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In-vitro Antioxidant and Cytotoxic Effects of *Physalis minima* Linn. in HeLa Cell Lines Against Cervical Cancer

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Abstract

In the present study, the shade-dried and powdered leaves of *Physalis minima* Linn. were subjected to Soxhlet extraction with methanol, concentrated, and further partitioned with n-hexane, chloroform, and ethanol. The *in-vitro* anti-oxidant activity was evaluated by DPPH radical scavenging assay and the cytotoxic potential by MTT assay in HeLa cell lines. The results of the study revealed that among the three fractions, the chloroform fraction of the methanolic extract showed better antioxidant activity (IC_{50} = 38.6 µg/mL) compared to standard Ascorbic acid (IC_{50} = 42.6 µg/mL). Moreover, the results of the MTT assay carried out in HeLa cell lines showed that the chloroform fraction exhibited a promising cytotoxic activity (IC_{50} = 59.66 µg/mL). Further, the chloroform fraction when subjected to apoptosis assay using acridine orange -ethidium bromide dual staining displayed the morphological characteristics of apoptotic event including nuclear fragmentation and chromatin condensation with orange red and reddish nuclei. The results of our study support the protective effect of chloroform fraction of methanolic extract of leaves of *Physalis minima* Linn., in the treatment of cervical cancer.

Key words: Cervical cancer, HeLa, *Physalis minima*, chloroform fraction

Introduction

Natural product research appears to be unlimited and endless while the interest in this field of study has recently been resurfaced in a significant manner, owing to recent technological advancements in separation, spectroscopic techniques, and improved bioassays. There has been strong evidence that naturally occurring phytochemicals produced from higher plants have the potential to be developed into modern medications over the years. Nowadays, the quest for bioactive chemicals from plant-based medicines is recovering and increasingly marketed. Around 25% of the medications used in the world today come from higher plants, while taxol, morphine, quinine, caffeine, atropine, and reserpine are a few among them (1,2).

Cervical cancer occurs when precancerous cells transform into cancer cells and spread further into the cervix or other tissues and organs. Cervical cancer stands as the fourth most common cancer after breast cancer, colorectal cancer, and lung cancer. More than 5,70,000 new cases and 3,11,000 fatalities were recorded in 2018 related to cervical cancer. Furthermore, in eastern, western, middle, and southern Africa, cervical cancer accounted for most cancer-related deaths among women (3,4). HPV is a family

of sexually transmitted viruses that causes 90% of cervical cancer in women by the age of 45. A majority of cervical cancer cases are caused by HPV infection, with HPV-16 and HPV-18 being the most carcinogenic subtypes, accounting for nearly 50% and 10% of cases, respectively (5).

Various species of *Physalis*, such as *Physalis alkekengi*, *Physalis angulata*, *Physalis ixocarpa*, *Physalis longifolia*, *Physalis minima*, *Physalis philadephica*, *Physalis pubescens*, and *Physalis peruviana* have been scientifically proven for their cytotoxic property. Specifically, the withanolides found predominantly in the *Physalis* genus, have demonstrated a wide variety of biological and pharmacological effects such as anti-inflammatory, immunoregulatory, antimicrobial, and antiproliferation (6, 7).

The leaves of *Physalis minima* in the form of decoction were traditionally used as antitussive and in treating diarrhoea. The fruits and flowers were cooked for stomach pain and constipation. The roots were used in Ayurvedic practice to treat blood disorders, fever, and bronchial asthma. In Siddha, the whole plants were used in the treatment of diabetes, and to treat swellings and tumours (8). In our present study, we have attempted to investigate the antioxidant and cytotoxic effect of this medicinal plant against cervical cancer based on its promising traditional claims and wide availability.

Materials and methods

Collection and authentication of plant material

Fresh leaves of *Physalis minima* Linn., were collected from Kallakurchi District, Tamil Nadu, and further identified and authenticated by the botanist Prof. P. Jayaraman (PARC/2021/4567).

Chemicals and reagents

All the reagents and chemicals were of analytical grade and were purchased from commercial chemical suppliers (Sigma-Aldrich, Mumbai, India).

Extraction from leaves

The dried leaves were ground into powder using a homogenizer. The pulverised plant material was extracted by Soxhletation technique using methanol and further partitioned with the solvents, n-hexane, chloroform, and ethanol, dried, weighed and percentage yield was calculated (9-11).

