as a wound washing agent in *Ayurveda.* Although the plant is known for its wound healing activity, no in-vitro studies have been reported.

This study evaluated the efficiency of *P. reticulatus* Poir for antioxidant, anti-cancer and wound healing activities. Above experimental results suggest that the crude extract of plant possesses antioxidant activity, anti-cancer activity and wound healing activities.

Free radical scavenging activity of the aqueous extract of *P. reticulatus* at 1000 µg / ml by DPPH method was 90.503 whereas vitamin C was only 84.381. From 400µg/ml, it was observed increase in the Anti-oxidant activity of the test compound than vitamin C (Table1, Figure 1). Similarly, the plant extract showed increased anti-oxidant activity at 400 µg/ml comparing to BHT (Butylated Hydroxy Toulene) (21). In reducing power assay both PRL and standard vitamin C were analyzed; the higher absorbance of the reaction mixture indicates the greater reducing power activity. The reducing power of the aqueous extract of *P. reticulatus* was 0.05517 and vitamin C was 0.07277 in 10 µg / mL but in 1000 µg /mL it was 0.31093 and vitamin C was 0.29353. It is showing a dose dependent effectivity of plant extract on test sample (Table 2, Figure 2).

As per WHO, cancer is the leading cause of morbidity and mortality worldwide in which cervical cancer is the fourth most common cancer in women. The morphological feature of cervical carcinoma may present in 3 patterns; fumigating, ulcerating, and infiltrating. The advanced stage of the disease is characterized by widespread destruction and infiltration into the adjacent structures and lymph nodes. It is very important to prevent the further spreading of ulcers in cervical carcinoma.

The human tumor cell lines have been used in cellular screening by in-vitro research. In this experiment, different concentration of PRL was screened for their anti-cancerous activity on SiHa cell lines at different concentrations ranging from 1 to 5000 µg /ml. Cytotoxic activity of *P. reticulatus* on SiHa cell line at different concentrations ranging from 1-5000 µg/mL was done. At 1µg /mL aqueous extract showed 62.9 % viable cells. As the concentration of the extract increased from lower to higher (1 to 5000 µg / mL) % viability gradually decreased up to 5.3 %. The concentrations from 1 µg / mL to 10 µg / mL showed moderate cytotoxic activity. The values of % of cell viability ranges from 62.922 ± 2.539 to 40.109 ± 0.917 at these concentrations. Effective cytotoxicity observed from 20 to 5000 µg /mL. Aqueous extract of *P. reticulatus* showed 5.309 ± 2.630 value and the positive drug cisplatin showed 1.137 ± 0.007 at 5000 µg / mL respectively. The inhibitory dose of aqueous extract of the present drug was found less (IC50 = 2.456 µg / mL) whereas the cytotoxic study of *P. reticulatus* showed IC 50 value of HeLa Cell and Vero Cell were 1.00 mg/ml and 8.09 mg/ml(22). Present experimental study suggested that *P. reticulatus* aerial parts showed efficient cytotoxic activity on SiHa cells (Table 3, Figure 3).

Wound healing is a complex process that aims to achieve the anatomical and functional integrity of disrupted tissues. Wound healing activity was carried out using in-vitro scratch assay which is an expedient technique to obtain first insights into how secondary metabolites of plants can positively influence the formation of new tissue (23). HEK 293 cells are human embryonic kidney cells in which the wound healing activity was carried out (24). The values obtained before and after treatment were measured and analyzed using paired t-test; the data were represented as mean value and SE. Probability of $p < 0.05$ is considered as significant. The p-value was observed less than 0.05, proving the effectiveness of the medicine in wound healing. After incubation time for 72 h when compared to the control group as the concentration of the drug increased wound healing capacity was gradually increased from a lower to a higher dose. After carrying out the experiment, statistical analysis was carried out before and after cell lines, which showed a mean difference of 0.5011, which was statistically different from zero. P-value was less than 0.05, which indicates the rejection of the null hypothesis. This provided evidence for the wound healing activity of PRL on HEK293 cell lines. The concentration of the extract increases the wound healing capacity. The same field photographs show increased cell migration after treatment with PRL at 10, 20, 40, 80,100, and 500 mg/ml of the drug which was taken before and after treatment with PRL (Figure 4). There was a significant increase observed in wound healing activity, which proved its efficacy. This study proved the efficacy of PRL as an antioxidant, anticancer, and wound healing activity agent. The total Aqueous extract of aerial parts of *P. reticulatus* poir. is used in this study, purified fractions may give more effective activities when compared to the crude. Further research is needed for the identification of which particular chemical moieties are responsible for these activities by doing individual studies of purified compounds.

Conclusion

Drugs possessing antioxidant, wound healing, and anticancer activities is the need of the hour to prevent lethal diseases like malignancy. Aqueous extract of aerial parts P. reticulatus was assessed through in-vitro model. Both free radical scavenging activity and reducing power assay have shown positive results, compared to Vitamin C, in higher concentrations. In the MTT assay, as the concentration of test drug extract increased from lower to higher (1 to 5000 µg/mL), % viability was gradually decreased up to 5.3 %, thus showing better cytotoxic activity. This study proved the efficacy of PRL as an antioxidant, anticancer, and wound healing activity agent, which can be taken for further study.

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