Quantitative estimation of phenolic content

About 1 mL of extract or standard gallic acid, 5 mL of distilled water, and 0.5 mL of Folin Ciocalteu's reagent was added into test tubes and kept for 5 minutes. About 1.5 mL of 20% sodium carbonate was added, followed by 10 mL of distilled water. The absorbance was measured at 750 nm after incubation for 2 hours at room temperature and the total phenolic content was expressed as gallic acid equivalents (GAE) per 100g dry extract (12).

Quantitative estimation of flavonoid content

The extract solution (0.4 mg/mL) was treated with 5 mL of 2% aluminium trichloride ($AlCl_3$) in methanol and absorbance was measured at 415 nm in a UV-VIS spectrophotometer after 10 minutes. The total flavonoid content was expressed as quercetin equivalents (QE) per 100 g dry extract (13).

DPPH radical scavenging activity

The free radical scavenging activity was measured in terms of hydrogen donating and radical scavenging capacities using the stable radical DPPH. About 3 mL of the n-hexane, chloroform, and ethanol fraction (50-250 mg/mL) was added to 1 mL of 0.1 M DPPH solution in methanol in separate test tubes and kept under darkness for 30 minutes. Absorbance was measured at 517 nm using ascorbic acid as standard. A reduction in the absorbance of the reaction mixture indicates increased free radical scavenging activity (14).

$$\text{DPPH radical scavenging activity} = \frac{\text{Control Absorbance} - \text{Extract Absorbance}}{\text{Control Absorbance}} \times 100$$

In-vitro cytotoxic activity by MTT assay

About 100 µL of extracts at different concentrations were applied to the partial monolayer in microtiter plates containing 100µL of diluted cell suspension (1 x 10^4 cells/well) at 37°C in a 5% CO_2 atmosphere. To this 20 µL of MTT (2 mg/mL MTT in PBS) was added and further incubated for 4 hours at room temperature in a 5% CO_2 environment. About 100 µL DMSO was gently mixed into the plate to dissolve the formazan and absorbance read at 570 nm (15). The percentage of viability was calculated by using

$$\text{Percentage of viability} = \frac{\text{Sample absorbance}}{\text{Control absorbance}} x\ 100$$

Apoptosis assay

Control and treated cells were planted on a 6-well plate (3x10^4/well) and cultivated for 24 hours in a CO_2 incubator after being treated with different doses of medication. The cells were fixed for 30 minutes at room temperature in a 3:1 combination of methanol and glacial acetic acid, washed in PBS, and then stained with AO/EtBr in a 1:1 ratio. The stained cells were rinsed in PBS and inspected at a magnification of 40x using a fluorescence microscope (16).

Results and Discussion

Extraction and preparation of Physalis minima leaf fractions

The percentage yield of n-hexane, chloroform, and ethanolic fractions of the methanolic extract of *Physalis minima* Linn leaves was found to be 12.62 %w/w, 10.95 %w/w, and 7.65 %w/w respectively (Table 1).

Table 1. The percentage yield of various fractions of methanolic extracts of *Physalis minima* leaves.

S. No	Extract	Method of Extraction	Solvent Used for Fraction	Physical Nature	Colour	Yield (%W/W)
1.	Methanol extract	Soxhlet extraction	n-Hexane	Semi-liquid	Dark Green	12.62%w/w
			Chloroform	Semi-liquid	Brown	10.95%w/w
			Ethanol	Semi-liquid	Dark yellowish brown	7..65%w/w

Total Phenolic and flavonoid content of different fractions of Physalis minima

The total phenolic contents of n-hexane, chloroform, and ethanol fractions of methanolic leaf extract of *Physalis minima* were found to be 181.75 ± 70.55, 620 ± 238.21, and 876 ± 49.47 mg GAU/g respectively and the same with flavonoid content were found to be 262.55 ± 83.78, 35 8.01 ± 423.98 and 556.52 ± 96.55 mg RU/g respectively (Table 2).

Table 2. Estimation of total phenolic and flavonoid content in various fractions of *Physalis minima*

S. No.	Fraction	Total phenolic content (mg GAU/g)	Flavonoid content (mg RU/g)
1	n-hexane	181.75 ± 70.55	262.55 ± 83.78
2	Chloroform	620 ± 238.21	358.01 ± 423.98
3	Ethanol	876 ± 49.47	556.52 ± 96.55

Each value denotes the average of three analyses ± standard deviation (SD)

In-vitro antioxidant potential of different fractions of *Physalis minima*

The results of the DPPH assay are presented in Figure 1. The various fractions showed potential antioxidant activity with an IC_{50} value ranging from 38.6 to 158.4 µg/mL. The *in-vitro* antioxidant activity of n-hexane, chloroform, and ethanol fractions of the methanolic extract

was investigated by the DPPH radical scavenging assay. Ascorbic acid was used as the standard and methanol was used as the blank. The IC_{50} values of the various fractions of methanolic extract of *Physalis minima* Linn., in ascending order were found to be chloroform fraction (38.6 µg/mL) < ethanol fraction (104.8 µg/mL) < n-hexane fraction (158.4 µg/mL) respectively. The IC_{50} value of the standard ascorbic acid was found to be 42.6 µg/mL. The chloroform fraction that exhibited higher antioxidant potential was selected for further cytotoxicity studies.

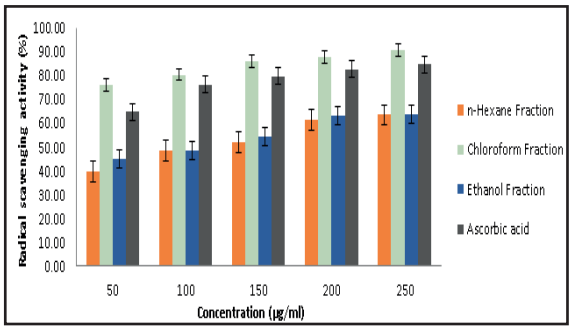

Fig. 1. DPPH radical scavenging activity of various fractions of total methanolic extract of *Physalis minima* Linn.

Cytotoxic effect of different fractions of *Physalis minima*

The cytotoxic activity of chloroform fraction of methanolic extract of *Physalis minima* Linn., leaves by MTT assay using cisplatin as standard was shown in Table.3. The percentage cell viability was found to be declining with the increase in the concentration of the chloroform fraction of *Physalis minima*. When compared with the standard cisplatin, the chloroform fraction showed a promising concentration-dependent cytotoxic effect. At a higher concentration of 160 µg/mL, the chloroform fraction showed a percentage cell death of 97.77 ± 0.425 compared to the standard (96.61 ± 0.002). The IC_{50} value of the chloroform fraction was found to be 59.66 µg/ml, while that of the standard Cisplatin resulted to be 3.44 µg/ml.

Table 3: Anticancer effect of Chloroform fraction of *Physalis minima* Linn., leaves on HeLa Cells.

Concentration	Percentage Cell Death (%)	
	Chloroform fraction	Cisplatin
10 µg/mL	15.96 ± 0.220	54.58 ± 0.002
20 µg/mL	24.73 ± 0.320	70.38 ± 0.002
40 µg/mL	45.39 ± 0.228	88.24 ± 0.002
80 µg/mL	60.31 ± 0.629	93.94 ± 0.001
160 µg/mL	97.77 ± 0.425	96.61 ± 0.002
IC_{50} Value	59.66 µg/mL	3.44 µg/mL

Observation of apoptosis upon acridine orange / Ethidium bromide staining

The degree of chloroform fraction-induced apoptosis, upon AO /EB dual staining, was observed. The control showed intact green nuclei whereas the chloroform fraction and cisplatin showed nuclear fragmentation and chromatin condensation with orange red and reddish nuclei as shown in Figure 2.

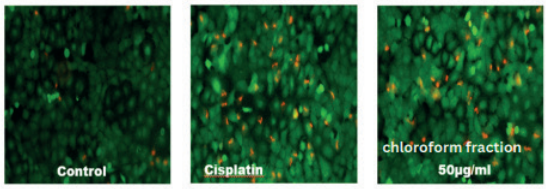

Fig. 2. Apoptotic morphological changes of HeLa cells upon treatment with chloroform fraction of *Physalis minima* (50 µg/mL)

Conclusion

The anti-oxidant, cytotoxic, and apoptotic potential of various extracts of *Physalis minima* Linn., leaves were studied. Eventhough, the total phenolic and flavonoid content was found to be higher in the ethanolic fraction, the DPPH assay revealed that among the three fractions of methanolic extract, the chloroform fraction exhibited promising antioxidant activity. Furthermore, the chloroform fraction showed a concentration-dependent cytotoxic potential against HeLa cell lines and displayed apoptotic

morphological observations. Further molecular studies can be directed towards the development of the chloroform fraction of methanolic extract of *Physalis minima* in treating cervical cancer.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships.

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